

รายงานวิจัยฉบับสมบูรณ์

ภาษาไทย	นิเวศน์วิทยาของยุงและแมลงวันคอกสัตว์และนัยยะความเป็นไปได้ในการใช้สารสกัดจากพืชเพื่อควบคุมแมลงพาหะนำโรค
ภาษาอังกฤษ	**ECOLOGY OF MOSQUITOES AND STABLE FLIES ANS POSSOBLE IMPLICATIONS FOR VECTOR CONTORL BY PLANT-DERIVED COMPOUNDS**

โดย

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ภาษาอังกฤษ **ECOLOGY OF MOSQUITOES AND STABLE FLIES ANS POSSOBLE IMPLICATIONS FOR VECTOR CONTORL BY PLANT-DERIVED COMPOUNDS**

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

The primary objective of this proposal was to develop a research program in conjunction with a group of young entomologists in Thailand to study various bionomics and control aspects of mosquitoes and stable flies. The first subproject was to study the repellent, irritant, and toxic effects of various plant essential oil compounds on different mosquito species by using the two noval screening test systems. The second characterized the efficacy of different stable fly attractants at four dairy cattle farms in Muak Lek district, Saraburi province, Thailand. Dry ice, octenol, a mixture of cow dung and urine, a combination of dry ice plus octenol and no attractants (control) were tested with Vovoua traps. The third obtained the diurnal activity, seasonal abundance and species diversity of Stomoxyine flies in two different ecological habitats, a local beef farm and the Open Zoo. The fourth subproject determined pattern of nucleotide variation of the partial immune genes (TOLL6 and CLIPA6) in *Anopheles minimus* populations, and to evaluate whether natural selection shapes pattern of sequence variation on the immune genes. The fifth obtained the susceptibility status of Anopheles species collected from endemic malaria areas in Mae Hone Son Province. The sixth identified the blood feeding/biting behavior, host preference and seasonal abundance of individual species of Anopheles mosquitoes using a PCR-based molecular in a small village of Sai Yok District, Kanchanaburi Province, Thailand. The seventh investigated a fragment of 400 bp of tim gene whether it can be used as a molecular marker to assess intraspecific variability among populations of main malaria vector founded in Asia. The eight obtained the information about the diversity of mosquitoes which are vectors of dengue viruses and filarial parasites of human in Thong Pha Phoom district, Kanchanaburi province, Thailand. Overall, the eight subprojects beautifully described their project activities and outcome.

Keywords: Mosquitoes, Stable flies, Ecology, Biology, DNA, Insecticides, Essential oils

บทคัดย่อ

โครงการวิจัยนี้มีวัตถุประสงค์เพื่อช่วยพัฒนาเครือข่ายการวิจัยของนักวิจัยรุ่นใหม่ในด้านชีววิทยา ชีวนิสัย นิเวศวิทยาและการควบคุมยุงและแมลงวันคอกสัตว์ที่พบในประเทศไทย โครงการวิจัยแรกศึกษาคุณสมบัติในการไล่ยุงของน้ำมันหอมระเหยที่สกัดได้จากพืช (ตะไคร้หอม แมงลัก หญ้าแมวและหญ้าแฝก) โดยผ่านการทดสอบจากเครื่องมือสองชนิด โครงการที่สองทำการทดสอบประสิทธิภาพของสารล่อชนิดต่างๆ เพื่อนำมาใช้ในการดึงดูดแมลงวันคอกสัตว์และพบแมลงวันคอกสัตว์จำนวน 7,000 ตัว พบว่าน้ำแข็งแห้งอย่างเดียวหรือน้ำแข็งแห้งร่วมกับผลิตภัณฑ์สารออกเทอนอลมีประสิทธิภาพในการดึงดูดแมลงวันคอกสัตว์ โครงการที่สามพบแมลงวันคอกสัตว์สี่ชนิดจากสวนสัตว์เปิดเขาเขียวและมีการกระจายตัวทั้งปี พบมากในฤดูร้อนและ *Stomoxys calcitran* พบมากที่สุด โครงการที่สี่ ศึกษาความผันแปรนิวคลีโอไทด์ของชิ้นส่วนยีน TOLL6 ซึ่งเป็นยีนที่เกี่ยวข้องกระบวนการส่งสัญญานในระบบภูมิคุ้มกันของแมลง และยีน CLIPA6 ซึ่งเป็นยีนในกลุ่มขยายกระบวนการขยายสัญญานในระบบภูมิคุ้มกัน โครงการที่ห้าศึกษาการต้านทานสารเคมีฆ่าแมลงในยุงก้นปล่องที่แนวชายแนวไทยพม่าและไม่พบว่ายุงพาหะนำโรคมาลาเรียต้านต่อสารเคมี โครงการที่หกศึกษาความหลากหลายและพฤติกรรมของยุงก้นปล่องมินิมัสที่อำเภอไทรโยค จังหวัดกาญจนบุรีพบว่ายุงก้นปล่องมินิมัสซับซ้อนมีสองชนิดและ Harrisoni จะพบมากว่า Minimus โครงการที่เจ็ดได้พัฒนาระบบโคลนนิ่งเพื่อตรวจหาเชื้อ *P. vivax* and *P. falciparum* ในยุงก้นปล่อง โครงการที่แปด มีจุดประสงค์หลัก เพื่อศึกษาถึงความหลากหลายและจำนวนประชากรยุงแต่ละชนิดที่ออกหากินตลอดปี และตลอด 24 ชั่วโมง ในท้องที่ป่าสวนยางพารา ในเขตอำเภอทองผาภูมิ จังหวัดกาญจนบุรี โดยสรุปทั้งแปดโครงการย่อยได้บรรลุวัตถุประสงค์ครบถ้วน

คำสำคัญ: ยุง, แมลงวันคอกสัตว์, นิเวศวิทยา, ชีวิวทยา, ดี เอ็น เอ, ยาฆ่าแมลง, น้ำมันหอมระเหย

Executive Summary

The primary objective of this proposal was to develop a research program in conjunction with a group of young entomologists in Thailand to study various bionomic and control aspects of mosquitoes and stable flies on which we have very limited or incomplete information. This select elite group included four researchers from Kasetsart University, one from Mahidol University, one from Khonkaen University, one from the Ministry of Public Health, and one from the Ministry of Agriculture Cooperative and Extension. The project successfully described the biology and ecology of various species of important mosquitoes, stable flies, and pests of humans and animals. Three important outcomes regard the two pest groups were obtained. Using GIS mapping techniques, we depicted for the first time the distribution of malaria vectors identified by molecular techniques, addressed the distribution of insecticide resistance patterns in mosquito vectors, and described the distribution of stable fly species in Thailand. The first two findings were published in the Trends in Parasitology (6.34 IF) and Parasites & Vectors (3.46IF), respectively, whereas the last task regard stable flies is under preparation. We also successfully developed a modified trap design to collect stable flies in the field. Lastly, 4 screened essential oils were found to be excellent candidates as potential mosquito repellents that could be commercially developed in the near future. In total, 11 Ph.D. and 12 Master-level students were directly assisted on these project topics.

วัตถุประสงค์ของโครงการ

Main objective

The main objective of this proposal is to develop a research program in conjunction with a young group of entomologists in Thailand to study various aspects of mosquitoes and stable flies on which we have very few information and knowledge

Objective of Subproject 1 (Dr. Theeraphap Chareonviriyaphap)

To investigate the active properties of synthetic and plant derived compounds for the control of mosquitoes

Objective of Subproject 2 (Dr. Junnongjit Phasuk)

To evaluate the efficiency of attractants in sampling stable flies on diry farms

Objective of Subproject-3 (Dr. Suprasada Sukhonthabhirom)

To survey adult stabel fly, population dynamic of the different species, evaluate the circadian activity of different species and identify host sources

Objective of Subproject-4 (Dr. Uraiwan Arunyawat)

To estimate level and pattern of gentic diversity of immune genes in the main malaria vector (e.g. *Anopheles dirus* and *An. minimus*) in Thailand and to evaluate whether natural sekection shapes pattern of molecular variation on the immune genes

Objective of Subproject-5 (Dr. Wannapa Suwondkerd)

To determine the mosquito behavior, biting pattern and their habitat in the selected areas

Objective of Subproject-6 (Dr. Monthathip Kongmee)

To investigate the *Anopheles* species and a seasonal abundance of dominant malaria vectors

Objective of Subproject-7 (Dr. Waraporn Juntarajumnong)

To develop *P. vivax* and *P. faciparun* system for detection of both *Plasmodium* species in *Anopheles* mosquitoes

Objective of Subproject-8 (Dr. Jinrapa Phothikasikorn)

To investigate vectorial ability of potential vectors susceptible to dengue and filarial disease disease in the villages and rubber plantation forest of Kanchanaburi province

เนื้อหางานวิจัย

Subproject 1: Evaluation of Repellent and Toxic Properties of Plant Essential Oils against Mosquitoes

(Theeraphap Chareonviriyaphap)

Abstract

Repellent property of plant essential oils obtained from four plant species, citronella (*Cymbopogon nardus*), hairy basil (*Ocimum americanum*), catnip (*Nepeta cataria*) and vetiver (*Vetiveria zizanoides*) were characterized on various mosquito species using two assay systems, excito-repellency (ER) test system and high throughput screening system (HITSS). The ER test system (contact irritant and non-contact repellent) was performed on the four essential oils compared with two standard repellents (DEET and picaridin) and two synthetic pyrethroids (deltamethrin and permethrin) against *Aedes aegypti*, *Ae. albopictus, Anopheles minimus* and *Culex quinquefasciatus*. Results revealed that *Cx. quinquefasciatus* and *An. minimus* exhibited much stronger behavioral responses to all test compounds compared to *Ae. aegypti* and *Ae. albopictus*. Synthetic pyrethroids displayed stronger contact irritant response than non-contact repellents across all four mosquito species. While the HITSS was used to determine the different concentrations of plant essential oils compared with synthetic repellents (DEET and picaridin) to *Ae. aegypti* and *An. minimus*. Results indicated that the two mosquito species exhibited significantly different escape responses between contact treatment and control across all concentrations of test compounds. Spatial repellent responses were found in both mosquito species when exposed to all compounds but percent escape responses depend on compounds and their concentrations. The study showed that the higher concentrations of test compounds had greater toxicity effects to mosquitoes but vetiver had no toxic effect on *Ae. aegypti*.

Keywords: Plant essential oils, Mosquitoes, Excito-repellency, High throughput screen system

Introduction

Mosquitoes (Order: Diptera, Family: Culicidae) are the most important disease vectors worldwide. Blood feeding female mosquitoes have been implicated in the transmission of many diseases including dengue fever, yellow fever, chikungunya, malaria, and Japanese encephalitis (Rattanarithikul and Panthusiri 1994). Repellents as personal protection against mosquitoes have been used for decades. DEET (N,N-diethyl-3-methylbenzamide) was developed by the U.S. Army in 1946 and marketed for public use in 1957. DEET is still considered the most effective and common ingredient in insect repellent products but some people have reported side effects including rash, skin irritation, blisters, and eye irritation (Miller 1982, Amichai et al. 1994, Patel et al. 2012). There are many reports revealed that some plant-derived essential oils are considered non-toxic, alternative insect repellents for humans. Good naturally derived mosquito repellents can be safer with no long-term or serious side effects when used properly. Citronella is the most common ingredient used in plant-based mosquito repellents. Phasomkusolsil and Soonwera (2010) reported that citronella had repellent activity against *An. minimus*, *Culex quinquefasciatus,* and *Ae. aegypti* when applied on human skin. Moreover, Bernier et al. (2005) revealed catnip was a good spatial repellent against *Ae. aegypti*, *An. albimanus,* and *An. quadrimaculatus*. Various plant essential oils have been proven to be effective against many mosquito species (Tawatsin et al. 2001, Zhu et al. 2006, Phasomkusolsil and Soonwera 2010). Thus essential oils obtained from plants can potentially play an important role in mosquito repellency.

In this subproject, besides using excito-repellency (ER) test system to characterize behavioral responses of four different mosquito species (*Ae. aegypti*, *Ae. alboppictus*,
An. minimus, and *Cx. quinquefasciatus)* to various essential oils according to the previous reports, we also used the high throughput screening system (HITSS) to investigate the escape response and mortality rate of *Ae. aegypti* and *An. minimus* to four plant essential oils (citronella, hairy basil, catnip, and vetiver). The ER chambers and HITSS can effectively help selecting plants that work well for repelling mosquitoes.

Materials and Methods

1. Behavioral responses of mosquitoes to plant essential oils, synthetic repellents and pyrethroid insecticides using excito repellency test system

Test mosquitoes

Laboratory test populations of *Ae. aegypti*, *Ae. albopictus*, *An. minimus* and *Cx. quinquefasciatus* were used in this study. The *Ae. aegypti*, insecticide susceptible test population (U.S. Department of Agriculture laboratory [USDA]) was obtained as eggs from the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. The USDA colony of *Ae. aegypti* has been reared in the laboratory for many years. *Aedes albopictus* has been maintained in the entomological laboratory at Kasetsart University for 6 years. A colony of *Cx. quinquefasciatus* were obtained from the National Institute of Health (NIH), Ministry of Public Health, Nonthaburi, Thailand where the colony has been established since 1978. The *An. minimus* colony was originated from the Malaria Division, Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand in 1998. The colony has been maintained in the Department of Entomology, Kasetsart University for more than 15 years.

All four species of mosquitoes were reared in the insectary of the Department of Entomology, Faculty of Agriculture, Kasetsart University. All larvae and adults were held under laboratory conditions of 25 ± 5 $^{\circ}$C and 80 ± 10% RH with a photoperiod of LD 12:12 h. Larvae were fed with fish food pellets whereas *An. minimus* larvae were fed finely ground fish food flakes (TetraMin®). Adult mosquitoes were reared in a screened cage and provided 10% sugar solution as food. Female mosquitoes aged 3-5 days old were starved for 24 h before testing.

Plant essential oils

Four plant essential oils were used in behavioral response study - citronella (*Cymbopogon nardus*), hairy basil (*Ocimum americanum*), catnip (*Nepeta cataria*), and vetiver (*Vetiveria zizanoides*). Two essential oils were provided by Thai-China Flavours and Fragrances Industry Co., Ltd. and catnip oil (*Nepeta cataria*) was received from the Chemicals Affecting Insect Behavior Lab, United States Department of Agriculture, Beltsville, MD.

Synthetic repellents

DEET and picaridin were selected as representative synthetic repellents. DEET was obtained from the USDA, Beltsville, MD (Lot No: 0326/2009). Picaridin was obtained from Bayer Thai Co., Ltd. (Lot No: CHCAEN0020).

Pyrethroid insecticides

Deltamethrin ([Cyano-[3-(phenoxy)phenyl]methyl] 3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate) (98% purity) was obtained from BASF (Lot No: HDDLTK034). Permethrin (3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) (97.6% purity) was provided by Sherwood Chemicals Public Co., Ltd. (Lot No: ABJFPAP118).

Paper impregnation

Deltamethrin and permethrin were dissolved in a mixture of acetone and silicone (Dow Corning 556) to obtain doses of 25 mg/m^2 and 500 mg/m^2, the highest doses of insecticidal treated nets (ITNs), respectively (WHO 2006). DEET, picaridin and four essential oils were diluted in absolute ethanol to a concentration of 2.5% active ingredient, based on optimal concentrations identified from previous mosquito behavioral studies (Polsomboon et al., 2008; Suwansirisilp et al., 2012). A 2.8 ml of the solution was dropped with a micropipette on Whatman No.1 filter paper, 14.7 × 17.5 cm^2. Impregnated papers were allowed to air dry one hour before testing (Licciardi et al., 2006). Control papers were treated with only ethanol or solvent mixture for insecticide tests.

Excito repellency test system

The excito-repellency test assay used in this study has already been well described previously (Chareonviriyaphap et al., 2004 Mongkalangoon et al., 2009) as shown in Figure 1. Briefly, a set of four test chambers was used to evaluate both non-contact repellent and contact irritant behaviors (Figure 2). Each test chamber was connected with a receiving box for collecting mosquitoes exiting from the test chamber. A matched control was performed for each chemical treatment evaluation. Fifteen unfed female mosquitoes of 3-5 days old were released into each of four test chambers and mosquitoes were allowed to adjust themselves to environmental conditions inside the test chamber for 3 minutes before the exit door open. The number of escaping mosquitoes was recorded every minute for 30 minutes during exposure to test repellent compounds. At the end of the exposure period, escaped and non-escaped mosquitoes were transferred to individual containers and provided 10% sugar solution. Knockdown was observed after 30 minutes and mortality after 24 hours in both treatments and controls for both escaped and non-escaped cohorts. Each repellent compound was tested in four replicates between 0800 am and 1600 pm.

Statistical analysis

Kaplan-Meier survival analysis was used to evaluate escaping mosquitoes from each test chamber of the excito-repellency test system (Roberts et al., 1997). The time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of assay populations to escape was calculated for each product assessed and the log-rank test used to compare the escape responses of test populations. Observed percentage escape was corrected with Abbott's formula (Finney, 1971).

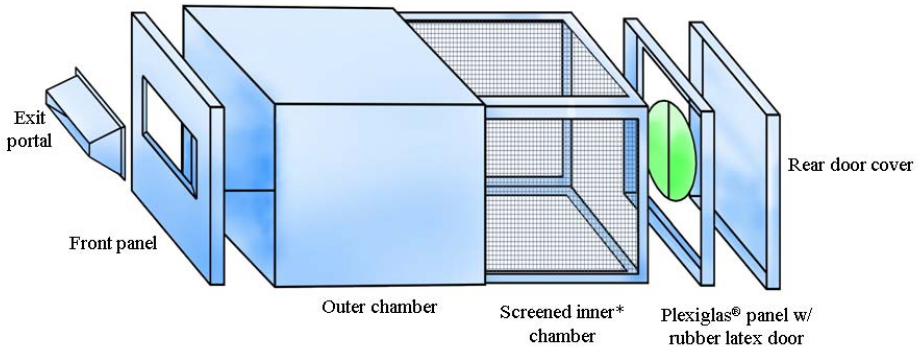


Figure 1 Blow-up diagram of the excito-repellency test chamber showing key components in apparatus design, including the screened inner chamber to measure spatial repellency

* Screened inner chamber: our treated papers were placed inside of the screened surfaces (top, bottom, 2 sides) of the inner chamber for contact trials, wherein mosquitoes were able to make direct contact with the trated surface of the papers and alternatively, papers were placed on the outside of the screens for non-contact trials, wherein mosquitoes were denied direct access to make physical contact the treated surfaces.

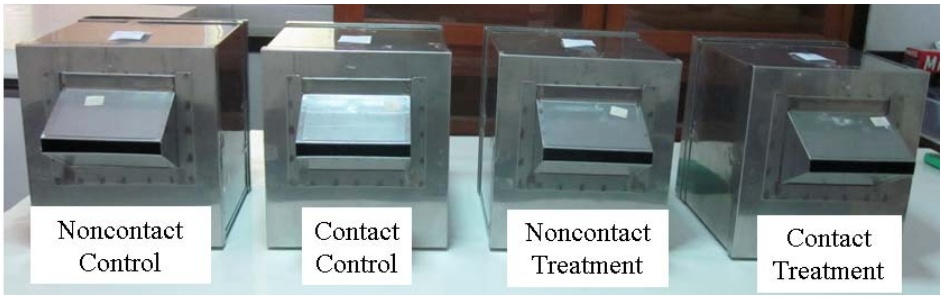


Figure 2 Excito-repellency test system showing the four chambers aligned in sequence for each trial run, one pair as contact and matched control, the other set as non-contact and control chamber.

2. Behavioral responses of *Aedes aegypti* and *Anopheles minimus* to plant essential oils and synthetic repellents using high throughput screening system

Mosquito populations

Two laboratory colonies of *Ae. aegypti* and *An. minimus* were used in the study. The colony of *Ae. aegypti* is a long-standing (~70 years) laboratory colony originating from the USDA (U.S. Department of Agriculture), Gainesville, FL, USA. *Anopheles minimus* colony has been maintained in the Department of Entomology, Kasetsart University for more than 15 years without exposure to any insecticide*s.* The colony originated from the Malaria Division, Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand, in 1998. Both mosquito species were reared in the insectary of entomological laboratory at Kasetsart University, Bangkok, Thailand under rearing conditions of 25 + 5 $^{\circ}$C and 80 ± 10% RH with a photoperiod of LD 12:12 h (Figures 1A and 1B). *Aedes aegypti* larvae were fed with fish food pellets and *An. minimus* larvae were fed finely ground TetraMin® fish food flakes. Adult mosquitoes were kept in clean screen cages and 10% sugar solution was provided. Three to five day-old female mosquitoes were used in the susceptibility and behavioral tests. For behavioral tests, mosquitoes were provided no access to sugar solution for approximately 24 hours before conducting the assays.

Plant essential oils

Citronella oil, *Cymbopogon nardus* (Lot No: NO5410008-1/1910), hairy basil oil*,* *Ocimum americanum* (Lot No: 5308093/0408), catnip oil, *Nepeta cataria* (Lot No: 01022011) and vetiver, *Vetiveria zizanoides* (Lot No: 5506713/2706) were selected for the tests. Citronella, hairy basil and vetiver oils were supplied in 100% purity from Thai-China Flavours and Fragrances Industry (TCFF) Co. Ltd., Thailand. Catnip oil was received from the Chemicals Affecting Insect Behavior Lab, United States Department of Agriculture, Beltsville, MD.

Synthetic repellents

DEET (N*,* N-diethyl-3-methylbenzamide) and picaridin (2-(2-hydrozyethyl-1-piperidincarboxylic acid 1-methylpropyl ester) were used as standard repellents for comparison purposes. DEET (97% purity, Lot No: 0326/2009)) was obtained from the USDA, Beltsville, MD, USA. Picaridin (98.4% purity, (Lot No: CHCAEN0020) was obtained from Bayer Thai Company Limited.

A novel high-throughput screening system (HITSS)

HITSS device (Grieco et al., 2005) consists of three parts. The first part is spatial repellency assay, second part is contact irritancy assay and another part is toxicity assay (Figure 3).

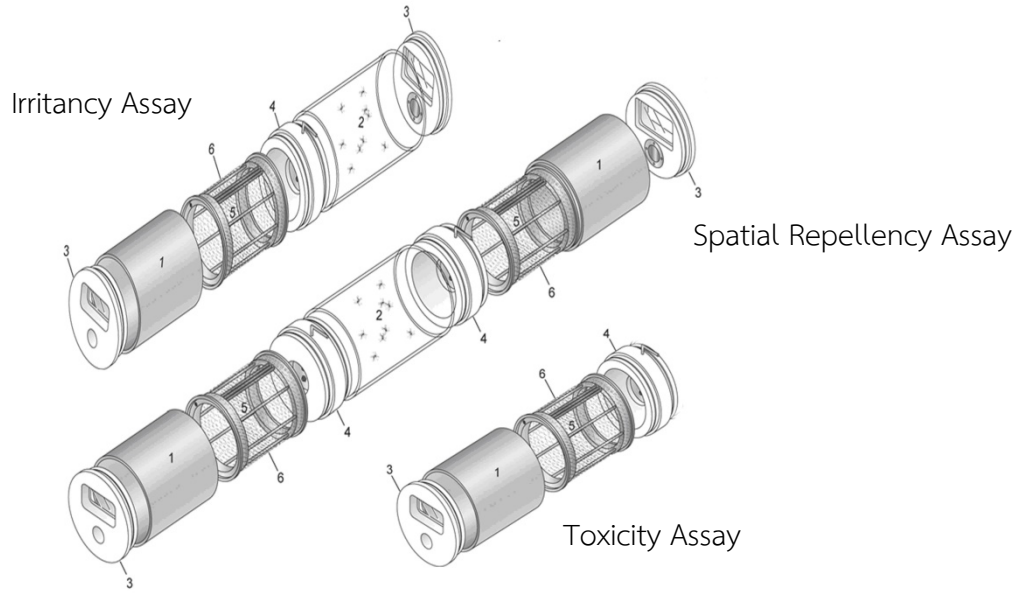


Figure 3 The high-throughput screening system (HITSS) showing the configuration for contact irritancy assay on the top, spatial repellency assay in the middle and toxicity assay on the bottom. The metal treatment cylinder can be used alone to evaluate chemical toxicity. Major components include: 1) treatment chamber 2) clear chamber 3) end cap 4) linking sections 5) inner metal spool and 6) treatment net (Thainispong et al., 2010)

Net impregnation

Essential oils and repellents were tested at concentration in the ranges 0.5-3.0% active ingredient in absolute ethanol. Nylon netting was cut into 11 × 25 cm^2 strips. Volume of 1.5 ml test solution was treated on a net strip by using a 1000 µl micropipette with a tip. The treated nets were dried in air for 15 minutes before attaching it into the metal test cylinder. Control nets were treated with ethanol only.

HITSS test assays

The HITSS was used to determine three actions of test compounds, contact irritant, spatial repellent and toxicity. The protocol and system was developed and optimized by Grieco et al. (2005, 2007).

Contact irritancy assay (CIA)

This assay consisted of two opposite placed cylinders- a clear cylinder and a metal cylinder with both ends capped. Both cylinders were connected to each other by a butterfly valve on linking section. Ten female mosquitoes were released into the metal cylinder which having the treated net. The mosquitoes were allowed to rest for 30 second then a butterfly valve was turned in the open position for 10 minute. The valve was turned back to the closed position. The number of mosquitoes escaping into the clear cylinder and those remaining in the metal cylinder as well as knocked down mosquitoes from each side was recorded. All tested mosquitoes were removed from the clear and metal cylinders. Six replicates were conducted for treatments and controls.

Spatial repellency assay (SRA)

The SRA consisted of one clear and two metal cylinders. The each side of clear cylinder was connected to metal test chamber (control and treatment). Twenty female mosquitoes were introduced into the clear cylinder and allowed to rest for 30 second then the butterfly valves on linking sections were open for 10 minute. After the valves being close, number of mosquitoes in each cylinder was recorded including knocked down specimens. All tested mosquitoes were removed from the clear and metal cylinders. Nine replicates of each treatment and control were required to complete this assay.

A spatial activity index (SAI), based upon the oviposition activity index as described by Kramer and Mulla (1979), was applied to evaluate the escape response of mosquitoes in the spatial repellency assay.

$$SAI = \frac{(N_c - N_t)}{(N_c + N_t)}$$

N_c = the number of mosquitoes in the control cylinder

N_t = the number of mosquitoes in the treatment cylinder.

The SAI value ranges from -1 to 1, with zero represented no response. The SAI value of -1 is referred to those higher number mosquitoes appeared in the treatment cylinder indicating an attractant response. SAI value of 1 is referred to that a higher number of mosquitoes remained in the control cylinder indicating a repellent response.

Toxicity assay (TOX)

The TOX assay was only used metal cylinder with end cap and butterfly valve for each side of cylinder. The treated net was rolled inside the cylinder. Twenty female mosquitoes were transferred into the test cylinder for control and treatment for an hour. After the exposure period, knocked down mosquitoes were noted and all mosquitoes were moved to clean plastic cups with a 10% sugar solution. Mortality was monitored and recorded after the 24 hour holding period. Six replicates were required for treatment and control in this assay.

Statistical analysis

Contact irritancy data were analyzed using the two-sample Wilcoxon test to evaluate the different number of escape mosquitoes between treated and control cylinders. Spatial repellency data were analyzed by the nonparametric Wilcoxon signed ranks test to calculate the SAI for each test treatment. Toxicity data, percentage of knockdown and mortality at 24 hour was corrected using Abbott's formula and transformed to arcsine square root for analysis of variance (ANOVA). The knock down and mortality of treatment at each concentration was compared and separated using Tukey's honestly significant difference test at P=0.05 (SAS Institute, 1999).

Results and Discussion

1. Behavioral responses of *Aedes aegypti*, *Aedes albopictus*, *Anopheles minimus* and *Culex quinquefasciatus* to plant essential oils, synthetic repellents and pyrethroid insecticides using excito repellency test system

Escape response rates of mosquitoes

Results in contact trials indicate that responses of *Ae. albopictus* to the synthetic pyrethroids (deltamethrin and permethrin) were significantly greater than DEET, picaridin or essential oils (P<0.05). For *Ae. aegypti*, deltamethrin showed greater escape responses than others, except hairy basil (P=0.0892). Permethrin showed no significant difference in repelling effect as compared to all essential oils, $P>0.05$ (Tables 8 and 9). All compounds were found to elicit escape responses in both *Cx. quinquefasciatus* (69.2% to 96.6%) and *An. minimus* (65.4% to 97.8%), except picaridin in that comparatively low escape responses were observed (3.4% and 12.2% respectively). Moreover, two test populations, *Cx. quinquefasciatus* and *An. minimus* showed high escape responses to three essential oils, citronella, hairy basil and catnip in contact and non-contact trials (72.4% to 94.4%). Vetiver displayed extremely high escape responses from both species in the contact chambers (>95%) (Tables 1 and 2).

Mortality rates of mosquitoes

Overall, mortality rates were low, except when *An. minimus* was exposed to catnip as 76.7% and 89.9% mortality was reported in contact and non-contact, respectively (Tables 1 and 2).

Escape time

Escape time in minutes (ET) 25%, 50% and 75% during the 30-min exposure assay is shown in Table 3. *Aedes aegypti* showed the greatest escape response to permethrin with an ET_{25} value of 1 min and an ET_{50} value of 4 mins in contact trial. No response was found in the non-contact trials. *Aedes albopictus* had low ET_{25} values in both contact and non-contact trials ($\leq$1 min) against deltamethrin, DEET, catnip and vetiver. For ET_{50}, this species displayed the value of $<$1 min only in contact trials for deltamethrin and permethrin. ET_{75} values of 2 mins and 4 mins appeared for deltamethrin and permethrin in contact treatment chambers, respectively. *Culex quinquefasciatus* displayed ET_{25} values of $<$1 min in contact trials against all four essential oils tested and $\leq$1 min in non-contact trials for citronella, hairy basil and catnip. *Anopheles minimus* showed fast escape responses of $\leq$1 min at ET_{25} to DEET, hairy basil and catnip. For *An. minimus,* catnip was found to have an ET_{25}, ET_{50} and ET_{75} for both contact and non-contact trials $\leq$4 mins. *Anopheles minimus* had a delayed escape response to permethrin, DEET, and citronella with ET_{50} values of 6, 6 and 7 mins, respectively (Table 3).

Escape patterns

The escape patterns during 30-min exposures for the four mosquito species in response to four essential oils or two repellents or two pyrethroid insecticides in contact and non-contact trials are presented in Figures 4 and 5. A comparison of escape responses among the mosquito test populations between contact and non-contact trials. All species exhibited significant differences in response patterns between trials for deltamethrin and permethrin ($P<0.0001$). *Aedes aegypti* showed slightly different in patterns of escape among DEET ($P=0.0460$), hairy basil ($P=0.0166$) and catnip ($P=0.0201$), but there were no significant differences for picaridin, citronella and vetiver ($P>0.05$). A significant difference was found between DEET and vetiver ($P<0.0001$) trials using *Cx.*

quinquefasciatus. Escape responses were also significantly against citronella and vetiver trials using *An. minimus.*

The log-rank tests for comparisons between two species of test mosquitoes in either contact or non-contact trials were conducted. The study found that there were very significant differences between *Ae. albopictus* and other three species in both test conditions of deltamethrin ($P<0.0001$). The significant differences in escape responses between two species were most found in citronella, hairy basil, catnip and vetiver ($P<0.05$). While synthetic pyrethroids and repellents indicated some significant differences between species.

Statistical tests for comparisons between pyrethroids and repellents/essential oils against four mosquito species were conducted. *Anopheles minimus* and *Ae. albopictus* showed most comparison data significant differences ($P<0.05$) in contact trials. There were very significant differences between deltamethrin and other compounds except when paired with hairy basil in contact trials but no statistically significant differences between permethrin and all four essential oils in the same trials for *Ae. aegypti*.

Table 1 Knockdown, mortality, and escape response of 4 mosquito species exposed to deltamethrin (0.025 g/m^2), permethrin (0.5 g/m^2), DEET, picaridin, citronella, hairy basil, catnip and vetiver each at 2.5% (2.72 g/m^2) in contact trial.

Compound	*Aedes aegypti*					*Aedes albopictus*					*Culex quinquefasciatus*					*Anopheles minimus*				
	%	% KD		%Mort (24h)		%	% KD		%Mort (24h)		%	% KD		%Mort (24h)		%	% KD		%Mort (24h)	
	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs
Deltamethrin	67.9	46.6	22.4	46.6	27.6	94.4	36.7	5.0	13.3	5.0	72.4	0	0	0	0	65.4	5.1	5.1	15.3	10.2
Deltamethrin-C	8.6	0	0	0	0	10.0	0	0	0	0	3.3	0	0	0	0	10.0	0	0	0	0
Permethrin	72.2	27.8	24.1	24.1	24.1	85.2	25.0	8.3	33.3	10.0	85.9	0	1.7	0	0	97.8	0	3.4	6.9	0
Permethrin-C	7.0	0	0	0	0	10.0	0	0	0	0	3.4	0	0	0	0	10.0	0	0	0	0
DEET	21.4	0	0	0	0	47.3	0	1.7	0	1.7	69.2	0	0	0	0	80.7	0	0	0	0
DEET-C	6.7	0	0	0	0	6.7	0	0	0	0	3.3	0	0	0	0	6.7	0	0	0	0
Picaridin	3.7	0	0	0	0	28.6	0	0	0	0	3.4	0	0	0	0	12.2	0	1.7	0	1.7
Picaridin-C	5.0	0	0	0	0	5.0	0	0	0	1.7	3.4	0	0	0	0	5.0	0	0	0	0
Citronella	37.9	0	0	0	0	5.6	0	76.3	0	0	80.8	0	0	0	0	86.3	0	0	0	0
Citronella-C	0	0	0	0	0	6.7	0	0	0	0	5.0	0	0	0	0	6.7	0	0	0	0
Hairy basil	47.3	0	0	0	0	3.5	0	88.3	0	1.7	89.3	0	0	0	0	90.8	0	6.9	0	3.4
Hairy basil-C	6.7	0	0	0	1.7	6.7	0	0	0	0	5.1	0	0	0	0	6.7	0	0	0	0
Catnip	32.8	5.2	32.8	0	0	61.1	0	8.3	0	1.7	80.7	0	8.6	0	5.2	87.9	0	10.0	76.7	0
Catnip-C	5.1	0	0	0	0	10.0	0	0	0	0	1.7	0	0	0	0	10.0	0	0	3.4	0
Vetiver	37.8	0	0	0	0	42.9	0	0	0	1.7	96.6	0	0	0	0	96.5	0	0	0	0
Vetiver-C	1.7	0	0	0	0	6.7	0	0	0	1.7	1.7	0	0	0	0	6.7	0	0	0	0

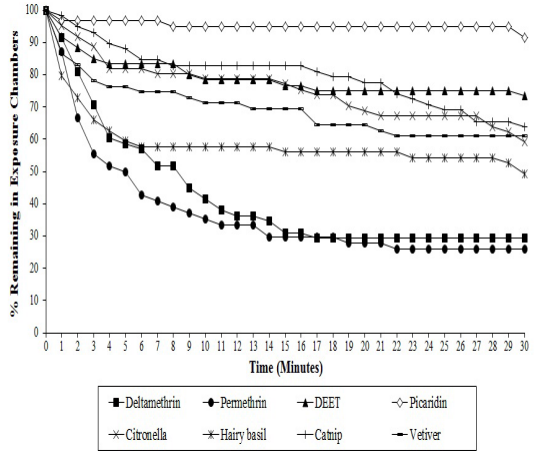
Table 2 Knockdown, mortality, and escape response of 4 mosquito species exposed to deltamethrin (0.025 g/m^2), permethrin (0.5 g/m^2), DEET, picaridin, citronella, hairy basil, catnip and vetiver each at 2.5% (2.72 g/m^2) in non-contact trial.

Compound	*Aedes aegypti*					*Aedes albopictus*					*Culex quinquefasciatus*					*Anopheles minimus*				
	%	% KD		%Mort (24h)		%	% KD		%Mort (24h)		%	% KD		%Mort (24h)		%	% KD		%Mort (24h)	
	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs	Esc	Es	NEs	Es	NEs
Deltamethrin	3.7	0	10.7	0	12.5	62.3	0	0	0	0	0	0	0	0	0	16.2	0	0	3.4	3.4
Deltamethrin-C	3.5	0	0	3.5	0	3.3	0	0	0	0	0	0	0	0	0	3.3	0	0	0	0
Permethrin	0	0	0	0	3.6	40.8	0	0	0	0	0	0	0	0	0	50.8	0	1.7	1.7	3.3
Permethrin-C	8.9	0	0	0	1.8	15.3	0	0	0	0	1.7	0	0	0	0	15.3	0	0	0	1.7
DEET	8.7	0	0	0	0	57.9	0	0	0	1.7	21.1	0	0	0	0	77.7	0	0	0	0
DEET-C	5.0	0	0	0	0	10.3	0	0	0	0	3.3	0	0	0	0	10.3	0	0	0	0
Picaridin	0	0	0	0	0	14.8	0	0	0	0	1.8	0	0	0	0	2.1	0	0	1.7	0
Picaridin-C	6.7	0	0	0	0	10.0	0	0	0	0	1.7	0	0	0	0	10.0	0	0	0	0
Citronella	30.4	0	0	0	0	14.6	0	52.5	0	1.7	85.9	0	1.7	0	1.7	73.2	0	0	0	0
Citronella-C	3.4	0	0	0	0	6.6	0	0	0	0	3.3	0	0	0	0	6.6	0	0	0	0
Hairy basil	30.4	0	17.5	1.8	1.8	11.2	0	72.9	0	5.1	89.6	0	0	0	0	78.1	1.8	17.5	1.8	3.5
Hairy basil-C	1.7	0	0	0	1.7	11.7	0	0	0	1.7	1.7	0	0	0	0	11.7	0	0	0	0
Catnip	18.6	1.7	35.0	0	0	60.0	0	6.7	0	0	72.4	0	3.4	0	0	94.4	18.6	5.1	89.8	0
Catnip-C	1.7	0	0	0	0	8.3	0	0	1.7	0	0	0	0	0	0	8.3	0	0	5.0	0
Vetiver	31.7	0	0	0	0	63.7	0	0	3.3	0	24.1	0	0	0	0	66.0	0	0	3.3	4.9
Vetiver-C	0	0	0	0	0	3.4	0	0	1.7	1.7	0	0	0	0	0	3.4	0	0	0	0

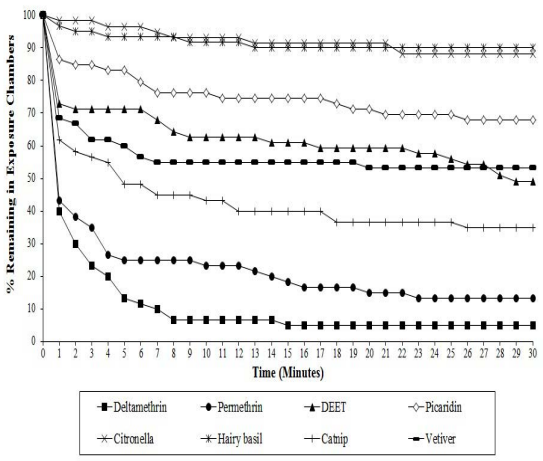
Table 3 Time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of 4 mosquitoes test populations to escape from test chambers treated with deltamethrin, permethrin, DEET, picaridin, citronella, hairy basil, catnip and vetiver

Compound	*Aedes aegypti*			*Aedes albopictus*			*Culex quinquefasciatus*			*Anopheles minimus*		
	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact												
Deltamethrin	2	8	-	<1	<1	2	4	9	-	7	18	-
Permethrin	1	4	-	<1	<1	4	2	7	14	2	6	12
DEET	17	-	-	<1	28	-	2	12	-	1	6	21
Picaridin	-	-	-	10	-	-	-	-	-	-	-	-
Citronella	15	-	-	-	-	-	<1	4	19	5	7	22
Hairy basil	1	29	-	-	-	-	<1	3	8	<1	2	4
Catnip	21	-	-	<1	15	-	<1	3	18	<1	2	4
Vetiver	6	-	-	<1	-	-	<1	2	5	3	5	11
Non-contact												
Deltamethrin	-	-	-	<1	24	-	-	-	-	-	-	-
Permethrin	-	-	-	5	-	-	-	-	-	10	22	-
DEET	-	-	-	<1	6	-	-	-	-	5	16	27
Picaridin	-	-	-	-	-	-	-	-	-	-	-	-
Citronella	26	-	-	-	-	-	<1	6	22	8	10	30
Hairy basil	15	-	-	-	-	-	<1	2	6	<1	1	16
Catnip	-	-	-	1	4	-	1	7	-	<1	2	4
Vetiver	9	-	-	<1	2	-	-	-	-	3	19	-

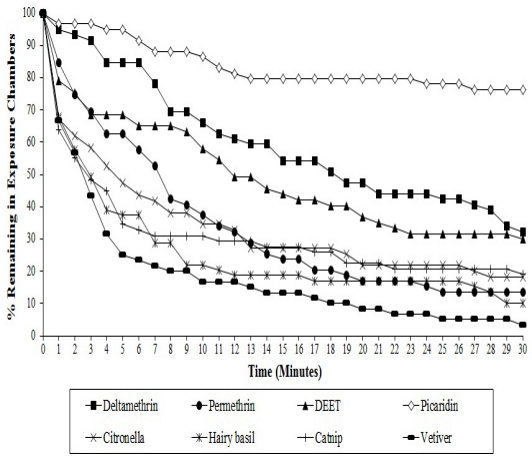
Aedes aegypti



Aedes albopictus



Culex quinquefasciatus



Anopheles minimus

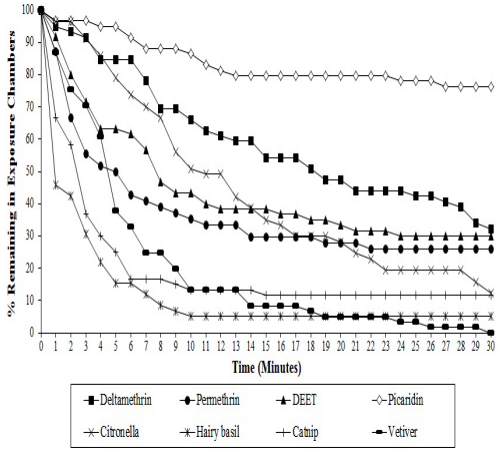
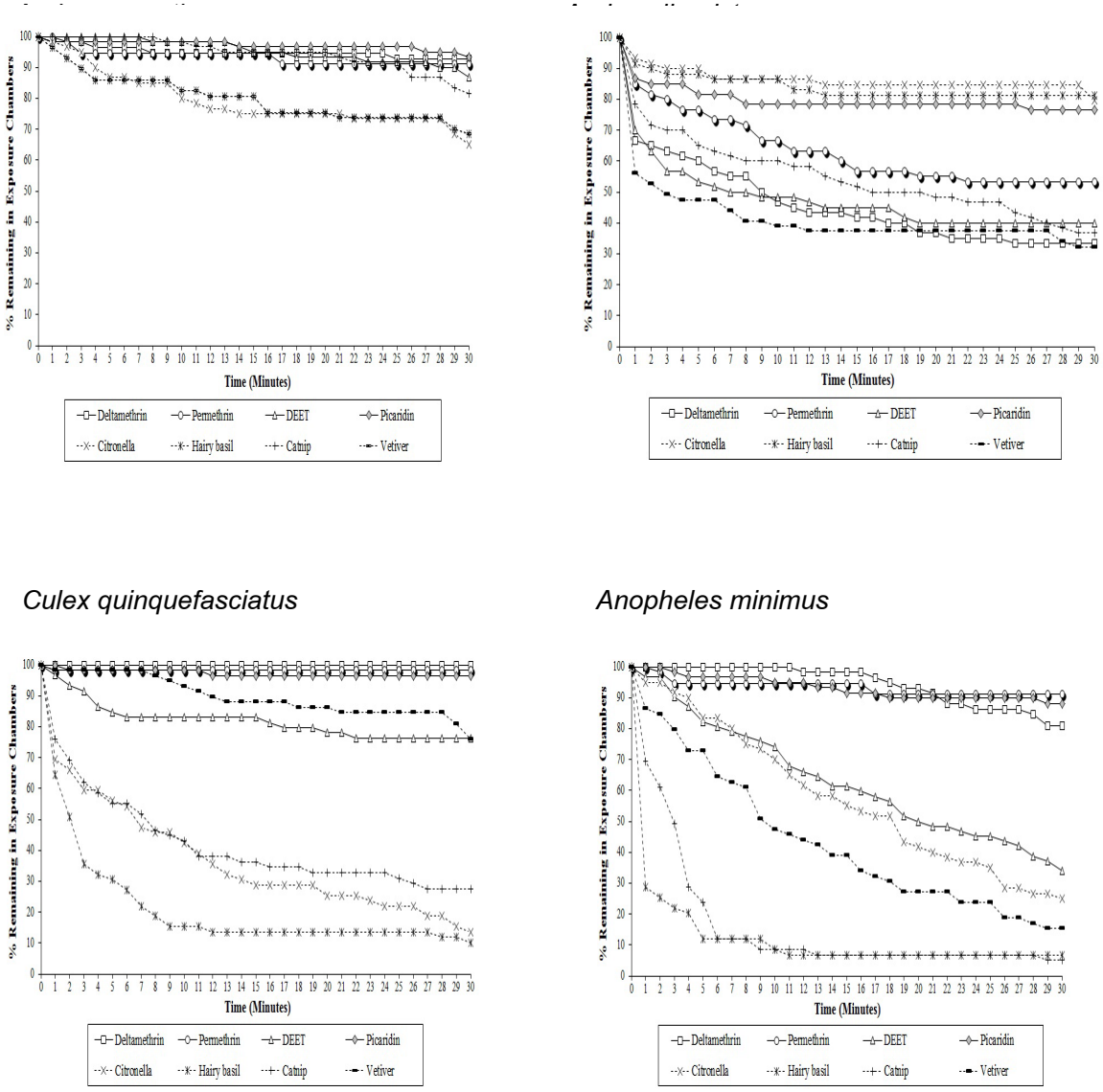


Figure 4 Kaplan-Meier survival curves indicating the percent of 4 mosquito species remaining inside a treated chamber during 30-minute exposure to eight test compounds in contact behavioral assays



Culex quinquefasciatus *Anopheles minimus*

Figure 5 Kaplan-Meier survival curves indicating the percent of 4 mosquito species remaining inside a treated chamber during 30-minute exposure to eight test compounds in non-contact behavioral assays

2. Behavioral responses of *Aedes aegypti* and *Anopheles minimus* to essential oils and synthetic repellents using the high throughput screening system

Contact irritancy assay

Escape response of *Ae. aegypti* and *An. minimus* vary dramatically depending on the compound tested, concentration (0.5-3%), and mosquito species (Tables 4 and 5). Most *Ae. aegypti* data indicated there were significant differences between the treatment and control escape responses, but no significant difference was observed at 0.5% picaridin ($P > 0.05$). All *An. minimus* data showed statistically significant ($P < 0.05$) differences between the treatment and control groups (Figure 6).

Spatial repellency assay

Significant differences ($P<0.05$) in escape responses were found between all treatment and control pairs for catnip, vetiver, and DEET against *Ae. aegypti*. Citronella and hairy basil showed significant differences at 2 and 3% concentrations (Table 6). No significant difference in spatial repellent response was observed at 0.5 and 1% citronella and hairy basil or with 0.5% picaridin. DEET exhibited SAI values between 0.97 to 1 for all concentrations whereas 0.5% picaridin had SAI negative value of -0.33.

Anopheles minimus results indicated no significant difference ($P>0.05$) in 2 and 3% catnip, which also was found in picaridin 0.5% (Tables 7). All concentrations of DEET had SAI values = 1, while picaridin had values of SAI less than 1. Citronella at 1-3% indicated SAI = 1 and hairy basil had SAI values between 0.96 to 1. Catnip showed SAI close to 1 only at 0.5% while 3% catnip produced a negative SAI value (Figure 7).

Toxicity assay

In general, knockdown and mortality rates for both mosquito species increased with higher compound concentrations. Citronella, hairy basil, and catnip produced high mortality rates (Tables 8 and 9). DEET exhibited the highest knockdown and mortality rates with all concentrations for both mosquito species. Vetiver showed toxicity in *An. minimus* with 59-94% mortality but was non-toxic with no mortality in *Ae. aegypti*.

In *Ae. aegypti* 2-3% citronella and catnip showed 100% knockdown and 76-100% mortality. Hairy basil at 2-3% gave high knockdown (98-100%) and mortality rate (99-100%) in *Ae. aegypti*. The results of *An. minimus* indicated that at 1-3% citronella, hairy basil, and catnip displayed between 99-100% knockdown and 82-100% mortality. No toxicity, knockdown or mortality, was detected with picaridin in either mosquito species (Figure 8).

Table 4. Escape response of *Aedes aegypti* in the contact irritancy assay to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Number escaping (mean ± SE)		Percent escaping (mean ± SE)	Mean %KD in the treated metal chamber	*P*-value
				Treated	Control			
Citronella	0.5	6	60	7.2 ± 0.8	0.3 ± 0.2	70.0 ± 6.1	0	0.0022
	1	6	60	6.5 ± 0.6	0.3 ± 0.2	63.3 ± 6.6	0	0.0022
	2	6	60	3.5 ± 0.4	0.3 ± 0.2	54.8 ± 4.9	21.7 ± 11.7	0.0022
	3	6	60	2.3 ± 0.3	0.7 ± 0.2	71.7 ± 4.2	50.0 ± 15.5	0.0022
Hairy basil	0.5	6	60	8.0 ± 0.4	0	80.0 ± 3.7	0	0.0022
	1	6	56	5.8 ± 0.9	0.7 ± 0.2	62.8 ± 7.0	0	0.0022
	2	6	60	3.8 ± 0.7	0.3 ± 0.2	69.4 ± 8.0	31.7 ± 21.4	0.0022
	3	6	60	3.5 ± 0.6	0	65.0 ± 5.0	30.0 ± 17.9	0.0022
Catnip	0.5	6	60	7.3 ± 0.9	0	73.3 ± 8.8	0	0.0022
	1	6	60	8.3 ± 0.4	0	83.3 ± 4.2	0	0.0022
	2	6	59	0.8 ± 0.4	0	100 ± 2.6	90 ± 8.9	0.0022
	3	6	60	0.3 ± 0.2	0.3 ± 0.2	93.0 ± 4.5	90 ± 8.9	0.0022

Table 4. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Number escaping (mean ± SE)		Percent escaping (mean ± SE)	Mean %KD in the treated chamber	*P*-value
				Treated	Control			
Vetiver	0.5	6	60	6.5 ± 0.2	0.3 ± 0.2	63.7 ± 2.5	0	0.0022
	1	6	60	8.2 ± 0.5	0	81.7 ± 4.8	0	0.0022
	2	6	60	7.3 ± 0.3	0.3 ± 0.2	72.4 ± 3.4	0	0.0022
	3	6	60	7.5 ± 0.6	0	75.0 ± 6.2	0	0.0022
DEET	0.5	6	60	4.3 ± 0.8	0.3 ± 0.2	56.9 ± 5.4	15 ± 10.5	0.0022
	1	6	56	6.3 ± 0.8	0	78.3 ± 7.0	15 ± 10.5	0.0022
	2	6	60	4.3 ± 0.4	0.3 ± 0.2	61.9 ± 2.7	20 ± 11.0	0.0022
	3	6	60	4.0 ± 0.9	0	65.0 ± 6.7	25 ± 13.8	0.0022
Picaridin	0.5	6	60	1.0 ± 0.4	0	10.0 ± 4.5	0	0.0606
	1	6	60	3.6 ± 0.6	0.7 ± 0.2	31.7 ± 7.4	0	0.0108
	2	6	59	3.8 ± 0.8	0.7 ± 0.4	33.3 ± 8.6	0	0.0130
	3	6	60	2.2 ± 0.5	0.3 ± 0.2	18.9 ± 5.1	0	0.0130

P-value were from Wilcoxon two-sample test for difference between the number escaping in a treatment and a control chambers

Table 5. Escape response of *Anopheles minimus* in the contact irritancy assay to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Number escaping (mean ± SE)		Percent escaping (mean ± SE)	Mean %KD in the treated metal chamber	*P*-value
				Treated	Control			
Citronella	0.5	6	60	3.5 ± 0.6	0	40.0 ± 4.5	6.7 ± 10.3	0.0022
	1	6	60	4.7 ± 0.5	0.7 ± 0.2	77.0 ± 4.8	31.7 ± 19.4	0.0022
	2	6	60	1.8 ± 0.4	0.3 ± 0.2	86.1 ± 2.3	68.3 ± 11.7	0.0022
	3	6	59	1.2 ± 0.3	0	96.7 ± 3.3	84.8 ± 5.3	0.0022
Hairy basil	0.5	6	60	8.5 ± 0.7	0.3 ± 0.2	84.8 ± 6.7	0	0.0022
	1	6	56	3.3 ± 0.7	0	88.3 ± 3.1	55.0 ± 18.7	0.0022
	2	6	60	2.5 ± 0.7	0.7 ± 0.2	100 ± 3.8	75.0 ± 16.4	0.0022
	3	6	60	2.2 ± 0.5	0.5 ± 0.2	94.4 ± 5.0	73.3 ± 13.7	0.0022
Catnip	0.5	6	60	5.7 ± 0.2	0	83.3 ± 9.2	26.7 ± 20.7	0.0022
	1	6	60	5.7 ± 0.7	0.7 ± 0.2	100 ± 0	43.3 ± 16.3	0.0022
	2	6	60	3.0 ± 0.3	0.7 ± 0.4	100 ± 0	70.0 ± 6.3	0.0022
	3	6	60	3.0 ± 0.7	0.7 ± 0.4	100 ± 0	70.0 ± 16.7	0.0022

Table 5. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Number escaping (mean ± SE)		Percent escaping (mean ± SE)	Mean %KD in the treated chamber	*P*-value
				Treated	Control			
Vetiver	0.5	6	60	6.8 ± 0.4	0.7 ± 0.2	66.3 ± 3.7	0	0.0022
	1	6	60	7.3 ± 0.5	0.7 ± 0.4	71.3 ± 5.1	0	0.0022
	2	6	60	5.7 ± 0.6	0.3 ± 0.2	58.2 ± 6.1	3.3 ± 5.2	0.0022
	3	6	60	5.3 ± 0.7	0	61.7 ± 6.0	8.3 ± 4.1	0.0022
DEET	0.5	6	60	6.0 ± 0.6	0.3 ± 0.2	78.9 ± 7.7	20.0 ± 17.9	0.0022
	1	6	60	4.8 ± 0.5	0.3 ± 0.2	84.3 ± 5.9	36.7 ± 5.2	0.0022
	2	6	60	4.2 ± 0.5	0	81.7 ± 7.5	40.0 ± 17.9	0.0022
	3	6	60	3.8 ± 0.8	0.7 ± 0.2	79.1 ± 5.7	41.7 ± 14.7	0.0022
Picaridin	0.5	6	60	6.0 ± 0.7	0.7 ± 0.4	55.0 ± 9.8	0	0.0022
	1	6	60	5.5 ± 0.9	0.3 ± 0.2	53.5 ± 8.7	0	0.0022
	2	6	60	3.3 ± 0.5	0.3 ± 0.2	30.9 ± 5.1	0	0.0065
	3	6	60	1.8 ± 0.3	0	18.3 ± 3.1	0	0.0022

P-value were from Wilcoxon two-sample test for difference between the number escaping in a treatment and a control chambers

Table 6. Escape response of *Aedes aegypti* in the spatial repellency to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent responding (SE)	Mean SAI[1](SE)	SR[2]	*P* > *S*
Citronella	0.5	9	179	14.6 (2.73)	0.31 (0.28)	7.0	0.3359
	1	9	180	25.0 (1.67)	0.26 (0.18)	11.0	0.2383
	2	9	180	27.2 (5.66)	0.84 (0.08)	22.5	0.0039
	3	9	180	29.4 (4.12)	1.00 (0)	22.5	0.0039
Hairy basil	0.5	9	180	26.7 (5.59)	0.05 (0.23)	0.5	0.9766
	1	9	179	20.8 (4.45)	0.24 (0.24)	5.5	0.4375
	2	9	180	30.0 (3.61)	0.60 (0.23)	12.5	0.0781
	3	9	180	31.7 (5.77)	0.95 (0.03)	22.5	0.0039
Catnip	0.5	9	179	21.8 (3.03)	0.46 (0.16)	15.5	0.0313
	1	9	180	41.7 (4.86)	0.64 (0.15)	20.5	0.0117
	2	9	180	75.6 (5.30)	0.99 (0.01)	22.5	0.0039
	3	9	180	65.0 (5.59)	0.94 (0.05)	22.5	0.0039

Table 6. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent responding (SE)	Mean SAI[1] (SE)	SR[2]	*P* > *S*
Vetiver	0.5	9	179	28.9 (3.89)	0.56 (0.13)	21.5	0.0078
	1	9	180	29.1 (3.36)	0.66 (0.07)	22.5	0.0039
	2	9	180	33.9 (5.26)	0.58 (0.10)	18.0	0.0078
	3	9	180	33.7 (3.81)	0.74 (0.09)	22.5	0.0039
DEET	0.5	9	180	37.8 (7.37)	1.00 (0)	22.5	0.0039
	1	9	179	32.4 (5.05)	1.00 (0)	22.5	0.0039
	2	9	180	28.9 (4.0)	0.97 (0.03)	22.5	0.0039
	3	9	180	28.3 (2.89)	1.00 (0)	22.5	0.0039
Picaridin	0.5	9	179	13.9 (2.61)	- 0.33 (0.24)	- 9.0	0.2578
	1	9	180	28.1 (3.34)	0.36 (0.12)	16.0	0.0313
	2	9	180	32.8 (3.64)	0.35 (0.1)	18.0	0.0078
	3	9	180	27.2 (5.08)	0.32 (0.16)	13.0	0.0781

[1] SAI = spatial activity index [2] SR = signed-rank statistic derived through PROC UNIVARIATE

Table 7. Escape response of *Anopheles minimus* in the spatial repellency to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent responding (SE)	Mean SAI[1] (SE)	SR[2]	*P* > *S*
Citronella	0.5	9	180	10.0 (2.50)	0.89 (0.11)	18.0	0.0078
	1	9	180	42.8 (4.80)	1.00 (0)	22.5	0.0039
	2	9	180	53.3 (1.67)	1.00 (0)	22.5	0.0039
	3	9	180	60.0 (2.36)	1.00 (0)	22.5	0.0039
Hairy basil	0.5	9	180	51.1 (3.71)	0.86 (0.07)	22.5	0.0039
	1	9	180	66.1 (3.71)	0.97 (0.02)	22.5	0.0039
	2	9	180	71.7 (4.25)	0.96 (0.03)	22.5	0.0039
	3	9	180	84.4 (5.56)	1.00 (0)	22.5	0.0039
Catnip	0.5	9	180	73.3 (4.64)	0.96 (0.02)	22.5	0.0039
	1	9	181	55.8 (3.95)	0.45 (0.07)	22.5	0.0039
	2	9	180	62.2 (4.94)	0.26 (0.14)	12.5	0.1641
	3	9	180	68.3 (3.63)	- 0.02 (0.09)	- 2.0	0.7656

Table 7. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent responding (SE)	Mean SAI[1] (SE)	SR[2]	*P* > *S*
Vetiver	0.5	9	180	27.2 (3.55)	0.91 (0.06)	22.5	0.0039
	1	9	180	62.8 (3.02)	0.96 (0.04)	22.5	0.0039
	2	9	180	42.2 (5.34)	0.79 (0.09)	22.5	0.0039
	3	9	180	48.3 (3.22)	0.54 (0.14)	20.5	0.0117
DEET	0.5	9	180	40.6 (6.15)	1.00 (0)	22.5	0.0039
	1	9	180	53.9 (7.67)	1.00 (0)	22.5	0.0039
	2	9	180	63.3 (5.34)	1.00 (0)	22.5	0.0039
	3	9	180	70.6 (5.10)	1.00 (0)	22.5	0.0039
Picaridin	0.5	9	180	29.4 (4.37)	0.38 (0.22)	11.5	0.1250
	1	9	180	38.9 (6.71)	0.53 (0.21)	17.0	0.0430
	2	9	180	45.6 (5.03)	0.76 (0.10)	22.5	0.0039
	3	9	180	46.7 (3.44)	0.88 (0.05)	22.5	0.0039

[1] SAI = spatial activity index [2] SR = signed-rank statistic derived through PROC UNIVARIATE

Table 8. Knockdown and mortality response of *Aedes aegypti* in the toxicity assay to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent 1 h knockdown (mean ± SE)	Mean percent 24 h Mortality (mean ± SE)
Citronella	0.5	6	117	12.5 ± 6.7 B	1.7 ± 1.7 C
	1	6	120	98.3 ± 1.7 A	20.8 ± 9.7 B
	2	6	119	100 ± 0 A	98.3 ± 1.1 A
	3	6	120	100 ± 0 A	100 ± 0 A
Hairy basil	0.5	6	121	1.7 ± 1.1 C	2.5 ± 2.5 B
	1	6	121	8.3 ± 3.8 B	4.2 ± 3.3 B
	2	6	118	98.3 ± 1.1 A	99.2 ± 0.8 A
	3	6	120	100 ± 0 A	100 ± 0 A
Catnip	0.5	6	117	0.8 ± 0.8 B	0 D
	1	6	117	100 ± 1.4 A	9.3 ± 3.6 C
	2	6	119	100 ± 0 A	75.7 ± 3.9 B
	3	6	120	100 ± 0 A	87.5 ± 5.3 A

Table 8. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent 1 h knockdown (mean ± SE)		Mean percent 24 h Mortality (mean ± SE)	
Vetiver	0.5	6	115	4.6 ± 2.2	A	0	A
	1	6	116	4.5 ± 1.8	A	0	A
	2	6	120	5.8 ± 2.0	A	0	A
	3	6	119	10.2 ± 4.8	A	0	A
DEET	0.5	6	118	100 ± 0	A	96.7 ± 2.8	B
	1	6	119	100 ± 0	A	100 ± 0	A
	2	6	118	100 ± 0	A	100 ± 0	A
	3	6	119	100 ± 0	A	100 ± 0	A
Picaridin	0.5	6	119	0	A	0	A
	1	6	118	0	A	0	A
	2	6	119	0	A	0	A
	3	6	120	0	A	0	A

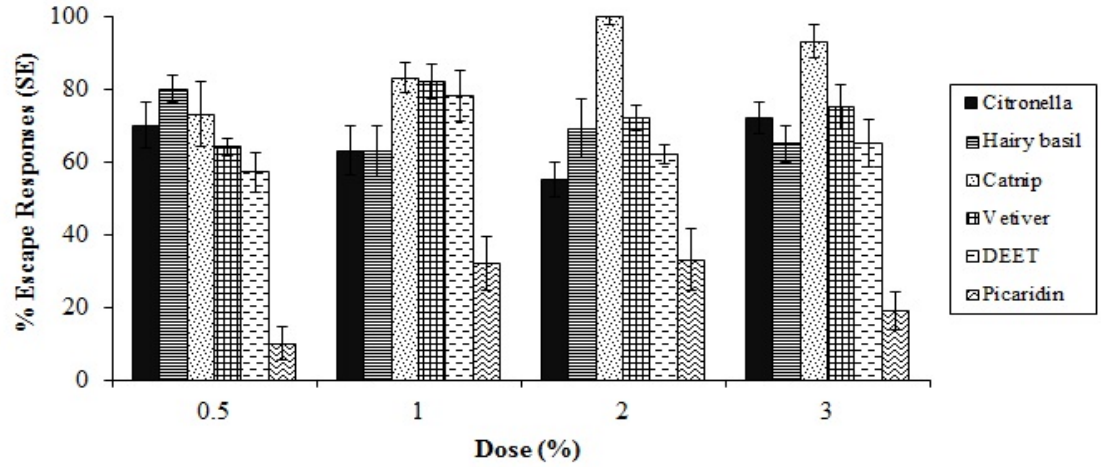
Table 9. Knockdown and mortality response of *Anopheles minimus* in the toxicity assay to citronella, hairy basil, catnip, vetiver, DEET and picaridin

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent 1 h knockdown (mean ± SE)		Mean percent 24 h Mortality (mean ± SE)	
Citronella	0.5	6	117	67.9 ± 9.0	B	31.8 ± 6.5	C
	1	6	116	100 ± 0	A	90.5 ± 4.9	B
	2	6	120	100 ± 0	A	100 ± 0	A
	3	6	116	100 ± 0	A	100 ± 0	A
Hairy basil	0.5	6	118	73.0 ± 8.9	B	14.3 ± 8.8	B
	1	6	116	99.1 ± 0.9	A	82.9 ± 10.8	A
	2	6	119	100 ± 0	A	100 ± 0	A
	3	6	120	100 ± 0	A	100 ± 0	A
Catnip	0.5	6	114	87.0 ± 7.0	B	5.7 ± 2.6	C
	1	6	118	100 ± 0	A	88.1 ± 3.7	B
	2	6	117	100 ± 0	A	96.5 ± 2.2	A
	3	6	119	100 ± 0	A	100 ± 0	A

Table 9. (Continued)

Compound	Dose (%)	No. of replicates	No. of mosquitoes	Mean percent 1 h knockdown (mean ± SE)		Mean percent 24 h Mortality (mean ± SE)	
Vetiver	0.5	6	114	92.9 ± 2.5	B	59.1 ± 9.0	B
	1	6	119	94.4 ± 1.5	B	91.1 ± 4.7	A
	2	6	114	100 ± 0	A	89.7 ± 2.1	A
	3	6	113	99.2 ± 0.8	A	93.7 ± 2.6	A
DEET	0.5	6	114	100 ± 0	A	98.9 ± 1.1	A
	1	6	115	100 ± 0	A	97.3 ± 1.9	A
	2	6	120	100 ± 0	A	100 ± 0	A
	3	6	119	100 ± 0	A	100 ± 0	A
Picaridin	0.5	6	119	0	A	0	A
	1	6	119	0	A	0	A
	2	6	117	0	A	0	A
	3	6	117	0	A	0	A

Aedes aegypti



Anopheles minimus

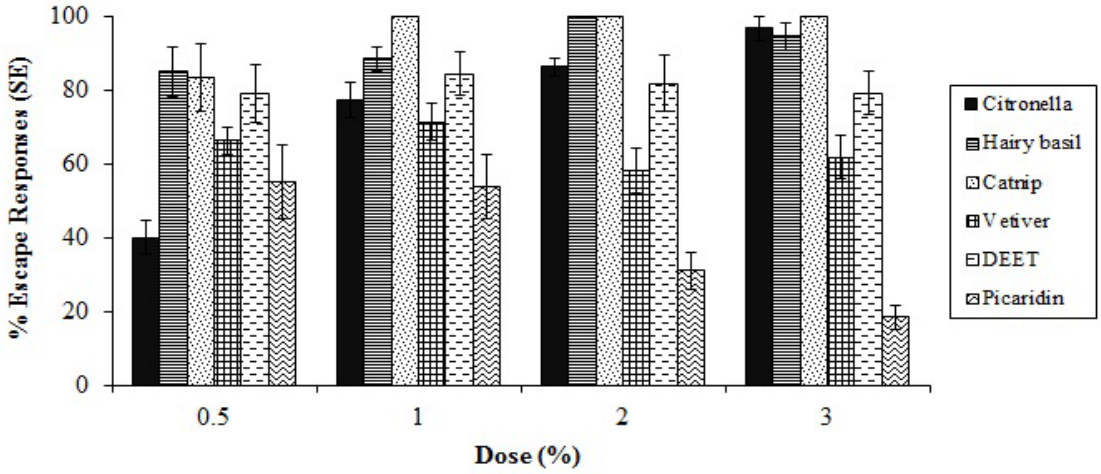
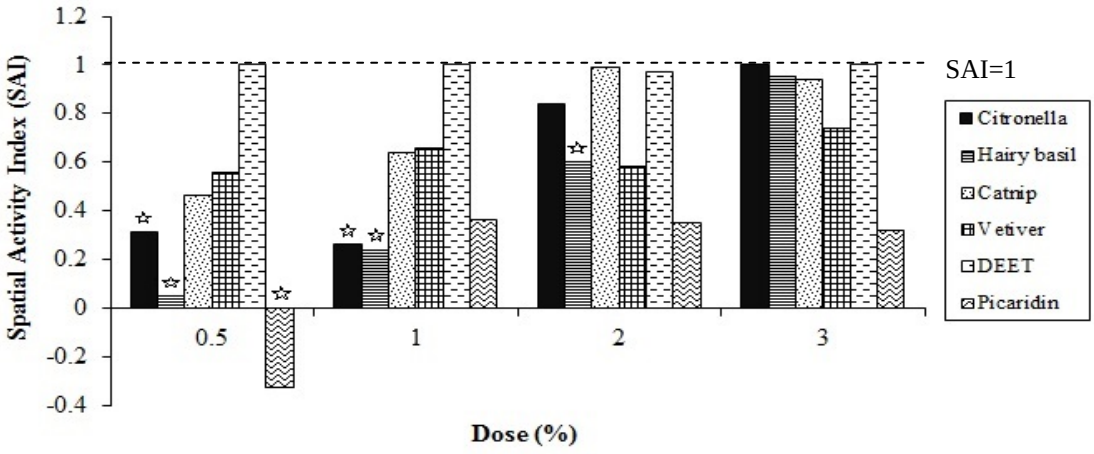


Figure 6 Contact irritant responses (CIA) with S.E. of *Aedes aegypti* and *Anopheles minimus* laboratory colonies exposed to four concentrations of citronella, hairybasil, catnip, vetiver and DEET and picaridin

Aedes aegypti



Anopheles minimus

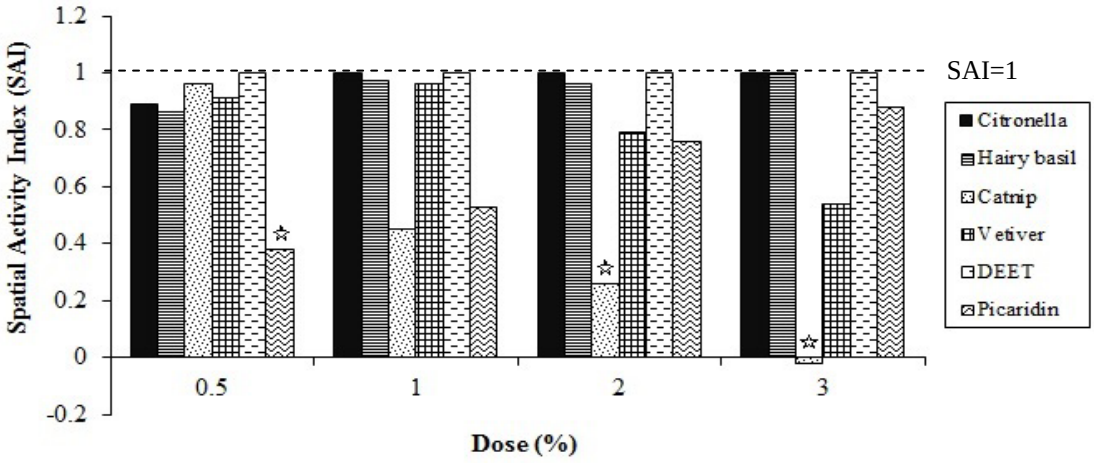
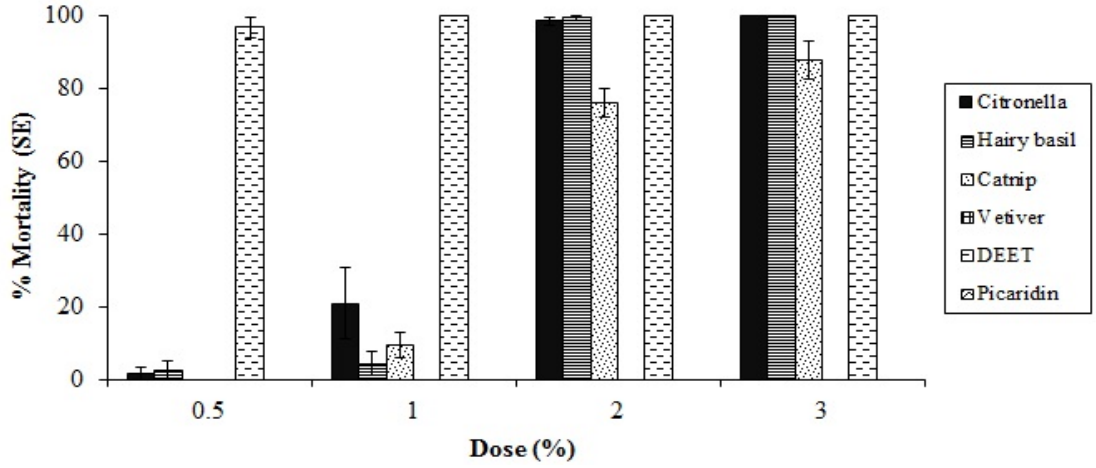


Figure 7 Spatial repellent responses (SRA) of *Aedes aegypti* and *Anopheles minimus* laboratory colonies exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET and picaridin

☆ Denoted not statistically significant (*P*> 0.05) repellent response compared between treatment and control

Aedes aegypti



Anopheles minimus

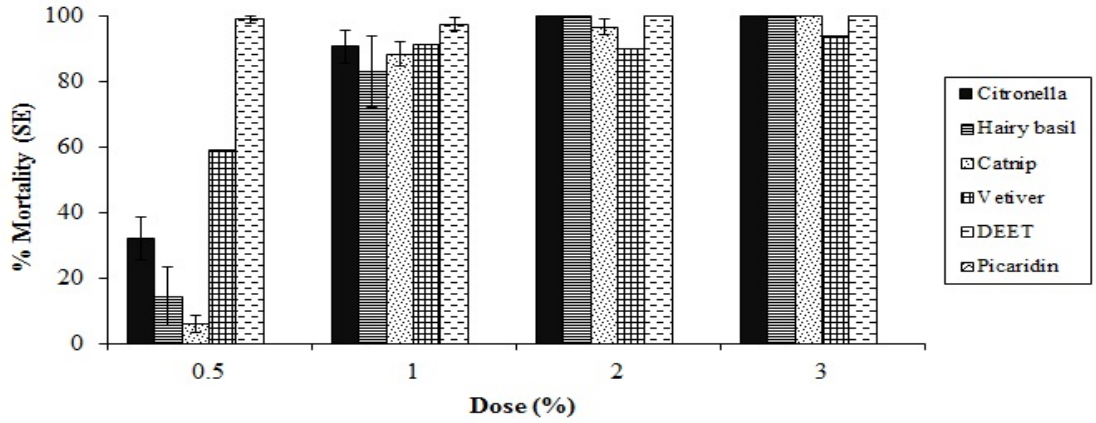


Figure 8 Percentage of 24-h mortality (TOX) with S.E. (Standard error) of *Aedes aegypti* and *Anopheles minimus* laboratory strains exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET and picaridin

As a result of the current study, citronella, hairy basil, catnip and vetiver essential oils could be served as potential mosquito repellent products against all four mosquito species tested. Further testing with the higher concentrations of all essential oils should be performed to find an effective repellent dose against *Ae. aegypti*. According to Phasomkusolsil and Soonwera (2011), increased concentration of essential oil can increase the repellent activity. Due to insecticide resistance phenotypes in many important vector species will continue to pressure the scienctific

community to develop new and improved vector control strategies. Thus combination approaches are a good way to ensure the beneficial qualities of the various tools implemented (Gratz 1993; Yap et al. 1994; Lee et al. 2010). Essential oils from plants are one such area for exploration. However, development of a natural repellent formulation with long lasting protection is necessary to be comparable to the effectiveness of the standard topical repellent DEET. The irritant and/or repellent effects of test compounds described here must be validated with tests on field mosquitoes for confirmation.

Discussion

Understanding of mechanistic actions of four plant essential oils, citronella oil, hairy basil oil, catnip oil and vetiver oil against mosquitoes is comepletely needed. The two test systems, high throughput screening (HITSS) and excito-repellency (ER) test systems are the appropriate designed for further study of behavioral response patterns in mosquitoes to volatile compounds. The HITSS was applied to evaluate through three behavioral modes of action (contact irritant, spatial repellent and toxic) of four plant essential oils on *Ae. aegypti* and *An. minimus*. While the excito-repellency test system was used in the testing of contact irritant action and non-contact repellent action of the plant essential oils against four mosquito species, *Ae. aegypti*, *Ae. albopictus*, *An. minimus* and *Cx. quinquefasciatus*. As a result, the behavioral responses of mosquitoes to four plant essential oils indicated that some plant essential oils had spatial repellent, contact irritant and/or toxic properties but some of these plant compounds performed better repellent than irritant to test mosquitoes. The study also found that each test mosquito species responded differently in escape patterns to the various test compounds. Therefore, the plant efficacy against mosquitoes depends on the type, chemical composition and concentration of the essential oils as well as the species of mosquito used in the test.

The recent study also found that the high throughput screening system and excito-repellency test system are useful tools to perform screening of plants for repellent activity. The high throughput screening system is suitable for working in the laboratory where temperature and humidity operating can be controlled because the materials are affected by high temperature (above 26 $^{\circ}$C). This system is required a small amount of test compound and each assay of the

HITSS is attained within a short time. In contrast, excito-repellency test system can be applied in both the laboratory and field experiments due to the whole tool set can be carried in convenient bags and materials are not affected by surrounding temperature. These two systems are effective tools to help evaluate the potential of plant essential oils or extracts as natural mosquito repellents. This will lead to developed formulations of plant based repellents with long-lasting mosquito protection.

The study provides supportive data on the mosquito-repellent property of plants against *Ae. aegypti*, *Ae. albopictus*, *An. minimus* and *Cx. quinquefasciatus*. This will be useful with the screening of plants for mosquito repellents. Testing under laboratory conditions found that *Cx. quinquefasciatus* and *An. minimus* were great responses to citronella, hairy basil, catnip, and vetiver oils by using excito-repellency system while *Ae. aegypti* and *An. minimus* showed high irritant responses to all the essential oils tested using high throughput screening system.

Recommendations for Further Study

The data that obtained from these laboratory tests should be confirmed in the field study to further support the effectiveness of the tested compounds because human and environmental factors could impact repellent efficacy.

The Output of this Subproject

The four essential oils of citronella, hairy basil, catnip, and vetiver have been proven to be effective in mosquito repellents, these have also been formulated as lotion, spray, balm, and liquid balm by the mosquito lab, Department of Entomology, Faculty of Agriculture, Kasetsart University. These mosquito repellent products containing new effective plant-based ingredients (hairy basil, catnip, vetiver) that will be alternative to the citronella-based products.

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Subproject 2: A comparison of attractants for sampling Stomoxys calcitrans (Diptera: Muscidae) on dairy farms in Saraburi province, Thailand (Dr.Jumnongjit Phasuk)

Abstract

The efficacy of different stable fly attractants was evaluated at four dairy cattle farms in Muak Lek district, Saraburi province, Thailand. Dry ice, octenol, a mixture of cow dung and urine, a combination of dry ice plus octenol and no attractants (control) were tested with Vovoua traps. A total of 7,000 individuals of *Stomoxys* species were collected during July 2013 to September 2014 of which 1,058 were trapped on farm 1,867 on farm 2, 1,274 on farm 3 and 3,801 on farm 4. Four species of *Stomoxys* were identified: *Stomoxys bengalensis* Picard, 1908, *Stomoxys calcitrans* (Linnaeus, 1758), *Stomoxys indicus* Picard, 1908 and *Stomoxys sitiens* Rondani, 1873. *S. calcitrans* was the predominant species comprising 99% of all samples collected. The number of male and female *S. calcitrans* collected differed significantly by attractant type. Significantly more *S. calcitrans* were attracted to dry ice or a combination of dry ice plus octenol-baited traps than to unbaited or octenol-baited traps. The Vavoua traps baited with dry ice alone or a combination of dry ice plus octenol were effective attractants for *S. calcitrans*.

Keywords: Attractants, dry ice, octenol, stable flies, *Stomoxys calcitrans*, Thailand

Introduction

Stable flies (*Stomoxys* spp.) belong to the subfamily Stomoxyinae in the family Muscidae (Diptera). Among the 18 *Stomoxys* species described, six species are recorded from Thailand, of which one is cosmopolitan, *Stomoxys calcitrans* (Linnaeus, 1758) (Zumpt, 1973, Tumrasvin and Shinonaga, 1978). Stable flies are important and widely distributed insect pests of livestock, wildlife and sometimes humans. Adult stable flies of both sexes are blood-sucking flies and cause painful bites and significant blood loss in some animals. High populations of biting activity can reduce animal productivity by reducing weight gain and milk production (Bruce and Decker, 1958, Campbell et al., 1987, Wieman et al., 1992, Catangui et al., 1997, Campbell et al., 2001). Stable flies may act as both mechanical and biological vectors of disease agents, including viruses, bacteria, protozoa and helminthes (Baldacchino et al., 2013) such as equine infectious anemia virus (EIAV) (Foil et al., 1983), trypanosomes *Trypanosoma evansi* and *Trypanosoma congolense* (Sumba et al., 1998) and the nematodes *Habronema microstoma* and *Habronema muscae* (Traversa et al., 2008).

Concern for public and animal health have been considered recently about chemicals for insect control with the development of resistance, food safety and the safety of chemicals to non-target organisms, including humans and the environment. To reduce risks from insecticides, insect attractants and traps offer effective alternatives for use in insect management.

Many types of traps have been developed for the survey and control of stable flies (Taylor and Berkebile, 2006, Gilles et al., 2007). Two types of Alsynite sticky traps (Williams trap and Broce trap) have been used for sampling stable flies (William 1973, Broce 1988). However, one disadvantage of these traps, is that they also catch many non-target insects (Gilles et al., 2007). The Vavoua trap (Laveissiere and Grebaut, 1990) is designed for tsetse flies and is used to sample *Stomoxys* spp. in many African countries and this trap is the best choice to use for stable fly control (Gilles et al., 2007).

Besides traps, attractants can be used in traps to reduce pest insect populations. The use of octenol (a component of cow breath) and CO_2 as attractants increases the trapping efficacy for

stable fly control and flight activity (Mihok et al., 1995, Schofield and Brady, 1997, Schofield et al., 1997). Both traps and attractants do not injure animals or humans and do not leave harmful residues on foodstuffs or feed. They are relatively safe to use, inexpensive and effective.

However, little is known about the efficacy of different attractants for collecting stable flies in Thailand. The present study compared attractants for *S. calcitrans* on dairy farms in Saraburi province, Thailand. The information obtained in this study will be benefit for fly control programs by improving their effectiveness.

Materials and Methods

Study sites

Four dairy farms were randomly selected in Muak Lek district, Saraburi province. Saraburi is located in the central highlands of Thailand, an area of relatively high plains and plateaus about 140 km northeast of Bangkok. Muak Lek located in the east of the province is the largest district and has the largest number of dairy farms. Thailand has three seasons: the rainy season from June to October, the dry cool season from November to February and the dry hot season from March to May (Youthao et al. 2007). Farm 1 (14° 45.084' N, 101° 14.592' E; 219 m a.s.l) had 113 cows in milk production, farm 2 (14° 45.156' N, 101° 14.336' E; 202 m a.s.l) had 30 cows, farm 3 (14° 47.319' N, 101° 17.290' E; 473 m a.s.l) had 77 cows and farm 4 (14° 47.487' N, 101° 17.278' E; 467 m a.s.l) had 39 cows.

Specimen collection

At each farm, stable flies were collected using five Vavoua traps (Laveissière and Grébaut, 1990). The study was carried out over 2 years (2013–2014) during two rainy seasons. In 2013, the experiment was conducted from July to October on farms 1 and 2 and in 2014 from June to September on farms 3 and 4. All trapping experiments were operated for five weeks. Each trapping week was performed over two consecutive days. The daily trapping period was between 10 a.m. and 4 p.m. The five traps were randomly installed about 1 m from the cattle pen and at least 10 m from one another. The traps were placed at 30 cm above the ground. After trapping, flies in the Vavoua traps were killed using ethanol spray. All collected flies were

preserved in 80% ethanol. The specimens were brought back to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Thailand for species identification using the key of Tumrasvin and Shinonaga (1978).

Attractants

The five treatments consisted of control (no attractant), 10 kg of dry ice (CO_2 source) only, octenol only, a mixture of cow dung and urine and a combination of 10 kg of dry ice plus octenol (Figure 1-4). The octenol lures (Envirosafe Technologies NZ Ltd., Ottawa, ON, New Zealand) contained octenol (1-octen-3-ol, 3.74 g per strip). The octenol strips were hung and left on traps for two consecutive days during each collection period. The mixture of cow dung and urine was collected during the morning from cattle pens at the farm before traps were baited. The mixture of cow dung and urine inside a black plastic container was placed on the ground under the traps and replaced each day. Dry ice inside a poly styrofoam box was placed on the ground under the traps for two consecutive days. To eliminate any position-specific effect, all attractant treatments were rotated to the next trap position each week.

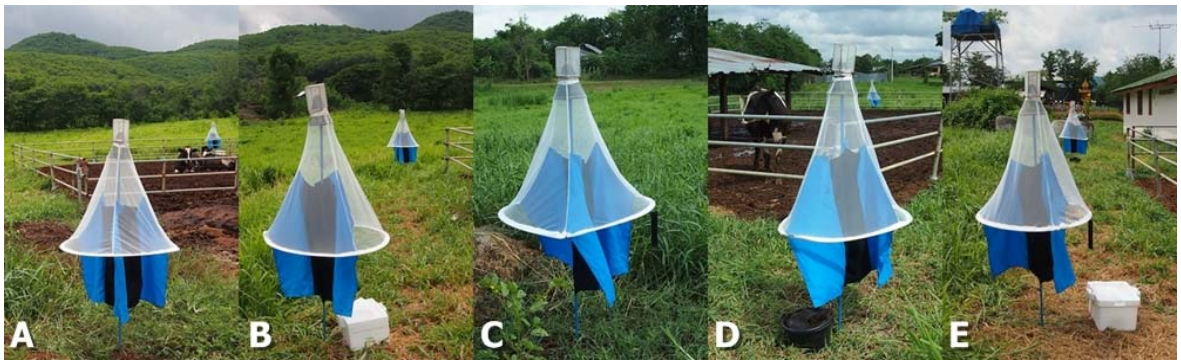


Figure 1 Vavoua traps with attractants used for collecting *Stomoxys* species on farm 1. (A) control (no attractant); (B) dry ice; (C) octenol; (D) a mixture of cow dung and urine; (E) octenol + dry ice.

Data analysis

Pearson chi-square analysis was used to evaluate the differences in numbers of males and females attracted to different attractant types at each farm. A one-way analysis of variance (ANOVA) was used to evaluate the treatment effects on the numbers of each sex of stable flies collected at each farm. Data on the number of stable flies collected were square-root transformed before analysis. When the ANOVA was significant, variations in the numbers of each sex among treatment types were separated using the least significant difference (LSD) *post hoc* analysis. The data were analyzed using SPSS (Version 17, SPSS Inc., Chicago, IL, USA). All statistical significance levels were set at $P < 0.05$.

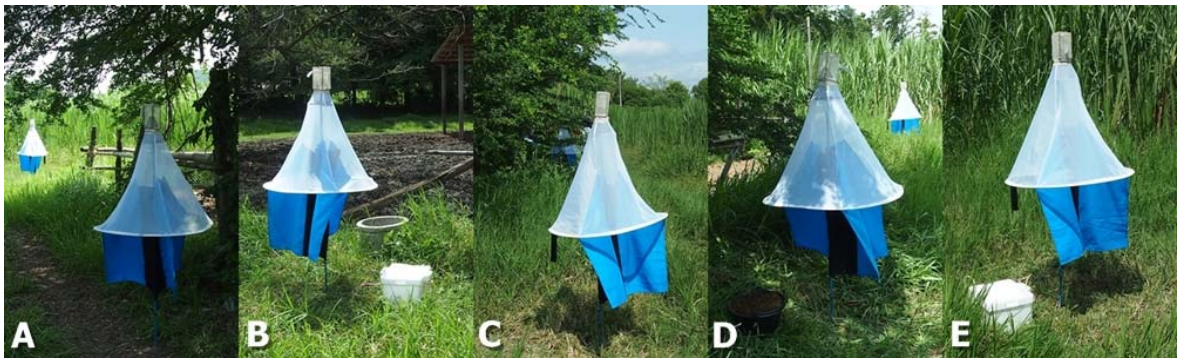


Figure 2 Vavoua traps with attractants used for collecting *Stomoxys* species on farm 2. (A) control (no attractant); (B) dry ice; (C) octenol; (D) a mixture of cow dung and urine; (E) octenol + dry ice.

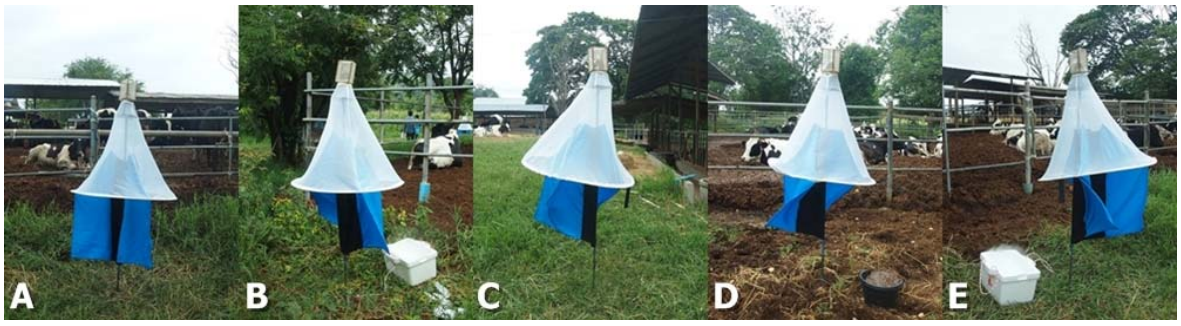


Figure 3 Vavoua traps with attractants used for collecting *Stomoxys* species on farm 3. (A) control (no attractant); (B) dry ice; (C) octenol; (D) a mixture of cow dung and urine; (E) octenol + dry ice.

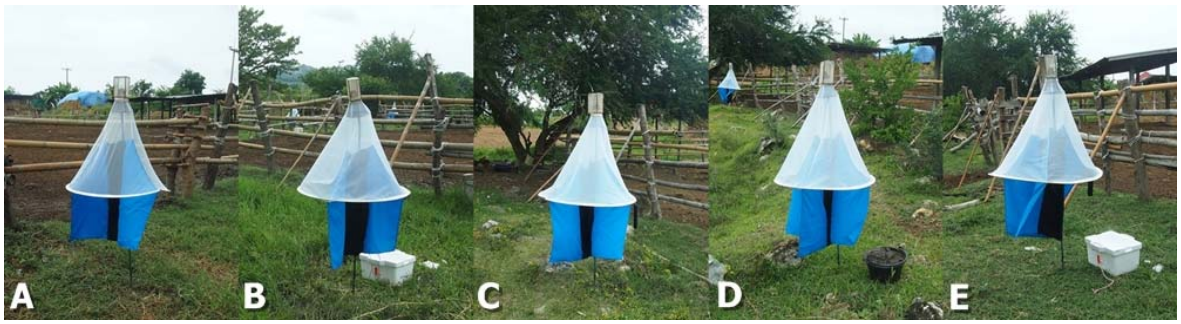


Figure 4 Vavoua traps with attractants used for collecting *Stomoxys* species on farm 4. (A) control (no attractant); (B) dry ice; (C) octenol; (D) a mixture of cow dung and urine; (E) octenol + dry ice.

Results

A total of 7,000 individuals of *Stomoxys* species were collected during July 2013 to September 2014 of which 1,058 were trapped on farm 1, 867 on farm 2, 1,274 on farm 3 and 3,801 on farm 4 (Tables 1 and 2). The following four species of *Stomoxys* were captured: *Stomoxys bengalensis* Picard, 1908, *Stomoxys calcitrans* (Linnaeus, 1758), *Stomoxys indicus* Picard, 1908 and *Stomoxys sitiens* Rondani, 1873. The most predominant species was *S. calcitrans* comprising 99% of the total collected. For the remaining 3 species were less abundant (0.01-0.8%) and were too low for statistical analysis.

Pearson chi-square analysis showed that differences between the numbers of male and female *S. calcitrans* collected were influenced by attractant types (farm 1: χ^2 = 22.75, df = 4, $P < 0.05$, farm 2: χ^2 = 63.19, df = 4, $P < 0.05$, farm 3: χ^2 = 15.89, df = 4, $P < 0.05$, farm 4: χ^2 = 50.02, df = 4, $P < 0.05$). All attractants captured more males than females on farms 2, 3 and 4. However, the number of captures between males and females varied with different attractants on farm 1, with dry ice, a mixture of cow dung and urine and a combination of dry ice plus octenol capturing more females while the control or octenol captured more males (Figure 5).

On farm 1, the number of *S. calcitrans* males was not significantly different among treatments. (F = 1.66, df = 4, 45, P = 0.176). However, traps baited with only dry ice captured the highest number of males. The number of females was significantly different among treatments (F = 2.87, df = 4, 45, P = 0.034). A higher number of females was captured in traps baited with dry

ice compared with octenol, a mixture of cow dung and urine or the control, but traps baited with a combination of dry ice plus octenol did not capture significantly different numbers of females than either the dry ice, octenol, a mixture of cow dung and urine or the control (Table 3).

On farm 2, there were no significant differences between the numbers of both males and females among treatments (F = 0.76, df = 4, 45, *P* = 0.557, F = 2.09, df = 4, 45, *P* = 0.098), respectively. However, the highest numbers of both males and females were captured in traps baited with dry ice alone (Table 3).

On farm 3, there were no significant differences between the numbers of both males and females among treatments (F = 0.42, df = 4, 42, *P* = 0.791, F = 1.50, df = 4, 42, *P* = 0.220), respectively. However, the highest number of both males and females were captured in traps baited with dry ice alone (Table 4).

On farm 4, there was a significant difference between the numbers of both males and females among treatments (F = 5.49, df = 4, 43, *P* = 0.001, F = 13.81, df = 4, 43, *P* = 0.000), respectively. A higher number of males was captured in traps baited with a combination of dry ice plus octenol when compared with octenol, a mixture of cow dung and urine or the control but the numbers of males captured using traps baited with dry ice was not significantly different to either a combination of dry ice plus octenol or the control and there were no differences in the numbers of males captured in traps baited with octenol, a mixture of cow dung and urine or the control. A higher number of females were captured in traps baited with a combination of dry ice plus octenol than those baited with dry ice, octenol, a mixture of cow dung and urine or the control. However, traps baited with dry ice captured significantly more females when compared with octenol, a mixture of cow dung and urine or the control but there were no differences in the numbers of males captured in traps baited with octenol, a mixture of cow dung and urine or the control. The highest numbers of both males and females were captured in traps baited with dry ice plus octenol (Table 4).

Discussion

Of the four species of *Stomoxys* collected, *S. calcitrans* was the most abundant species on the dairy cattle farms. This finding is also in agreement with previous reports (Masmeatathip et al. 2006, Muenworn et al. 2010, Phasuk et al. 2013). Moreover, this species was present in large numbers at different zoos in Thailand (Tanasak et al. 2012). In the current study, there was a significant difference in the numbers of captures between *S. calcitrans* males and females with the different attractants. Males were reported to be more attracted to Vavoua traps than females (Masmeatathip et al. 2006, Muenworn et al. 2010, Phasuk et al. 2013). Those findings were similar to the results from the present study. Males are responding to the baited traps as both assembly sites and waiting stations for mating with females or potential hosts (Beresford and Sutcliffe, 2008). However, the number of captures between males and females varied with different attractants on farm 1. The situation of this farm (close to a mountain and without any trees around the cattle pen) differed from the other farms. This suggested that trees in the feedlot environment provide favorable resting habitats for adult stable flies in the shade where they can digest blood (Guo et al. 1998). Further studies are needed to evaluate whether these differences are associated with the environment around the trap or other factors that influence the number of males and females captured.

The Vavoua traps baited with the CO_2 alone or a combination of CO_2 plus octenol, collected significantly more *S. calcitrans* compared with octenol alone or no attractant. For example, CO_2 from dry ice has been reported as an effective attractant for increasing adult stable fly abundance on various traps (DeFoliart and Morris 1967, Hoy 1970, Cilek 1999). Generally, in the present study, there were no significant differences among trap collections when the combination of CO_2 plus octenol was compared with CO_2 alone. Cilek (1999) reported similar results from Alsynite cylinder traps. The present study showed traps baited with octenol caught more *S. calcitrans* than control traps but the difference was not significant. Similar findings were reported from *S. calcitrans* collections with Alsynite cylinders baited with octenol compared with no odor (control) in North Florida (Cilek 1999) and in California, USA, although cylindrical blue cloth targets were used (Mullens et al. 1995). In Ontario, Canada, octenol increased Nzi trap catches of *S. calcitrans* at a residence in the absence of livestock, but did not increase catches

at a dairy farm with cattle present at or near trap sites (Mihok et al. 2007). Broce and Haas (1999) reported that aged cattle manure was shown to attract stable flies, especially gravid females searching for oviposition sites. The abundance of stable flies increased significantly in grasslands or crop fields when the odor associated with cattle manure slurry was applied (Tangtrakulwanich et al. 2015). In contrast to these results, traps baited with a mixture of cow dung and urine produced no significant difference in capturing *S. calcitrans* when compared with no attractant. These baited traps were located in close proximity to cattle pens. This result suggested that the effects of manure odors from cattle pens may have masked the attractiveness of the traps baited with a mixture of cow dung and urine. The efficacy of the natural attractants is still very poorly known for collecting stable flies in Thailand. Further studies are needed.

In conclusion, the Vavoua traps baited with only CO_2 from the dry ice or a combination of CO_2 plus octenol were effective attractants for collecting *S. calcitrans* and also the baited traps should be placed in sunny locations away from bushes, trees and cattle. However, both dry ice and octenol can be costly and inconvenient to use in remote areas where dry ice is not available. Additionally, octenol is difficult to obtain and so it may not be justified for use in combination with dry ice for routine stable fly sampling or large-scale trap out programs (Cilek 1999).

Table 1 Total numbers of *Stomoxys* spp. collected for each attractant treatment on two dairy farms, Muak Lek district, Saraburi province from July to October 2013.

		Farm 1					
Species	Sex	Control	Dry ice	Octenol	Cow dung and urine	Octenol + dry ice	Total
S. bengalensis Picard, 1908	Male	0	0	0	0	0	0
	Female	0	0	0	0	1	1
S. calcitrans (Linnaeus, 1758)	Male	59	148	77	43	127	454
	Female	48	242	56	47	178	571
S. indicus Picard, 1908	Male	0	4	0	0	3	7
	Female	0	6	3	1	3	13
S. sitiens Rondani, 1873	Male	1	3	2	0	0	6
	Female	0	4	1	0	1	6
Total		108	407	139	91	313	1,058
		Farm 2					
Species	Sex	Control	Dry ice	Octenol	Cow dung and urine	Octenol + dry ice	Total
S. calcitrans (Linnaeus, 1758)	Male	75	167	82	74	162	560
	Female	8	122	18	14	117	279
S. indicus Picard, 1908	Male	0	1	0	0	2	3
	Female	0	0	1	1	0	2
S. sitiens Rondani, 1873	Male	2	2	1	0	6	11
	Female	1	7	0	0	4	12
Total		86	299	102	89	291	867

Table 2 Total numbers of *Stomoxys* spp. collected for each attractant treatment on two dairy farms, Muak Lek district, Saraburi province from June to September 2014.

		Farm 3					
Species	Sex	Control	Dry ice	Octenol	Cow dung and urine	Octenol + dry ice	Total
S. calcitrans (Linnaeus, 1758)	Male	137	238	144	176	110	805
	Female	46	136	78	106	88	454
S. indicus Picard, 1908	Male	0	0	0	0	0	0
	Female	1	1	2	0	2	6
S. sitiens Rondani, 1873	Male	1	2	0	0	0	3
	Female	0	2	3	1	0	6
Total		185	379	227	283	200	1274
		Farm 4					
Species	Sex	Control	Dry ice	Octenol	Cow dung and urine	Octenol + dry ice	Total
S. calcitrans (Linnaeus, 1758)	Male	350	584	325	240	711	2210
	Female	166	427	224	118	645	1580
S. indicus Picard, 1908	Male	0	0	0	0	0	0
	Female	0	0	1	0	0	1
S. sitiens Rondani, 1873	Male	1	0	1	2	0	4
	Female	1	2	2	0	1	6
Total		518	1013	553	360	1357	3801

Table 3 Mean number (±SE) of *Stomoxys calcitrans* males and females collected by each attractant treatment in Muak Lek district, Saraburi province during July to October 2013.

Attractant	Farm 1		Farm 2	
	Male	Female	Male	Female
Control	5.9 ± 2.82 a	4.8 ± 1.93 b	7.5 ± 3.39 a	0.8 ± 0.29 a
Dry ice	14.8 ± 5.05 a	24.2 ± 9.67 a	16.7 ± 7.4 a	12.2 ± 6.91 a
Octenol	7.7 ± 2.61 a	5.6 ± 2.1 b	8.2 ± 4.28 a	1.8 ± 0.47 a
Cow dung and urine	4.3 ± 1.39 a	4.7 ± 1.8 b	7.4 ± 3.3 a	1.4 ± 0.6 a
Dry ice + octenol	12.7 ± 5.2 a	17.8 ± 7.28 ab	16.2 ± 7.84 a	11.7 ± 6.54 a
ANOVA df = 4, 45	$F = 1.66$	$F = 2.87$	$F = 0.76$	$F = 2.09$
	$P = 0.176$	$P < 0.05$	$P = 0.557$	$P = 0.098$

Column means followed by the same letter in each column are not significantly different, LSD test, $P < 0.05$. Untransformed data shown.

Table 4 Mean number (±SE) of *Stomoxys calcitrans* males and females collected by each attractant treatment in Muak Lek district, Saraburi province during June to September 2014.

Attractant	Farm 3**		Farm 4*	
	Male	Female	Male	Female
Control	13.7 ± 5.41 a	4.6 ± 1.22 a	38.89 ± 10.37 bc	18.44 ± 4.22 c
Dry ice	23.8 ± 8.57 a	13.6 ± 4.09 a	58.4 ± 11.03 ab	42.7 ± 7.37 b
Octenol	14.4 ± 4.4 a	7.8 ± 1.9 a	32.5 ± 5.72 c	22.4 ± 5.99 c
Cow dung and urine	19.56 ± 8.47 a	11.78 ± 4.18 a	24 ± 2.64 c	11.8 ± 1.19 c
Dry ice + octenol	13.75 ± 3.16 a	11 ± 2.12 a	79 ± 16.04 a	71.67 ± 11.6 a
ANOVA df = 4, 43*	$F = 0.42$	$F = 1.50$	$F = 5.49$	$F = 13.81$
ANOVA df = 4, 42**	$P = 0.791$	$P = 0.220$	$P < 0.05$	$P < 0.05$

Column means followed by the same letter in each column are not significantly different, LSD test, $P < 0.05$. Untransformed data shown. *, ** There were missing data during the experiment on farm 4 resuling in the following sample number: control (n=9) and dry ice + octenol (n=9) and on farm 3 for cow dung and urine (n=9) and dry ice + octenol (n=8), respectively.

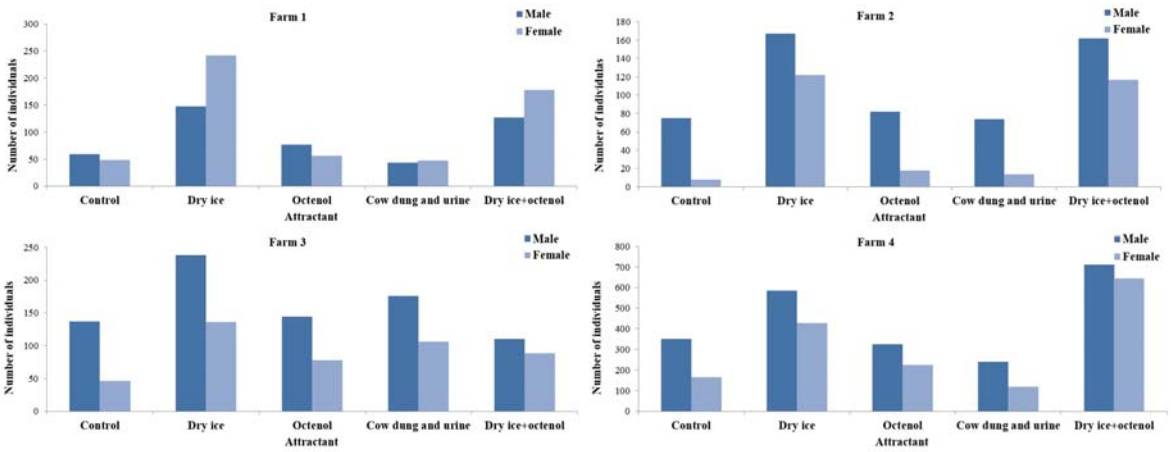


Figure 5 Total numbers of *Stomoxys calcitrans* males and females collected by Vavoua traps baited with different attractants on four dairy farms.

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Output

The output of this project is background information of the efficiency of attractants for *Stomoxys* species in Thailand. The use of the CO2 alone or a combination of CO2 plus octenol significantly increased the Vavoua trap catch of *S. calcitrans* than when unbaited trap was used. Attractant-baited trap is recommended for the use in monitoring or control efforts directed against this blood-sucking fly. The results can help farmers in understanding and making appropriate use of available attractant.

Subproject 3: Biology, ecology and epidemic importance of Stomoxys flies in Thailand (Suprada Sukhonthabhirom)

ABSTRACT

Four species of Stomoxyine flies were morphologically identification in this area, including *Stomoxys calcitrans* was found to be the most abounded species (79.19%), *S. indicus* was found as a second species (19.5%), *S. sitiens* (0.75%) and *S. uruma* (0.56%). It showed also that both species had their seasonal peak of abundance in August. The number of stable flies collected differed among different collection sites, with greater numbers from dairy farms (8,077 specimens) than the open zoo (1,276 specimens). Peak of daily flight activity of Stomoxyine flies was at 16.00-18.00 h, in two localities. *S. calcitrans* was the most abundant species, *S. indicus* was the second species most abundant, *S. sitiens* and *S. uruma* were very rare. Higher numbers of stomoxyine flies collected at the beef farm than the open zoo because the beef farm has a higher host density for blood feeding adults and more suitable habitat for larval development.

Keywords: Biology, Ecology, Stable flies, Trap

Introduction

The genus Stomoxys contains 18 species (Zumpt, 1973). They are blood sucking insects and considered as pests of livestock and other warm-blooded animals in many parts of the world (Zumpt, 1973; Mullens et al., 1988). Among these 18 species, 17 have an African and/or Asian distribution and only one is cosmopolitan: *S. calcitrans* (L. 1758), and known as stable fly. In addition *to S. calcitrans*, several other Stomoxyine flies can also attack domestic animals, including *S. niger, S. sitiens and S. indicus* (Freitas et al., 1984; Wall and Shearer, 1997). Both male and female stable flies feed on blood, generally once a day per fly. These insects are serious pests of humans, wild animals worldwide and they are also important pests of livestock (Kunz et al., 1991). The majority of stable flies prefer to feed on the lower side of large animals such as cattle, horses, pigs, sheep and donkeys (Dougherty et al., 1994). Furthermore, some Stomoxys species are known to obtain blood meals from human sources when cattle or other animal hosts are lacking, and they can be a major nuisance having major adverse effects on the tourism industry in areas prone to Stomoxys infestations (Hogsette et al., 1987). Flight range of *S. calcitrans* is more or less one mile. And its life cycle (egg to adult) can complete in 20 days in warm temperature. Adults can survive for months. Females feed for several days, at least three days, before laying the first batch of eggs. Biology of stable flies is described elsewhere (Newstead, 1906; LaBrecque et al., 1975; Berry et al., 1976; Smith et al., 1985). Flies remain severe in dairies and feedlots where they breed in moist soil and substrates (Meyer and Petersen, 1983). Severe biting activity causes reduction in body weight and milk production. The economic losses of up to a half pound a day in weight gain and 30-40% in milk yield were observed (Mullens et al., 1998). In the United States, the loss to beef and dairy industry was estimated about 399$ million annually (Smith et al., 1987). High number of flies biting cattle may have an impact on the epidemiology of transmitted diseases. Stable flies are served as mechanical vectors of many different pathogens, e.g. anaplasmosis, the major cause of death of dairy cattle, trypanosomosis (Well, 1972; Mihok et al., 1992, 1995; Torr et al., 2006) and bovine leucosis virus (Buxton et al., 1985). Surveys of adult stable fly populations can be assessed in several different techniques. Many studies have employed the use of direct counts or collections from animal, especially leg counts to access the flies (McNeal and Campbell, 1981; Berry and Campbell, 1985). Besides direct count, various

trapping devices have been developed to collect flies. In the United States, sticky traps (Broce trap and William trap) have been used for sampling stable flies (William, 1973; Broce, 1988). Years later, more sophisticated trap using electronic devices was developed for the control of stable fly populations (Pickens, 1991; Pickens and Mills, 1993). Many trap devices have exploited the attractiveness of UV-reflecting Alsynite panels following William (1993). Recently, a more field friendly trap which was originally designed for tsetse fly collection was used for stable fly collection. This trap was named “Vavoua”, from the name of a village in Africa. This trap has proved very efficient to sample *Stomoxys spp*. in many African countries (Holloway and Phelps, 1991; Mihok *et al*., 1995, 1996) and in La Reunion Island (Gilles, 2005). A survey performed on two complete years in La Reunion Island gave indications about the annual dynamics of the two species present there (*S. calcitrans* and *S. niger*) and on the main environmental parameters influent on the density of flies in the different seasons (Gilles, 2005). Comprehensive works on stable flies, essentially on *S. calcitrans*, have been documented in several areas throughout the world, especially in the African countries and the United States. In Thailand most studies on stomoxyine flies have been documented about the presence of different stomoxyine flies by species, their distribution, seasonal abundance, and biology in dairy farms. Recently, Muenworn *et al*. (2010) described the geographic distribution of stomoxyine flies and diurnal activity of *S. calcitrans* in dairy farming Promotion Organization of Thailand and Khao Kheow Open Zoo but the specimen collection was limited in Australia zone with some animals in this area including camels, kangaroos, and emus. Little is known regarding species diversity, distribution, and the biology of stomoxyine fly species at local beef farms and open zoos. In this study, stomoxyine flies were selected form a local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province and in around the Khao Kheow Open Zoo in Sriracha District, Chonburi Province. We will observe the diurnal activity, seasonal abundance and species diversity of Stomoxyine flies in two different ecological habitats, a local beef farm and the Open Zoo.

Materials and Methods

Study sites

In this study, two areas were selected for the field experiment. First site is a local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province and the second site is in the Open zoo, Khao Kheow Open Zoo in Chonburi Province.

1.1 A local beef cattle farm in Wang Nam Khiao district, Nakhon Ratchasima Province

A local beef cattle farm located in Moo 9 of Tumbun Thai Samakkee, Wang Nam Khiao District, Nakhon Ratchasima Province (N14º22'1.3", E101º55'31.6"), approximately 200 km northeast of Bangkok, is selected for the study site. The site is more or less 3 km near the Tub Lan National Park, one of the biggest national parks in Northeastern region Thailand. Local occupations are linked closely with the organic farms. Logging, hunting and forest preservation remains minor activities of villagers who reside close to the forest. Approximately 30 cows are being housed in this local farm with the area of 3 acre. Absolutely no insecticide has been treated to protect cows from insect bites.

1.2 The Khao Kheow Open Zoo

Khao Kheow Open Zoo (KKOZ) is situated at 235 Moo 7, Tumbun Bangpra, Sriracha District, Chonburi Province, approximately 150 km east of Bangkok. The area has been established as a unit of the Zoological Park Organization of Thailand since 1978 as an open zoo with an area of 2,000 acre. Khao Kheow Open Zoo belongs to the Zoological Park Organization under the Royal Patronage of Her Majesty the Queen in the Ministry of Natural Resource and Development and has been supported by the government since 1978. The four sustained environment operation policies of KKO are 1. wild animal conservation, 2. education, 3. research and 4. providing recreational areas. Todays, KKO has about 8,000 animals such as deer, gibbon, langur, bearcat, springbok, giraffe, white rhinoceros, oriental Small-Clawed Otters, asiatic black bear, reticulated python, crocodile, giant anteater, hyena, tiger, Birds, Serow, Elephant, Barbary sheep, Emu, Banteng, Guar, Sambar, Chital deer, Malayan tapir, Chimpanzee and Giant flamingo. Zoos are considered as an environment in which exotic and native vertebrates, arthropods, and humans

routinely coming where radically distinct genomes intersect and selection pressures transcend traditional borders. Zoos are unique environments in which to study stable fly foraging behavior and to use intense hypothesis testing to explain host adaptations and preferences of stable fly. For instance, zoos can be used to examine the role of genetic and environmental factors in shaping stable fly host selection, and to investigate the nastiness of ectoparasites and host networks.

2. Trapping design

2.1 A local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province

Ten Vavoua traps (Laveissière and Grébaut, 1990) were placed around the 3 Acre-farm along the animal fence, approximately 10 m far away from each one and left operational during the night before the first collection at 06.00 hr. Ambient air temperature and relative humidity were recorded every 2 hr. throughout the period of collection. Monthly rainfall data were obtained from Wang Nam Khiao District meteorogical station located approximately 3 km from the study site.

2.2 Khao Kheow Open Zoo

As cage animal species are comparatively diverse in the Khao Kheow Open Zoo, fourteen Vavoua traps were used to capture the stable flies in this study. These traps were placed in order to cover the potential sites for the stable fly. Each trap was placed close to the animal house all around the open zoo area.

3. Fly collections

The Vavoua traps were left operational during the night before the first collection at 0600 hr. Adult stomoxyine flies in the Vavoua traps were captured every 2 hr starting from 0600, 0800, 1000, 1200, 1400, 1600 and 1800 hr. Collections were made during 2 consecutive days per every other month from April 2013 to March in the year 2015. Ambient air temperature and relative humidity were recorded every 2 hr throughout the period of collection whereas rainfall data were obtained from the local Meteorological station located in the Zoo. All captured flies were preserved in vials, containing 95% ethanol and

recorded by date and hour of capture according to collection location. Specimens were subsequently brought back to the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand for the morphological identification following Zumpt (1973).

4. Data analysis

Seasonal abundance based on climatic factors (ambient temperature, rainfall, and humidity) and daily activity over two hour interval during the day (0600-1800) will be selected for analysis in trap landing collection. Seasons of the year are classified as dry (March to May), hot (June to October) and wet (November to February). Time intervals will be divided into three-morning collections (0600-1000), three late morning collections (1000-1400), and three afternoon collections (1400-1800). Diurnal activity will be tabulated by averaging the number of fly entering the trap per day. Captured flies will be comparing by a three-way analysis of variance (ANOVA) with year as blocked factor. Differences among seasons will be performed using Fisher's least-significant difference. The accept level of significance will be determined at 5% (P-value < 0.05). All data will be analyzed using SPSS program package (Ver 17, SPSS Inc., Chicago, IL, USA).

Results and discussion

A survey of stomoxyine flies was carried out at a local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand during a 11 months period from April 2013 to December 2014. Four species of stomoxyine flies were morphologically identification in this area, including *Stomoxys calcitrans* (Linnaeus, 1758), *S. indicus* Picard 1908, S. sitiens Rondani 1873 and S. uruma Shinonaga and Kanao 1966. A total of 8,077 flies were captured with the most abundant species being *S. calcitrans* with 6,278 specimens, two thousand and eight hundred nigh teen (2,819) specimens of *S. indicus*. *S. sitiens* and *S. uruma* were found in small specimens, *S. sitiens* were found in a relatively low number with 60 specimens and *S. uruma* were found 43 specimens respectively (Table 1).

Monthly fly collections were shown in table 1 during 11 months period. Two thousand and seventy flies (2,070) were captured the most in August 2013 from a total of 8,077. After these months the number of flies was decease and showed the lowest number of flies in April in which 81 specimens of 2 species (Table 1).

A survey of stomoxyine fly species was made in 10 traps within a local beef cattle farm. Collectively, 1,699 of stomoxyine flies were captured from trap 4 are the higher number of samples than other trap. Among the 10 traps, stomoxyine flies was found the most abundant represent to Table 2.

Diurnal activity of Stomoxyine flies during 11 months, collected from different periods of the day with 2 hr intervals, beginning from 06.00 to18.00 hr. Stomoxyine flies was observed with the most prominent activity right before sunset (16.00 to18.00 hr) (Table 3).

Table 1 Monthly captures of Stomoxyine flies (*Stomoxys* spp.) with Vavoua traps at local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from April 2013 to December 2014).

month	Species								Total
	S. calcitrans		*S. indicus*		*S. sitiens*		*S. uruma*		
	Male	Female	Male	Female	Male	Female	Male	Female	
Apr. 13	45	32	1	3	0	0	0	0	81
Jun. 13	444	100	77	127	8	2	8	9	775
Aug. 13	1,011	401	291	344	8	13	0	2	2,070
Oct. 13	305	199	47	78	1	1	0	1	632
Dec. 13	106	86	5	4	2	1	0	10	214
Feb. 14	278	177	11	19	0	3	4	1	493
Apr. 14	362	235	8	13	0	0	0	3	621
Jun. 14	322	206	49	47	0	0	0	0	624
Aug. 14	825	407	166	282	0	3	1	2	1,686
Oct. 14	272	163	41	58	2	2	2	0	540
Dec. 14	191	111	6	19	4	10	0	0	341
Feb. 15	318	159	0	1	0	0	0	0	478
Total	4,479	2,276	702	995	25	35	15	28	8,555

Table 2 Trap site captures of Stomoxyine flies with 10 Vavoua traps at local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from April 2013 to December 2014).

Trap site	Total
Trap 1	812
Trap 2	537
Trap 3	1,497
Trap 4	1,845
Trap 5	779
Trap 6	821
Trap 7	126
Trap 8	341
Trap 9	751
Trap 10	1,046
Total	8,555

Table 3 Numbers of Stomoxyine flies collected at a local beef cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from April to December 14) from 6 am to 6 pm.

Time	Total
18.00-06.00	1,111
06.00-08.00	723
08.00-10.00	856
10.00-12.00	788
12.00-14.00	866
14.00-16.00	1,798
16.00-18.00	2,413
Total	8,555

A survey of stomoxyine flies was carried out at Khao Kheow Open Zoo in Sriracha District, Chonburi Province, Thailand (from May 2013 to November 2014). Four species of stomoxyine flies were morphologically identification in this area, including *S. calcitrans* (Linnaeus, 1758), *S. indicus* Picard 1908, *S. uruma* Shinonaga and Kanao 1966 and S. sitiens Rondani 1873. A total of 1,276 flies were captured with the most abundant species being *S. calcitrans* with 1,129 specimens, one hundred and twenty eight (128) specimens of *S. indicus*. *S. uruma* and *S. sitiens* were found in small specimens, *S. uruma* were found in a relatively low number with 10 specimens and *S. sitiens* were found 9 specimens respectively (Table 4).

Monthly fly collections were shown in table 4 during 10 months period. Three hundred and fifty five flies (359) were captured the most in March 2014 from a total of 1,276 and showed the lowest number of flies in July 2014 in which 22 specimens (Table 4). A survey of stomoxyine fly species was made in 14 traps within an Open Zoo. Collectively, 416 of stomoxyine flies were captured from trap 2 near Eld's Deer female are the higher number the others trap. Among the 14 traps, stomoxyine flies was found the abundant of stomoxyine flies were represent to Table 5.

Diurnal activity of Stomoxyine flies during 10 months, collected from different periods of the day with 2 hr intervals, beginning from 06.00 to18.00 hr. Stomoxyine flies was observed with the most prominent activity right before sunset (16.00 to18.00 hr) (Table 6).

Table 4 Monthly captures of Stomoxyine flies (*Stomoxys spp*.) with Vavoua traps at Khao Kheow Open Zoo in Sriracha District, Chonburi Province, Thailand (from May 2013 to November 2014).

Month	Species								Total
	S. calcitrans		*S. indicus*		*S. uruma*		*S. sitiens*		
	Male	Female	Male	Female	Male	Female	Male	Female	
May 13	40	20	7	11	3	0	0	2	83
Jul. 13	35	19	5	5	4	0	0	1	69
Sep. 13	109	20	11	9	0	0	1	1	151
Nov. 13	184	17	20	14	0	0	0	0	235
Jan. 14	52	12	9	3	0	0	0	0	76
Mar. 14	273	81	0	2	0	0	0	3	359
May 14	94	20	2	0	0	0	0	0	116
Jul. 14	14	3	3	2	0	0	0	0	22
Sep. 14	41	6	6	10	0	0	0	1	64
Nov. 14	79	10	3	6	3	0	0	0	101
Jan. 15	39	15	2	0	0	0	0	0	56
Mar.15	53	10	0	1	0	0	0	0	64
Total	1,013	233	68	63	10	0	1	8	1,396

Table 5 Trap site captures of Stomoxyine flies with 14 Vavoua traps at Khao Kheow Open Zoo in Sriracha District, Chonburi Province, Thailand (from May 2013 to November 2014).

Trap site	Host	Total
Trap 1	Eld's Deer, male	55
Trap 2	Eld's Deer, female	456
Trap 3	Barking deer white	33
Trap 4	Giraffe	16
Trap 5	Meerkat	6
Trap 6	Ostrich	85
Trap 7	Camel	273
Trap 8	Elephant	6
Trap 9	Kangaroo	50
Trap 10	Wild pig	190
Trap 11	Tapir	207
Trap 12	Small Tiger	5
Trap 13	Big Tiger	0
Trap 14	Near pool	14
Total		1,396

Table 6 Numbers of Stomoxyine flies collected at Khao Kheow Open Zoo in Sriracha District, Chonburi Province, Thailand (from May 2013 to November 2014) from 6 am to 6 pm.

Time	Total
18.00-06.00	81
06.00-08.00	163
08.00-10.00	212
10.00-12.00	154
12.00-14.00	217
14.00-16.00	328
16.00-18.00	241
Total	1,396

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Subproject 4: Molecular evolution of innate immune genes in primary malaria vectors in Thailand (Uraiwan Arunyawat)

Abstract

Understanding evolutionary processes that govern susceptibility in mosquito vectors would have important public health implications for vector-borne diseases such as malaria. If the mosquito innate immune system is used as a principle blocking step in malaria transmission, then the immunity genes might have evolved under selection forces imposed by malaria parasite transmission. In this study, two partial immune genes including *TOLL6* which involves in signal transduction pathways of insect immune system and *CLIPA6* involving in signal modulation and amplification pathways were selected to estimate pattern of genetic diversity in *Anopheles minimus* population, the main malaria vectors in Southeast Asia. Primers were designed and tested to amplify the *immune gene* fragments, and the PCR products were directly sequenced and analyzed using single nucleotide polymorphism data. Low levels of nucleotide diversity were shown in the studied *An. minimus* populations. Statistical neutrality tests, including Tajima's *D* and Fu and Li's *D**, were used to measure deviation from neutral equilibrium expectation. No significant negative Tajima's *D* and Fu and Li's *D** values were detected in this study, indicating an excess of low frequency polymorphisms. The significant of mismatch distribution tests supported the population expansion model. In addition, Ka/Ks ration test indicated that the variation of the partial *CLIPA6* and *TOLL6* is most likely explained by the influence of mutation and genetic-drift interaction rather than the effect of directional selection.

Keywords: *Anopheles minimus,* immune gene, *TOLL6, CLIPA6,* sequence variation, evolutionary factors

Introduction

If the mosquito innate immune system is used as a principle blocking step in malaria transmission, then the immunity genes might have evolved under selection forces imposed by malaria parasite transmission. Examining evolutionary processes i.e. natural selection modes operating on immune genes might reveal the nature of host-pathogen interactions and evolutionary factors that govern susceptibility in mosquito populations. Moreover, Christophides et al. (2002) have identified 242 *Anopheles gambiae* genes from 18 gene families implicated in innate immunity. Four groups of immune-related gene families have been characterized including: 1) Recognition of Infectious Non-self (e.g. TEPs, GNBPs) 2) Signal Modulation and Amplification signal modulation (e.g. CLIPs family), 3) Signal transduction pathways (e.g. Toll genes) and 4) Effector Response Systems (e.g. PPO genes). Interestingly, innate immunity gene families in *An. gambiae* have detected significant diversification relative to *Drosophila melanogaster*, possibly reflecting selection pressures from different pathogens. It has been shown that many functional genes in various organisms are usually targeted by positive natural selection by population genetic analyses (Nielson 2005; Lehmann et al., 2009). In malaria system, for example, insecticide resistant genes in malaria vectors and genes responsible for malaria susceptibility in human host have been reported to evolve under positive selection model of molecular evolution (Hughes, 1990; Wang et al., 2003).

Anopheles species mostly exist in temperate, subtropical and tropical areas of the world. Moreover, they can be found in different geographies from hilly to coastal areas (Harbach, 2011). Most Anopheles mosquitoes are species complex which is difficult to distinguish from morphological characteristics. Six *Anopheles* mosquitoes are considered as malaria vectors in Thailand. Three species of these have been examined as primary vectors, i.e. *An. dirus*, *An. minimus*, and *An. maculatus*. And remains have been considered as secondary vectors, i.e. *An. aconitus*, *An. sundaicus*, and *An. pseudowillmori* (Ministry of Public Health, 2008). The Dirus Complex occurs in tropical evergreen rainforest, cultivated forests fringes along Southeast Asia and five Dirus mosquito species are found in

Thailand. Within the Minimus complex, *An. minimus* and *An. harrisoni* can be found in large sympatric areas in the Oriental Region (Manguin et al., 2008).

Molecular evolution of insect immunity genes has been studied primarily in *Drosophila*. However, studies on mosquito innate immune genes are still very limited, as well as the evolutionary factors that control pathogen susceptibility in host populations remains poorly understood. Increased understanding of host-pathogen relationships by investigating molecular evolutionary patterns of selected immune genes in the mosquito vectors would have important public health implications for vector-borne diseases such as malaria. Therefore, this study aims to describe genetic variation patterns of innate immune genes of the main malaria mosquitoes presented in Thailand, and to reveal the nature of host-pathogen interactions.

Objective

To estimate pattern of nucleotide variation of the partial immune genes (*TOLL6* and *CLIPA6*) in *Anopheles minimus* populations, and to evaluate whether natural selection shapes pattern of sequence variation on the *immune* genes

Materials and Methods

Population collection

Adult female *An. minimus* mosquitoes were collected from three different locations in Thailand, which approximately 9-10 mosquitoes each *An. minimus* population obtaining from Tak, Surathani and Chanthaburi provinces (Table 1). To resolve the problem of morphological identification, molecular technique was applied to confirm the identification between the two sibling species (*An. minimus* and *An. harrisoni)*, using primers in the ITS2 region developed by Garros et al. (2004)

Table1 Studied mosquito populations

Mosquito	Location	Code	Sample size
An. minimus	Gaeng Hang Meaw distinct, Chantaburi	Min-CB	9
An. minimus	Donsak distinct, Surathani	Min-ST	10
An. minimus	Mae Sod distinct, Tak	Min-TK	10

Choice of the immune genes and Primer design

Four groups of immune-related gene families have been characterized including: 1) Recognition of Infectious Non-self for example TEPs, GNBPs genes; 2) Signal Modulation and Amplification signal modulation e.g. CLIPs gene family; 3) Signal transduction pathways e.g. Toll genes; and 4) Effector response systems e.g. PPO genes (Christophides et al., 2002). In this study, two partial immune gene families including *TOLLs* which involves in signal transduction pathways of insect immune system and *CLIPs (*Clip-domain serine proteases) involving in signal modulation and amplification pathways were selected. The amplified primers were designed using EPIC (Exon Primed Intron Crossing) technique (Bierne et al. 2000). Briefly, primer pairs were designed in the conserved exons at least 20 bp away from the putative junction with the intron. The main advantage of EPIC primer design is that for cross-species amplification should be easier than when primers are designed in noncoding sequences because exon sequences are more conserved across species.

DNA amplification and Sequencing

Genomic DNA was isolated from single individual of *An. minimus* using Genomic DNA Mini kit (Tissue) (GeneAid, Biotech Ltd. Taiwan). Genomic DNA was quality checked on 1% agarose gel electrophoresis. Polymerase Chain Reaction (PCR) primers and conditions were tested to amplify the immune fragment. PCR products were checked quality on 1% agarose gel, and purified using two enzymes Exonuclease I and FastAP^{TM} Thermosensitive Alkaline Phosphatase (Fermentas, Canada) afterwards. PCR products were sequenced directly from both strands by the sequencing service unit (Macrogen Inc., Korea). Sequences were aligned and edited using Bioedit programs, and adjusted manually.

Sequence data analyses

Nucleotide diversity was estimated based on Single Nucleotide Polymorphisms (SNPs) for all populations by using θ_w estimator(Watterson, 1975) which is calculated based on the number of segregating sites in a sample, and π which is the average number of pairwisedifferences between sequences in a sample (Nei, 1987) as implemented in DNAsp version 5.0 program (Librado Rozas, 2009). Tajima's *D* test (1989) measures deviations from neutral expectation; a negative *D* values indicates an excess of rare frequency polymorphisms, and a positive *D* value suggests an excess of intermediate-frequency polymorphisms.

Results and Discussion

Testing primers for the immune fragments amplification

In this study, partial *TOLL6* and CLIPA6 immune genes were selected. *TOLL6* is a gene in TOLL gene family which involves in signal transduction pathways of insect immune system. Up to now, ten *TOLL* family genes were reported based on *An. gambiae* genome. *CLIPA6* is a member of the *CLIPs (*Clip-domain serine proteases) gene family involving in signal modulation and amplification pathways. In this study, we designed primers to amplify *TOLL6 and CLIPA6* fragments using sequence data of *An. gambiae* genome. The detail of selected fragment and primers is briefly presented in Table 2. Primers were tested to amplify PCR product using annealing temperature 54-60°C. The designed primers were able to amplify the *TOLL6*and *CLIPA6* fragments, showing PCR product size about 700 bp and 600 bp, respectively (Figure 1 and 2). The PCR products were sequenced directly from both strands. Figure 3 shows an example of sequence chromatogram of the partial TOLL6 genes from *An. minimus* mosquito. Sequences were aligned using computer programs, and adjusted manually afterward.

Table2 Summary of the partial *TOLL6*and *CLIPA6* immune genes and the designed primers

Gene	*TOLL6*	*CLIPA6* (Clip domain serine proteases)
Gene function class	3 (Immune signaling pathways)	2 (Signal modulation and amplification)
Chromosomal location	3L	3L
Forward primer	5’ CCA ACT CGT TCG TCC ATC TG 3’	5’AAG CGG TAT GCA TCC AGA AG 3’
Reverse primer	5’ CTG GCG AAA GCA CAC TGA T 3’	5’ CTC GTT CTT CGT CTG GGT GT 3’
Expected product size	755 bp	590 bp

Source: http://www.ncbi.nlm.nih.gov/

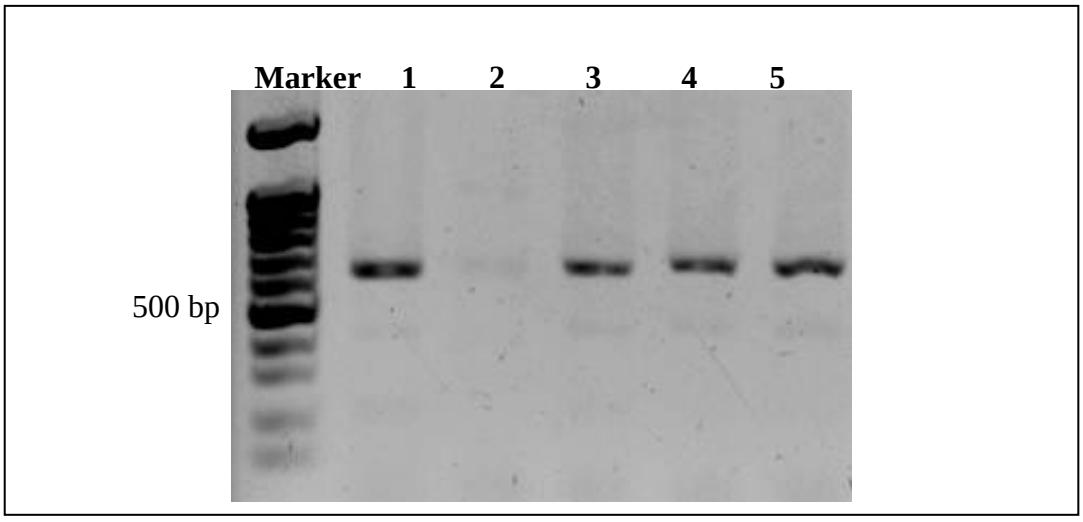


Figure 1 Testing primers for gene amplification of *TOLL6* in *An. minimus* mosquitoes

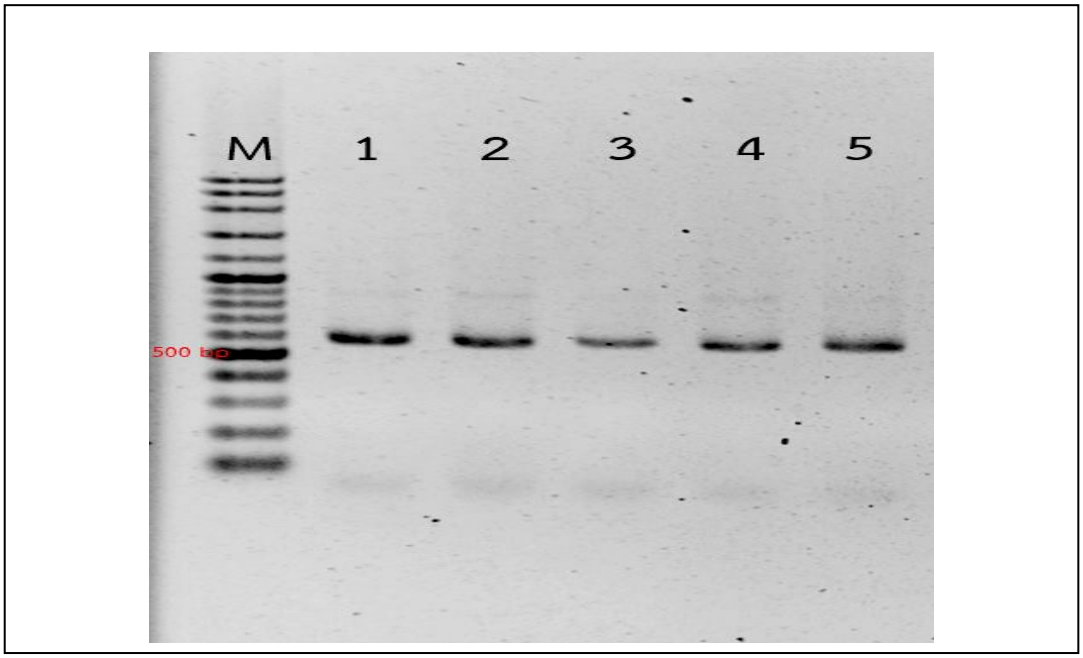


Figure 2 Testing primers for gene amplification of *CLIPA6* in *An. minimus* mosquitoes

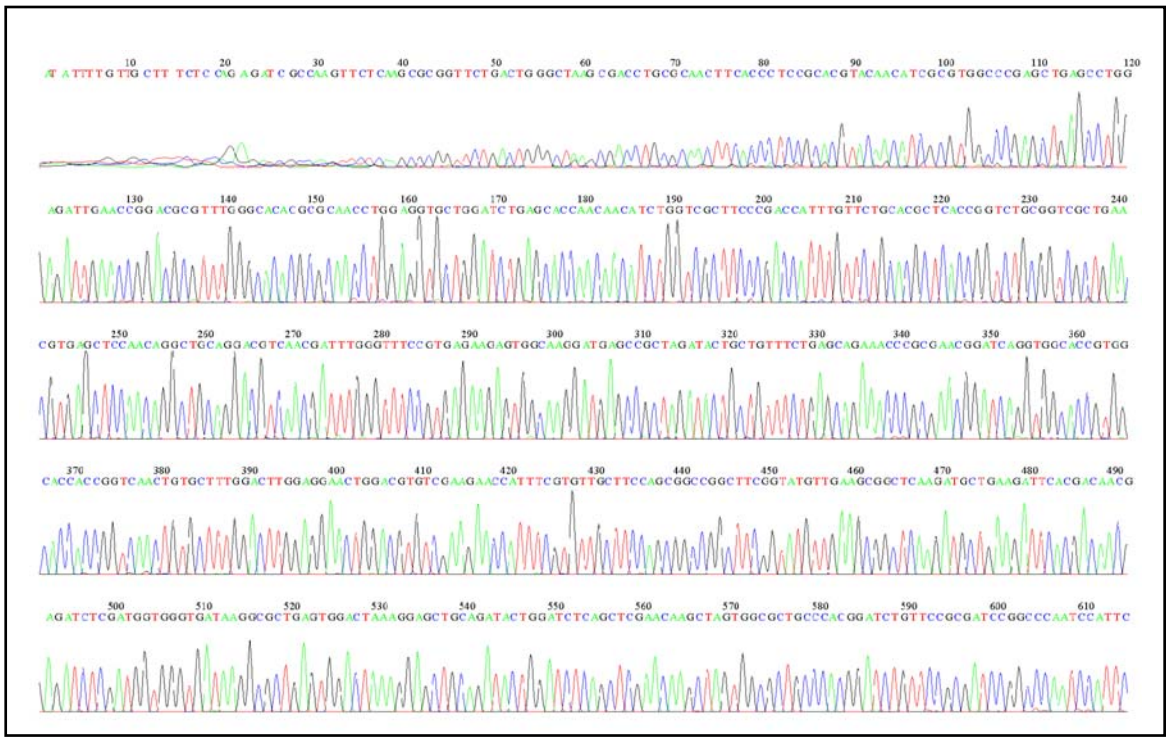


Figure 3 Sequence chromatogram of *TOLL6* in *An. minimus* mosquito

Sequence variation

A total length of the *TOLL6* fragment for sequence analysis is 590 base pair, in which 34 single nucleotide substitutions (SNPs) were identified in the combined samples of all populations. The highest nucleotide variation was found in *An. minimus* from Tak province (25 SNPs), while lowest polymorphism (17 SNPs) was shown in Surathani population. A moderate polymorphism was presented in Chantaburi population (22 SNPs). Details of polymorphic sites including the number of singleton sites as well as two and three variable sites for each population were shown in Table 3. For the analysis of *CLIPA6* sequences, a total number of 490 analyzed sites, 26 SNPs including 9 singletons, 16 parsimony informative sites and a three variable site were detected.

Table 3 Summary of sequence polymorphic sites

Gene	Population	Number of sequences	Analyzed sites	Segregating sites	Singleton sites	Parsimony informative sites	Three variable sites
TOLL6	Tak	20	590	25	10	14	1
	Surat Thani	20	590	17	10	7	0
	Chanthaburi	18	590	22	11	9	2
	All samples	58	590	34	12	20	2
CLIPA6	Tak	9	490	11	1	10	0
	Surat Thani	20	490	18	7	11	0
	Chanthaburi	18	490	19	8	11	0
	All samples	47	490	26	9	16	1

Moreover, we analyzed the frequency spectrum of polymorphic sites the combined populations using the constant population size model (Figure 4 and 5). The graph indicated that observed low frequency variants was higher than expected values, suggesting more singleton sites than expected under constant population size model.

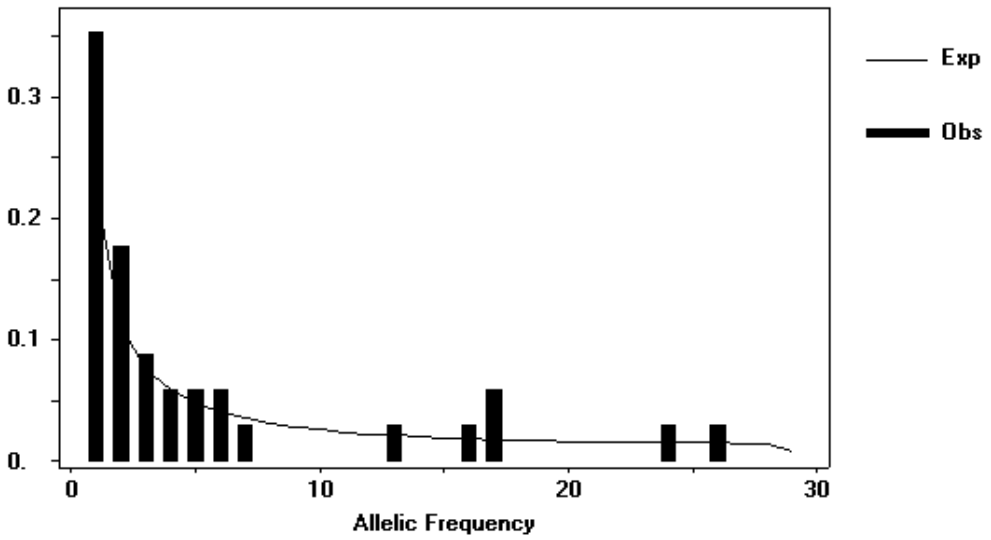


Figure 4 Frequency spectrums of observed polymorphic sites (Obs) of *TOLL6* sequences from *An. minimus* populations and expected values (Exp) based on constant population size model

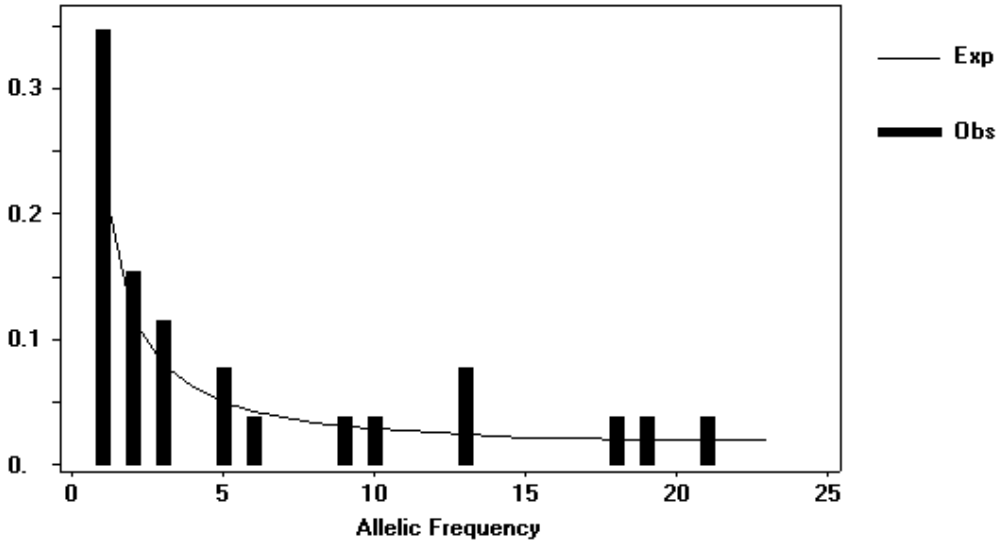


Figure 5 Frequency spectrums of observed polymorphic sites (Obs) of *CLIPA6* sequences from *An. minimus* populations and expected values (Exp) based on constant population size model

Levels of nucleotide variation and Test of Neutrality

The estimation of nucleotide diversity for each population was presented in table 4. The number of haplotypes indicates high haplotype diversity for *An. minimus* mosquitoes. In both immune fragment genes, low level of nucleotide variation was detected in all populations (θ_w and π varies from ~ 0.5-1.2 %). Mosquitoes from Tak province appear to be the most polymorphic population at locus *TOLL6* gene. However, less polymorphism was shown in TAK population at locus *CLIPA6*, which is most likely due to the small number of analyzed sequences for this population.

Table 4 Summary of Nucleotide variation and Neutrality test

Gene	Population	Number of sequences	Number of haplotypes	π (Nei,1983)	θ_w (Watterson,1975)	Tajima's *D* values	Fu and Li's *D** values
TOLL6	Tak	20	20	0.0104	0.0119	-0.494^{ns}	-0.566^{ns}
	Surat Thani	20	17	0.0058	0.0081	-1.054^{ns}	-1.520^{ns}
	Chanthaburi	18	18	0.0085	0.0118	-0.837^{ns}	-1.227^{ns}
CLIPA6	Tak	9	9	0.0094	0.0085	0.526^{ns}	1.163^{ns}
	Surat Thani	20	15	0.0092	0.0106	-0.285^{ns}	-0.485^{ns}
	Chanthaburi	18	15	0.0094	0.0116	-0.713^{ns}	-0.575^{ns}

ns = not significant

Tajima's *D* (1981) and Fu and Lu's D* (1993) statistics were used to test for deviation from neutrality. This Tajima's *D* test measures skews in the frequency spectrum based on two nucleotide variation estimators (θ_w andπ). Under standard neutral equilibrium, *D* value ~ 0 indicate an equal between θ_w and π estimators, assuming mutation and/or genetic drift forces shape the pattern of nucleotide variation. A significantly positive *D* value indicate an excess of intermediate frequency polymorphism ($\pi>\theta_w$), suggesting signature of balancing selection or demographic events (e.g. population bottleneck, population subdivision). A significant negative *D* value suggest an excess of low frequency polymorphism ($\pi<\theta_w$), assuming signature of negative selection or population expansion scenario. The Interpretation of Fu and Li's D* values is similar to Tajima's D test, which emphasize on

the excess of singleton. The values of neutrality tests for each and combined populations are given in table 4. None population showed statistically significant departure from neutral equilibrium, both Tajima's D and Fu and Li' D* tests value, suggesting the variation of the *TOLL6 and CLIPA6* fragments is most likely explained by mutation and drift evolutionary forces, rather than the effect of natural selection.

Population differentiation

Estimations of the standard F_{ST} statistic based on Hudson (1992) were performed in *An. minimus* samples. The average FST estimate was 0.0197, indicating low genetic differentiation among *An. minimus* populations. In addition, the average pairwise estimated population differentiation based on *TOLL6* fragment exhibited no differentiation between populations from TAK and Chanthaburi Provinces, and low level of population differentiation was found among TAK-Surat populations (F_{st} = 0.027) and Surat-Chantaburi populations (F_{st} = 0.038) as shown in Table 5. In addition, the estimation based on the sequence variation of *CLIPA6* gene indicated similar result of population differentiation as of the *TOLL6* fragment (data not shown).

Table 5 Pairwise F_{st} estimates (Hudson et al.,1992) among three *An. minimus* populations

Population	Tak	Surat Thani	Chanthaburi
Tak		0.027	0.000
Surat Thani			0.038
Chanthaburi			

Directional selection and Population expansion model

The analysis of Ka/Ks ratio (or dN/dS ratio) is the ration between the number of non-synonymous nucleotide substitution (Ka) and synonymous nucleotide substitution (Ks), which can be used as an indicator of direction selection acting on a studied gene. A high Ka/Ks ratio (Ka/Ks ratio> 1) implies positive selection, where Ka/Ks ratio < 1 implies purifying selection, and a ratio of one (Ka/Ks ratio ~1) indicating neutral (i.e. no selection) is most likely the evolutionary pressure evolving on a protein coding gene. Table 6 shows the parameters of Ka/Ks ratio of TOLL6 and CLIPA6 sequences that most of substitution

sites are synonymous substitution (Ks > Ka). Thus, Ka/Ks ratio is less than 1 inferring directional selective pressure is not major evolutionary force acting on the intra-nucleotide variation in both studied immune genes of *Anopheles minimus* populations.

Table 6 Summary of Substitution ratio, Linkage Disequilibrium and recombination tests

Gene	Analyzed sites	Ka	Ks	Ka/Ks ratio	Raggedness (r) index	R^2	Rm	Zns
TOLL6	590	0.026	0.052	0.506	0.0154^{**}	0.0919^{ns}	4	0.1137^{ns}
CLIPA6	490	0.032	0.124	0.258	0.0153^{*}	0.0833^{ns}	7	0.0583^{*}

ns = not significant; * significant *p*-value < 0.05computed based on 10,000 coalescent simulations

Ka = Nonsynonymous substitution rates per non-synonymous site; Ks = Synonymous substitution rate; Raggedness r = a statistic test of the pairwise differences distribution (Harpending, 1994); R^2= a statistic test of mismatch distribution (pairwise differences) model (Ramos-Onsins and Roza, 2002); Rm = minimum number of recombination events (Hudson and Kaplan, 1985); Zns = linkage disequilibrium parameter (Kelly, 1997)

Moreover, the mismatch distribution model was analyzed in *TOLL6* and *CLIPA6* genes as shown in figure 6 and 7, respectively. Two parameters i.e. Raggedness statistic (Harpending, 1994) and R^2statistic (Ramos-Onsins and Roza, 2002) were chosen to test population expansion model, which conceptually compare the frequency distribution of pairwise differences in observed sequence variation of the studied genes and the expected distribution under constant population size model. The results of both statistical tests are congruent, suggesting pattern of intra-specific sequence variation in the partial immune genes (*TOLL6* and *CLIPA6*) is influenced by population growth model i.e. population expansion.

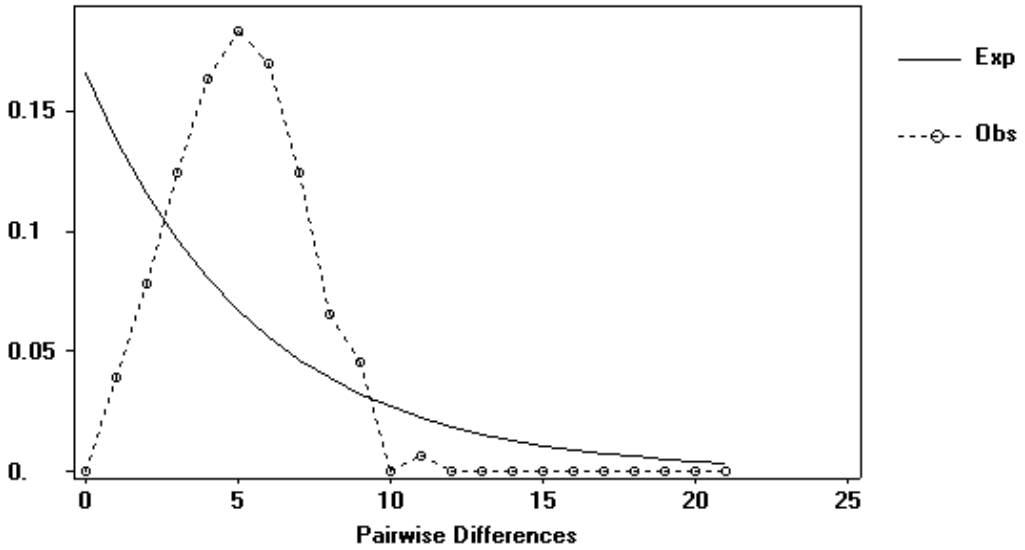


Figure 6 Pairwise Differences (mismatch distribution) of observed polymorphic sites (Obs) from *An. minimus* populations based on *TOLL6* sequences and expected values (Exp) based on constant population size model

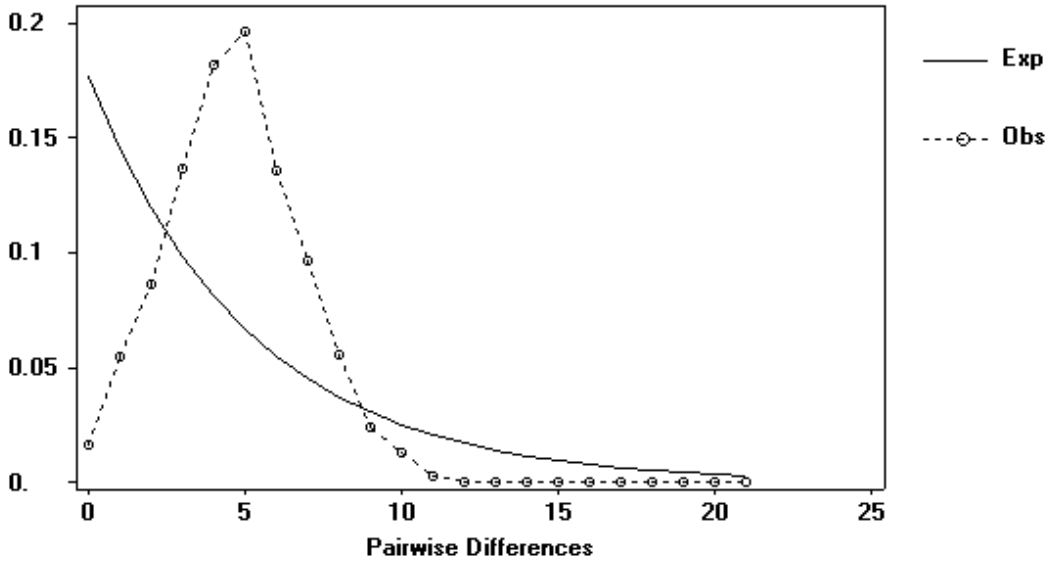


Figure 7 Pairwise Differences (mismatch distribution) of observed polymorphic sites (Obs) from *An. minimus* populations based on *CLIPA6* sequences and expected values (Exp) based on constant population size model.

Conclusion

Sequence variation of the selected *TOLL6* and *CLIPA6* immune fragments in three *An. minmus* populations was presented in this study. Overall low level of nucleotide variation was detected in all populations, and none population showed statistically significant departure from neutral equilibrium. The significant of mismatch distribution tests support the population expansion model. In addition, Ka/Ks ration test indicated that the variation of the partial immune genes (*CLIPA6* and *TOLL6*) is most likely explained by the influence of mutation and genetic-drift interaction rather than the effect of directional selection.

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Subproject 5: Current status susceptibility of Anopheles species collected from malaria endemic areas of Mae Hong Son province, Thailand (Wannapa Suwonkerd)

Abstract

Insecticides are one of the major tools for controlling vector populations which expected to reduce the mosquito disease transmission. In Thailand, pyrethroids and organophosphates have been introduced to public health purposes for decades against diseases vector mosquitoes. Country wide application of insecticides can be caused mosquitoes developed resistance, a proper surveillance and management of resistance is being utilized crucial for the success of control programs. Anopheles adult collected from three study fields from Mae Hong Son province were subjected to susceptibility test against ddt, deltamethrin, fenitrothion and propoxur. The test was performed following WHO standard procedure. *Anopheles minimus* s.l. and *An maculatus* group collected from all study sites showed high susceptible to four kinds of insecticides tested. Excepted *An. minimus* s.l., the other species, *An. sinensis, brabirostris, peditaenaitus* collected from Pang Ma Pha have developed resistance to all kinds of insecticide while *Anopheles* collected from Sob Moei district showed opposite of susceptible to all kind insecticides test. Widely used of pesticides and insecticide in agricultural and public health purposes are possibly affected to this phenomenal however further investigation is needed.

Key words: *Anopheles*, endemic malaria areas, susceptibility level, Thailand

Introduction

The development of resistance by mosquitoes to insecticides has posed a considerable threat to the success of public health efforts in combating mosquito-borne diseases in many part of the world. In Thailand, several kinds of insecticides have been widely used against agricultural pests and public health purpose in order to control disease insect vectors. DDT was applied for large scale particularly in the malaria endemic areas through out Thailand to control malaria vectors started in 1949 (Bathia &Notananda 1949). In 1958 DDT was also applied used for dengue vector control in Bangkok, metropolitan as well as the expanding to control of aedes larvae (Jatanasen & Mouheat1967). Until 1990s DDT was fate out due to its adverse affected and long persistent in the nature. Level of susceptibility to various kind of insecticides have been reported for some species of culicines and anopheline (Neely 1964, Jatanasen & Mouheat1967, Bang et al. 1969, Charoenviriyaphap et al 1999, Somboon et al. 2003, Suwonkerd et al 2009).

The management of insecticide resistance is complicated by the fact that resistance takes a variety of forms i.g.: *metabolic resistance* and *target-site resistance*. Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal, preventing the insecticide from reaching the intended site of action. In the case of malaria vectors, three enzyme systems are believed to be important: the esterases, the mono-oxygenases and the glutathione *S*-transferases. Target-site resistance occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation: when this happens the insecticide can no longer bind to the intended target site of the receptor and thus the insect is unaffected, or less affected, by the insecticide.

As to a new WHO guideline said if the observed mortality is between 90% and 97%, the presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/ or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then

resistance is confirmed. Resistance surveillance should be conducted in association with surveillance for control efficacy. Otherwise, the significance of changes in susceptibility can not be determined.

The aim of this study was to investigate the susceptibility status of Anopheles species collected from endemic malaria areas in Mae Hone Son province.

Materials and Methods

Anopheles **Field strains:** Mae Hong Son province where reported highest malaria incidence among 8 upper northern province of Thailand. Adult anopheles mosquito were collected from three districts: Muang, Sob Moei and Pang Ma Pha. (Fig.1). Mature stage of anopheles mosquito were collected from both animal bait and outdoor human landing collection, all mosquito were identified and kept in 25°C±5, 70-80% Relative humidity. Three days old unfed female were testes as per standard WHO (WHO 1989), using test paper each impregnated with DDT (4%), Deltamethrin (0.05%), Fenitrothion (1%) and Propoxur (0.1%) respectively.

Calculation of mortality:

The assessment of mortality, i.e. a count of the number of dead mosquitoes in both the exposure and the control tubes, is made 24 hours post-exposure. A mosquito is classified as dead if it is immobile or unable to stand or fly in a coordinated way.

Percentage of the total number of exposed mosquitoes:

$$\frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \text{ Observed mortality x } 100$$

If the control mortality is above 20%, the tests must be discarded. When control mortality is greater than 5% but less than 20%, then the observed mortality has to be corrected using Abbots formula, as follows: $\frac{(\%\text{ observed mortality} - \%\text{ control mortality}) \times 100}{(100 - \%\text{ control mortality})}$

Interpretation of susceptibility test results

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO bioassay has been revised. Mortality in the range 98–100% indicates susceptibility. A mortality of less than 98% is suggestive of the existence of resistance and further investigation is needed.

Results and Discussion

After 24 hours post-exposure, *Anopheles minimus* s.l a primary malaria vector collected from four study sites, showed susceptible to DDT. Moreover *An.minimus* s.l indicated high susceptible to the other three insecticides, Fenitrothion, Deltamethrin and Propoxur, a mortality rate range from 98.95-100% (Table 1 and Fig.3). While *Anopheles barbirostris*, *An. sinensis* and *An. peditaeniatus* collected from all study sites indicated a high level on DDT resistant (Table1). Among five anopheles species test *An. peditaeniatus* collected from three study sites were detected resistance to all kind of insecticides test, DDT Fenitrothion, Deltamethrin and Propoxur. Among the three study sites, anopheline collected from Pang Ma Pha district showed high frequency resistance to all insecticides tested, excepted *An.minimus* s.l.

The results of susceptibility test against Fenitrothion revealed that four anopheles field population except *Anophels peditaeniatus* (Pang Ma Pha strain) showed susceptible to this organophosphate, Fenitrothion. Levels of susceptibility have been reported for some species of culicines and anopheleine but the area of reporting may not represented all endemic malaria areas, particularly the area along the international borders where malaria transmission is still going on. Insecticide resistance has been a problem in all insect groups that serve as vectors of mosquito borne diseases. Although mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involved a complex pattern of resistance foci. Results of this study is the preliminary result indicated that DDT is still effective to use against malaria vector, *Anopheles minimus* s.l. in this Thai-Manymar borders, unfortunately in Thailand, DDT was banded to used long time ago since 1995. Our results indicated that

Fenitrothion is still one of insecticide of choice in these malaria endemic areas. Moreove, the insecticide monitoring is recommended to perform in a foci sentinel site to be a vector surveillance data supporting the Chemical committee and National Malaria Control Program for vector control cost effectively.

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Table 1: Susceptibility status of Anopheles species against DDT, Fenitrothiaon, Deltamethrin and Propoxur collected from Sob Moei district (N17 51.928 E97 57.874); Muang district (N19.385 E97.867) and Pang Ma Pha district, (N19.354 E98.130), Mae Hong Son province.

DDT			No. of mosquito		mortality	susceptibility
An.species	month/year	Locality	test	dead	rate	status
barbrirostris	Nov 2013-Aug 2014	Sob Moei	120	10	8.33	resistance
minimus	Nov 2013-Aug 2014	Muang	95	94	98.95	susceptible
peditaeniatus	Nov 2013-Aug 2014	Muang	75	65	86.67	resisitance
peditaenaitus	Aug 2014-Jan 2015	Pang Ma Pha	150	39	26	resistance
sinensis	Aug 2014-Jan 2015	Pang Ma Pha	110	26	23.63	resistance
minimus	Aug 2014-Jan 2015	Pang Ma Pha	100	100	100	susceptible
barbirostris	Aug 2014-Jan 2015	Pang Ma Pha	115	87	75.65	resistance

Fenitrothion			No. of mosquito		mortality	susceptibility
An. species	month/year	Locality	test	dead	rate	status
barbrirostris	Nov 2013-Aug 2014	Sob Moei	200	200	100	susceptible
pseudowillmori	Nov 2013-Aug 2014	Sob Moei	180	180	100	susceptible
minimus s.l.	Nov 2013-Aug 2014	Sob Moei	250	250	100	susceptible
sawadwongporni	Nov 2013-Aug 2014	Sob Moei	190	190	100	susceptible
peditaeniatus	Nov 2013-Aug 2014	Muang	50	49	98	susceptible
minimus s.l.	Nov 2013-Aug 2014	Muang	100	100	100	susceptible
peditaeniatus	Aug 2014-Jan 2015	Pang Ma Pha	100	12	12	resistance

Deltamethrin			No. of mosquito		mortality	susceptibility
An.species	month/year	Locality	test	dead	rate	status
minimus s.l.	Nov 2013-Aug 2014	Sob Moei	155	155	100	susceptible
sawadwongporni	Nov 2013-Aug 2014	Sob Moei	121	121	100	susceptible
minimus s.l.	Nov 2013-Aug 2014	Muang	125	125	100	susceptible
sinensis	Nov 2013-Aug 2014	Muang	30	28	93.33	resistance
peditaeniatus	Nov 2013-Aug 2014	Muang	85	73	85.88	resisitance
minimus s.l.	Aug 2014-Jan 2015	Pang Ma Pha	115	115	100	susceptible
sinensis	Aug 2014-Jan 2015	Pang Ma Pha	150	144	96	resisitance
peditaeniatus	Aug 2014-Jan 2015	Pang Ma Pha	200	26	13	resistance
barbirostris	Aug 2014-Jan 2015	Pang Ma Pha	110	105	95.45	resisitance

Propoxur			No. of mosquito		mortality	susceptibility
An. species	month/year	Locality	test	dead	rate	status
barbrirostris	Nov 2013-Aug 2014	Sob Moei	200	200	100	susceptible
pseudowillmori	Nov 2013-Aug 2014	Sob Moei	210	210	100	susceptible
minimus s.l	Nov 2013-Aug 2014	Sob Moei	202	202	100	susceptible
peditaeniatus	Nov 2013-Aug 2014	Muang	50	46	92	resistance
minimus s.l	Nov 2013-Aug 2014	Muang	100	100	100	susceptible
peditaeniatus	Aug 2014-Jan 2015	Pang Ma Pha	100	44	44	resistance
sinensis	Aug 2014-Jan 2015	Pang Ma Pha	125	20	16	resistance

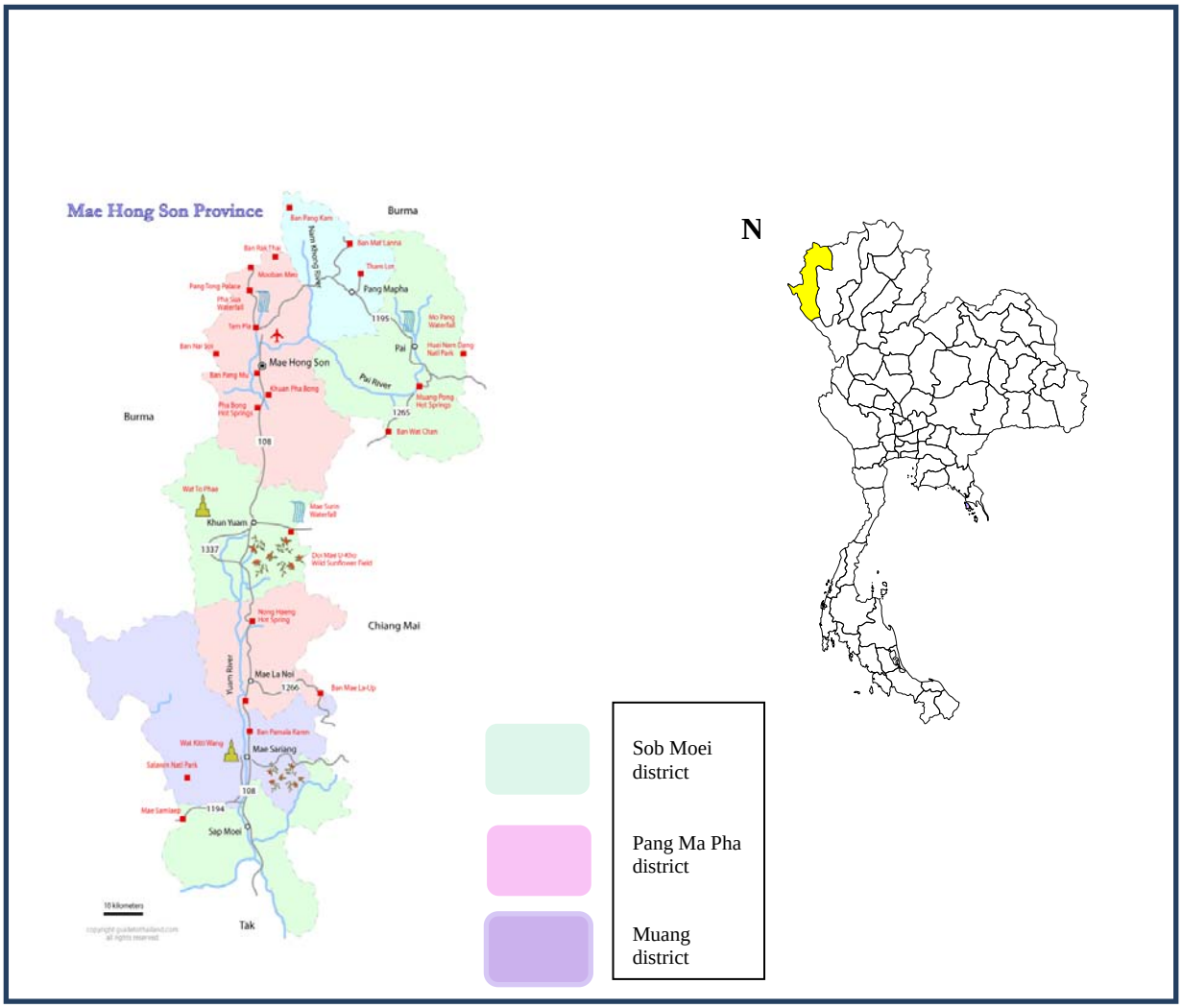


Figure 1 shows the places of origin of the strains tested, Sob Moie, Pang Ma Pha and Muang districts, Mae Hong son province, northern Thailand

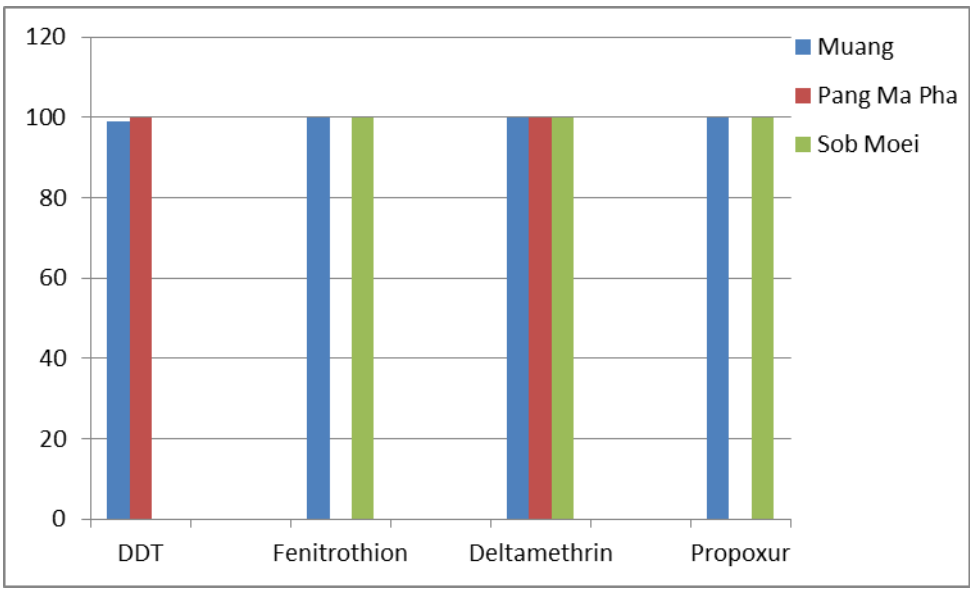


Figure 2 The mortality rate of *Anopheles minimus* s.l. test against DDT, Fenitrothion, Deltamethrin and Propoxur in Muang, Pang Ma Pha and Sob Moei districts , Mae Hong Son province

Subproject 6: Seasonal Abundance of Main Malaria Vectors and Species Diversity of *Anopheles* sp. in Malaria Endemic Area, Kanchanaburi Province, Thailand

(Monthathip Kongmee)

Abstract

The most common anopheline species in Ban Pu Teuy, Sai Yok District, Kanchanaburi Province during March 2013 to January 2015 was *An. harrisoni* (92.50%) followed by *An. dirus*, *An. minimus* and *An. maculatus*. The densities of *An. dirus* and *An. maculatus* were associated with the cumulative monthly rainfall. The effects of temperature and relative humidity were not found as a significant variable on the abundance of anophelines mosquitoes, except An. dirus that was associated with relative humidity, especially with high relative humidity in wet season. Significant differences in number during two-year period of *An. harrisoni*, *An dirus* and *An. maculatus* were found between seasons, with highest peaks in hot (*An. harrisoni*) and wet seasons (*An dirus* and *An. maculatus*). The biting activity and host preference of *An. harrisoni*, *An. minimus* and *An. maculatus* was shown as an exophagic and zoophilic, except An dirus was anthropophilic rather than zoophilic. However, there were no statistically significant differences between biting activity /host preference (collection methods). There was no significant difference between time period of collection, despite the number of captured mosquitoes in each hour of collection was different. For the pattern of host biting activity, *An. harrisoni* and *An. minimus* appeared immediately after sunset and fewer thereafter and occurred before midnight with high number. Outdoor activities of *An. dirus* more occurred before midnight. In contrast, the pattern of indoor (human) activity was higher activity after midnight. *An. maculatus* have two peaks of the biting activity pattern, the major peak between 18:00-20:00 and the minor peak between 23:00-01:00.

Keywords: Malaria, Vectors, Species, Abundance, Thailand

Introduction

Information on mosquito biology, especially host feeding activity and preference of individual species is essential to identify their respective roles in disease transmission and assist vector control operations to apply the most appropriate strategy of vector control management. Other observations on biting cycles and host preferences of *An. minimus* have been made in Thailand, however, nearly all ecological and behavioral studies to date have been based on populations identified by morphological traits alone. More recently, the seasonal abundance of *An. minimus* and *An. harrisoni* adults in Kanchanaburi Province, one of the most endemic areas of malaria, were identified using accurate molecular methods. Besdies, there are several malarious area in this province that need an additional study in terms of vector populations and biology. In this study area, the blood feeding/biting behavior, host preference and seasonal abundance of individual species of *Anopheles* mosquitoes will be determined using a PCR-based molecular method over a two-year period in a small village of Sai Yok District, Kanchanaburi Province, Thailand.

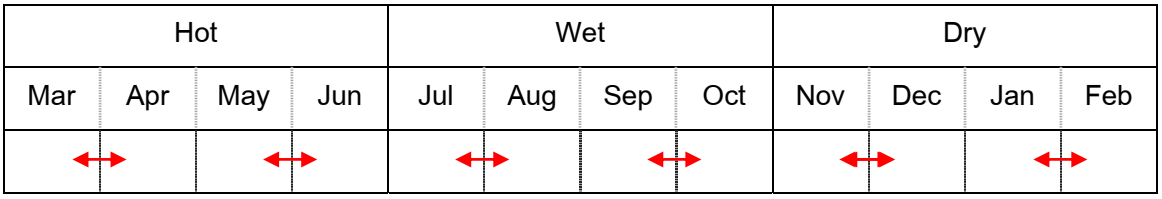
Materials and Methods

Collection site

Kanchanaburi is usually the one of top ten provinces in Thailand with highest malaria cases. The study will be conducted in Ban Pu Teuy, Tha Sao Subdistrict, Sai Yok District, Kanchanaburi Province is located in the western part of Thailand at the border with Myanmar. This area is a hilly zone and mostly surrounded by primary and secondary forest and with agricultural area. The main stream near the collection site is a small stream and covered with native vegetation along its margin.

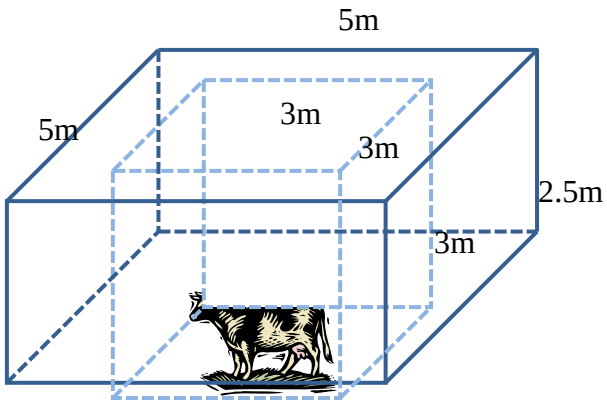
Collection method and Morphological identification

Anopheline mosquitoes will be collected two nights continuously in each site using a standard landing catch technique, including indoor and outdoor human baited landing, and cow baited dwelling, will be carried out. The collection will be conducted every two months, six collections each year.



Five people were in charge of live mosquito collections for each period. Collections were performed every hour from sunset to midnight (1800-2400 h; first period) and midnight to sunrise (2400-0600 h; second period). The first pair of collectors sat outside a house whereas the second pair sat inside a house. One hour landing collection consists of 45 minutes of collection and 15 minutes break. For the cow bait collection was made from the two-layers trap (inner layer is sized 3m x 3m x 3m and outer layer is sized 5m x 5m x 2.5m). One collector were prepared for mosquito captures on cow bed net in a gap between inner layer and outer layer of the trap each 15 minutes before the end of 1-hour intervals. The distance between collection points was approximately 100 m. The collectors observed the mosquitoes with flash lights, aspirate mosquitoes before they could bite and then place them in separate marked plastic cups covered with netting material, and changed every hour for recording the hourly biting peak for each species. Alive

mosquitoes were provided with 10% sugar solution. The temperature, humidity and rainfall will be recorded throughout the study period.



The following morning, each mosquito were sorted out using morphological criteria for a first identification at the complex or group level using morphological keys by Rattanarithikul *et al.* (2006). Mosquitoes were individually stored in labelled-tube (with the *Anopheles* group, type of bait and a number corresponding to the collection, and collection period). All specimen were bring back to the laboratory at Kasetsart University and kept at -20°C at laboratory of Department of Entomology, Kasetsart University for molecular identification.

Molecular identification of adult females

Anopheles mosquitoes were identified using the multiplex allele-specific polymerase chain reaction (AS-PCR) assay for distinguishes sibling species within the different complexes or groups.

Mosquito DNA extraction

The whole body of each *Anopheles* mosquito was placed in a separate labelled-tube and homogenized in 50 ml of extraction buffer (0.2 M sucrose, 0.1M Tris-HCl at pH 8.0, 50mM EDTA and 0.5% SDS). The tube is incubate at 65 °C for 30 min, add 7 µl 8mM KOAc (pH 9.0) and the tube place on ice for 30 min. Centrifuge at 12,000 rpm for 20 min and remove supernatant to a clean tube and add 100 µl of 100% ethanol then place into the 4°C refrigerator for 10 min. Centrifuge at 12,000 rpm for 20 min at 4°C to pellet the DNA. Wash the pellet with 150 µl of 70% ethanol and centrifuge at 12,000 rpm for 5 min at 4°C. Wash again with 100% ethanol and centrifuge at 12,000 rpm for 5 min at 4°C then the pellet dry at room temperature. Put 100 µl of deionized water (DNase, RNase-free) and store at -20 °C (Linton e*t al.,* 2001)

Amplification by PCR

Minimus Complex: The ITS2 region will be used to amplify the genomic DNA of Minimus Complex by the AS-PCR assay following the protocol of Garros *et al*. (2004). In a final volume of 25 µl, PCR amplification conditions are as follows: 1X of 5x reaction buffer, 200 µM of each dNTP, 64 nmol of each primer, 0.5 units of *Taq* DNA polymerase, and 1 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for two min, follow by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 sec, and extension at 72°C for 40 sec, and a final extension at 72°C for 5 min. Run electrophoresis of the PCR products on a 2% agarose gel.

Dirus Complex: The rDNA ITS2 will be used to amplify the genomic DNA of Dirus complex by the AS-PCR assay following the protocol of Walton *et al.* (1999). In a final volume of 25 µl, PCR amplification conditions are as follows: 1x of 5x reaction buffer, 200 µM of each dNTP, 1.5 mM MgCl2, 0.2 mM of each primer, 5 units of *Taq* DNA polymerase , 10% dimethylsulphoxide (DMSO) and 2 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for 5 min, follow by 32 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 10 min. Run electrophoresis of PCR products on a 2% agarose gel.

Maculatus Group: The rDNA ITS2 will be used to amplify the genomic DNA of Maculatus Group by the AS-PCR assay following the protocol of Walton *et al.* (2007). In a final volume of 25 µl, PCR amplification conditions are as follows: 5 µl of 5x reaction buffer, 200 uM of each dNTP, 1.5 mM MgCl2, 0.2 mM of primers 5.8F, MAC, DRAV, K and 0.1 mM of primers SAW and PSEU, 0.5 units of *Taq* DNA polymerase, 10% dimethylsulphoxide (DMSO) and 2 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for 5 min, follow by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. Run electrophoresis of the PCR products on a 2% agarose gel.

Data analysis

Three main study variables were selected for analysis, including seasons, time periods and different collection methods. Environmental parameters, temperature, and cumulative monthly rainfall, were used to determine association and affect on mosquitoes densities seen in collections. Seasons include hot wet and dry seasons. Collection time periods were subdivided into 3-h quarters: early evening (18:00-21:00), late evening (21:00-24:00), post-midnight (24:00-03:00), and pre-dawn (03:00-06:00). Collection categories were include indoor human-landing, outdoor human-landing, and cow baited collection. Differences in endophily, exophily, and number of species and individuals collected between sites were analyzed. Correlation analysis were used to investigate the interaction between the number of mosquitoes and environmental data. Comparison statistics were used for association between adult collection densities.

Results and Discussion

The results of this research during two-year period (March 2013 - January 2015) were shown in Figures 1-6 and Table 1. A total of 11,077 female anophelines were collected form location during this study period, representing 12 species. *Anopheles harrisoni* was the predominant species (92.50%) followed by *An. dirus* (2.65%), *An. minimus* (2.21%) and *An. maculatus* (1.44%).

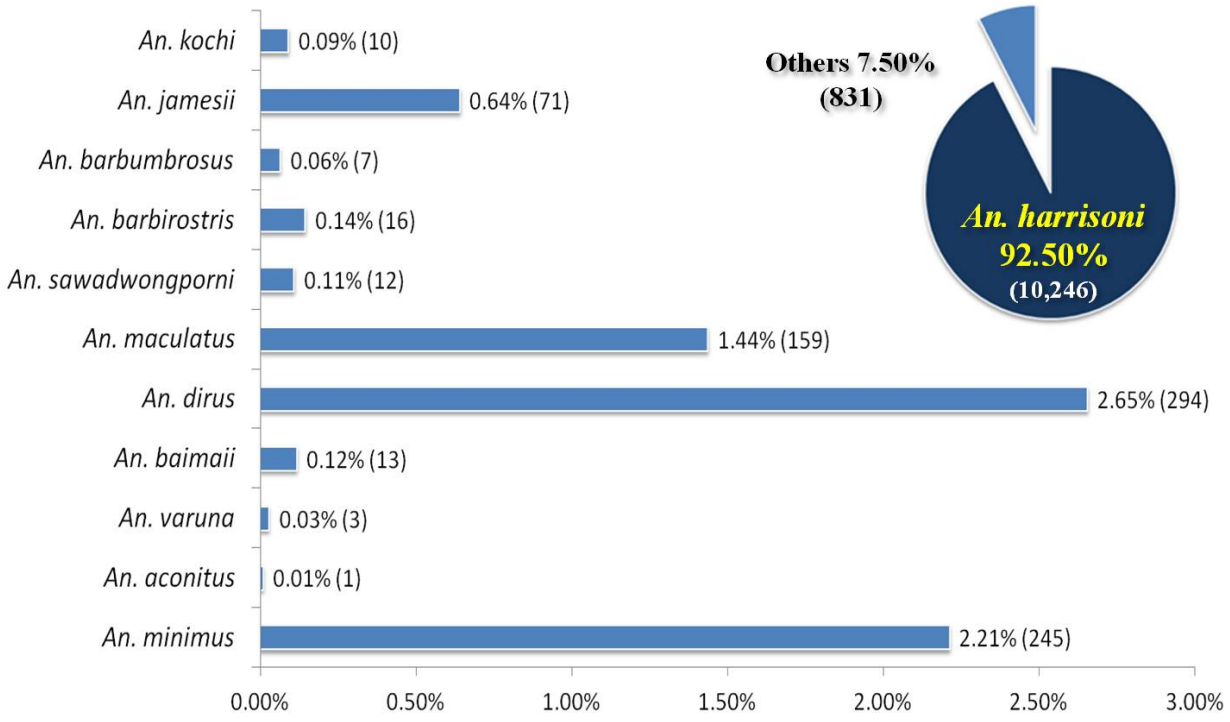


Figure 1 Overall relative proportion of anopheline mosquitoes by species collected from Ban Pu Teuy, Sai Yok District, Kanchanaburi Province during March 2013 to January 2015

As shown in the figure 2, the number of collected mosquito during the second year was very lower than first year, more than 75% of decrease number for *An. minimus*, *An. dirus* and *An. maculatus*. That could be caused by the pattern of rainfall is quite different. However, it was not include *An. harrisoni*. The pattern of rainfall had a small affect on the density of *An. harrisoni* (less than 25% of decrease number). The primary breeding habitat of *An. harrisoni* in this study area is the stream persists throughout the year which always presented of larva of this species with high density in all seasons (unpublished data). *Anopheles minimus* was also presented throughout the year but in very lower number. *Anopheles dirus* and *An. maculatus* populations had a presence in some months. From the results, *Anopheles dirus* and *An. maculatus* were obviously more abundant during the end of May to September. According to the previous studies (Muenworn et al., 2009; Sungvornyothin et al. 2009; Tananchai et al., 2012), the abundant of these two species at the end of hot season or the beginning of wet seasons through the end of wet seasons. In addition, this study found that there was positive association between the density of *An. dirus* with the cumulative monthly rainfall (R^2 =0.689, *P*<0.01), similar to *An.*

maculatus (R^2 =0.339, *P*<0.05). All four top species were not associated with ambient air temperature and relative humidity (*P*>0.05), except *An. dirus* that was also associated with relative humidity (R^2 =0.310, *P*<0.05).

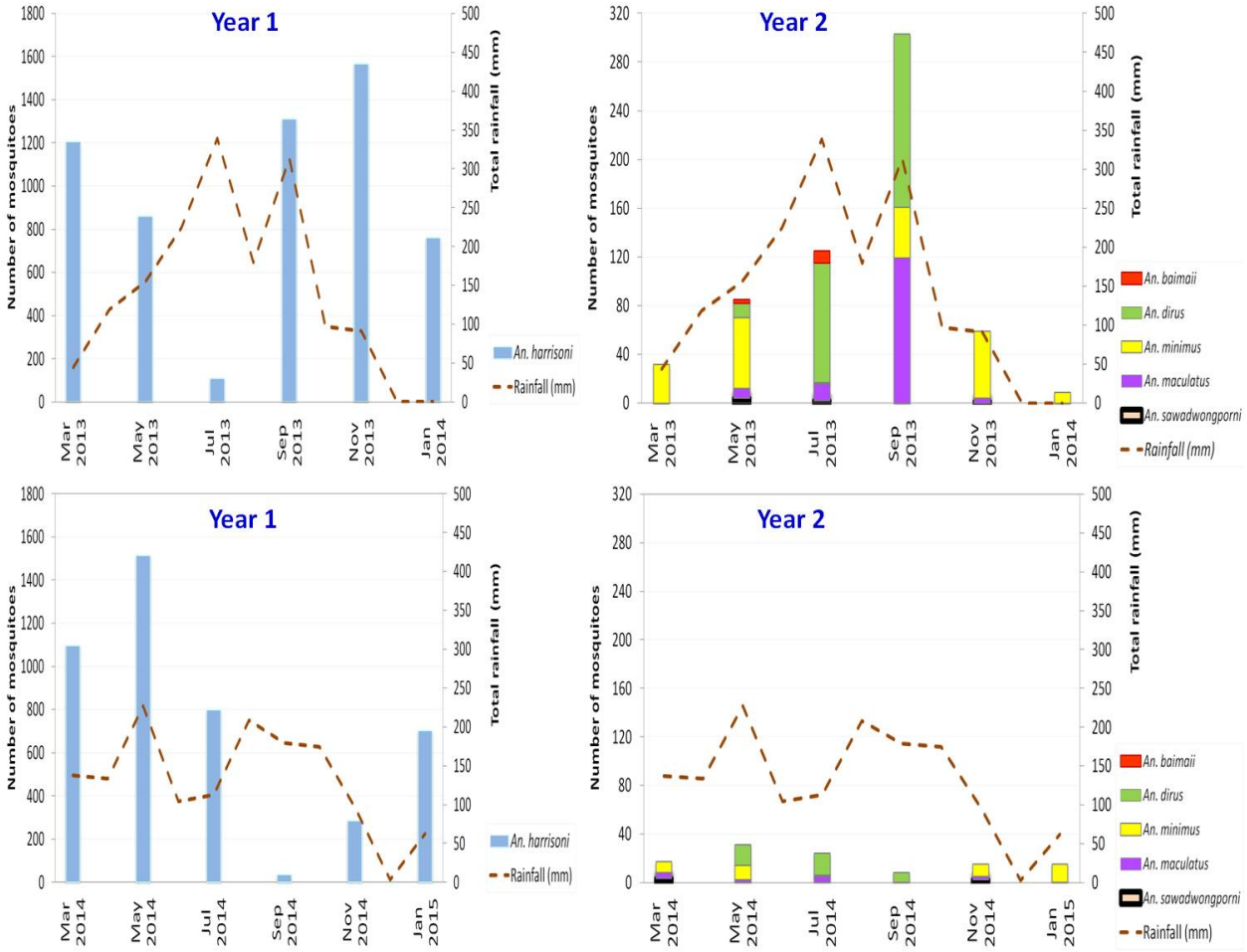


Figure 2 The total number by month of collected anopheline mosquito and rainfall pattern during two-year period

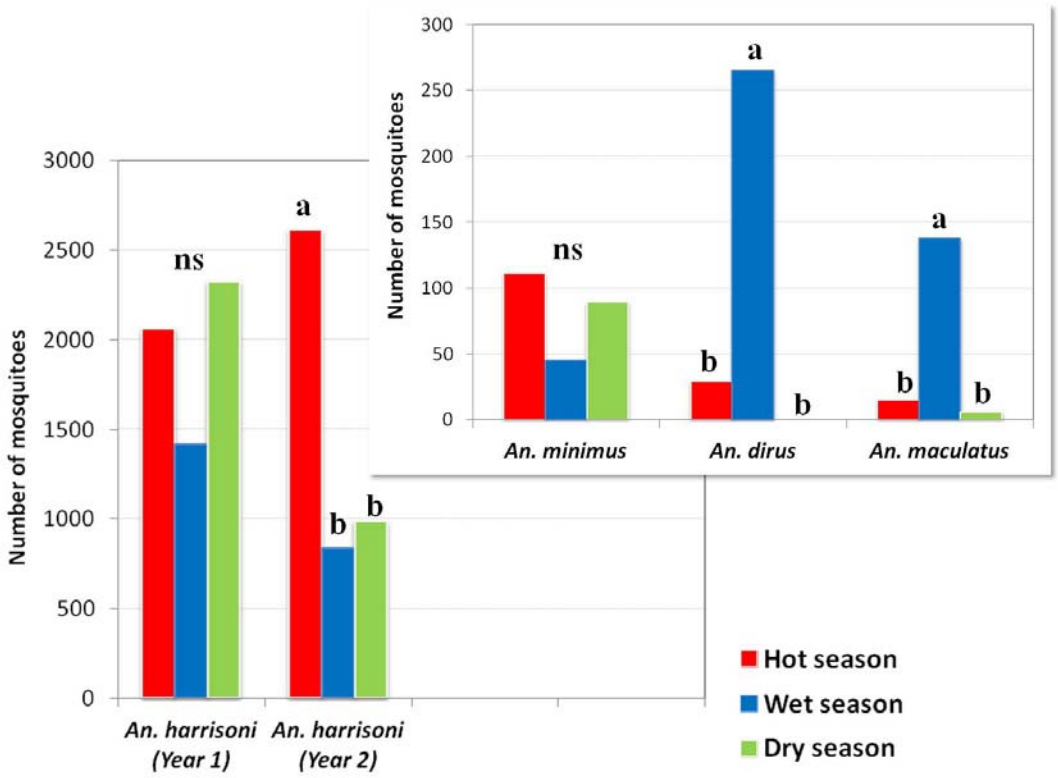


Figure 3 Total number of the top four most abundant *Anopheles* mosquitoes during different seasons

Significant differences in number of *An. harrisoni* was not found between seasons of first year, whereas the second year had significant difference between seasons. The highest number in the second year of *An. harrisoni* was occurred in hot season. The total abundance of *An dirus* and *An. maculatus* were significantly higher in the wet season.

Table 1 Ratio of host biting activity of anopheline mosquitoes

Species	Total number	Ratio
	Indoor : Outdoor : Cow	Indoor : Outdoor : Cow
Anopheles harrisoni	2833 : 3311 : 4102	1.0 : 1.2 : 1.4
Anopheles minimus	51 : 45 : 149	1.1 : 1.0 : 3.3
Anopheles dirus	109 : 124 : 61	1.8 : 2.0 : 1.0
Anopheles baimaii	4 : 5 : 4	1.0 : 2.3 : 3.5
Anopheles maculatus	21 : 54 : 84	1.0 : 1.2 : 1.4

As shown in table 1, all species were prefer both of outdoor activities than indoor activity especially biting activity on cow, except *An. dirus* rather prefer human blood meal than cow blood meal. However, there were no significant difference between host biting activities (or collection methods).

The pattern of host biting activity of three species groups for 3 collection methods are provided in figures 4-6. The biting activity of *An. harrisoni* and *An. minimus* occurred throughout the night, but the higher number of mosquito occur before midnight for all collection methods. The mosquitoes was appeared immediately after sunset and fewer thereafter. In the same way, as the outdoor activities of *An. dirus*. In contrast, the pattern of indoor human landing of *An. dirus* was higher activity after midnight.

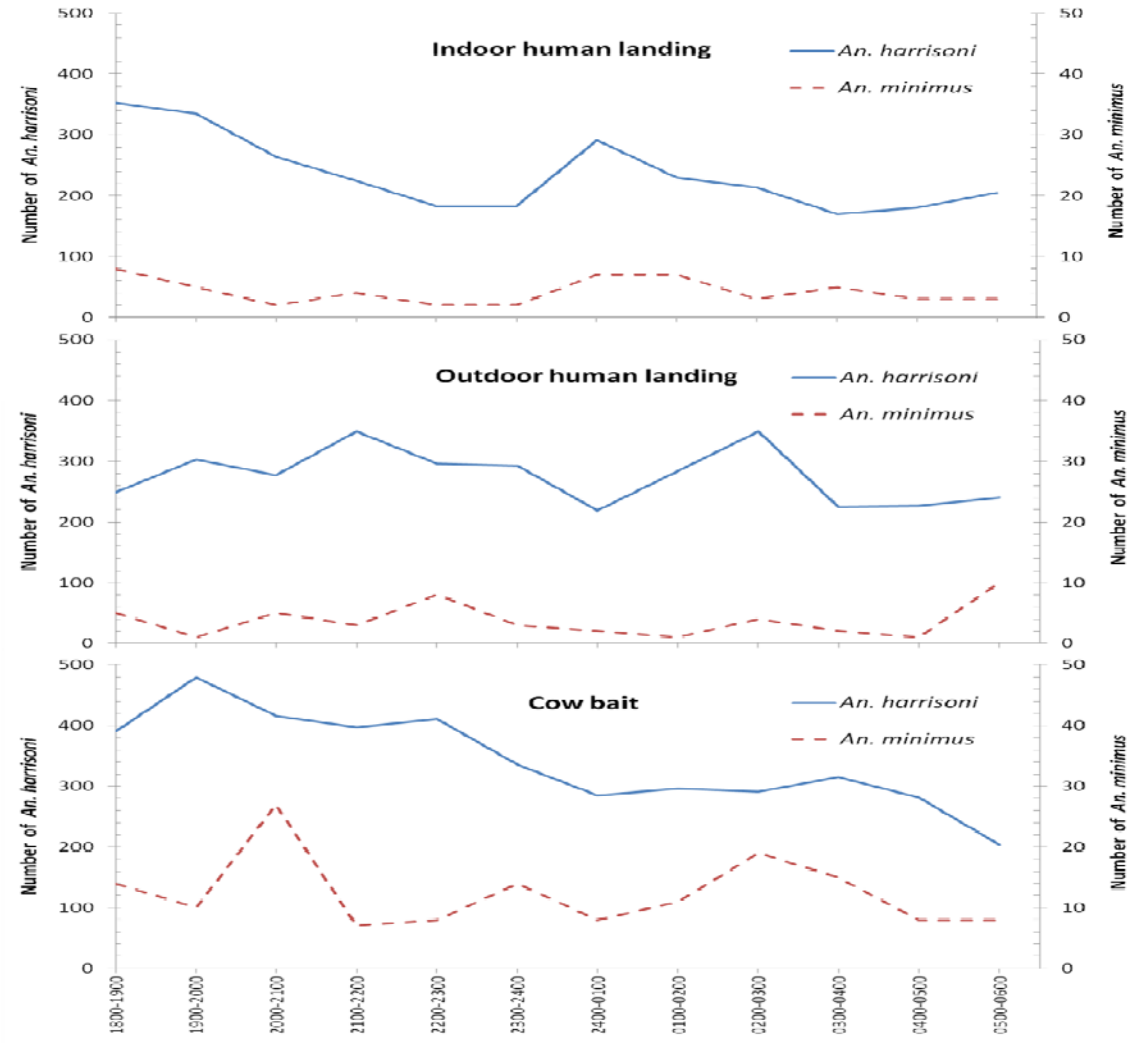


Figure 4 The host biting activity pattern of *An. harrisoni* and *An. minimus* for three collection methods

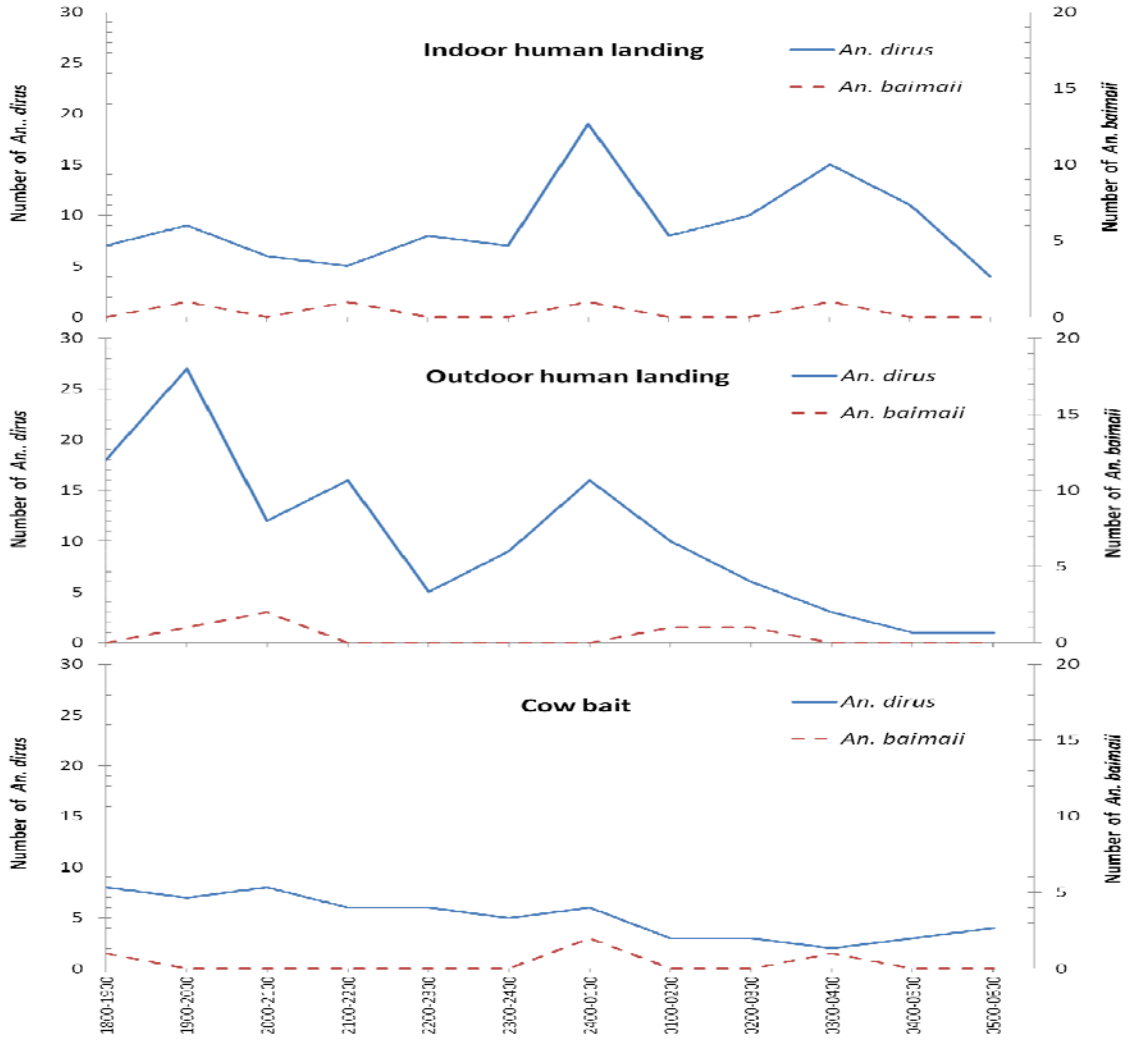


Figure 5 The host biting activity pattern of *An. dirus* for three collection methods

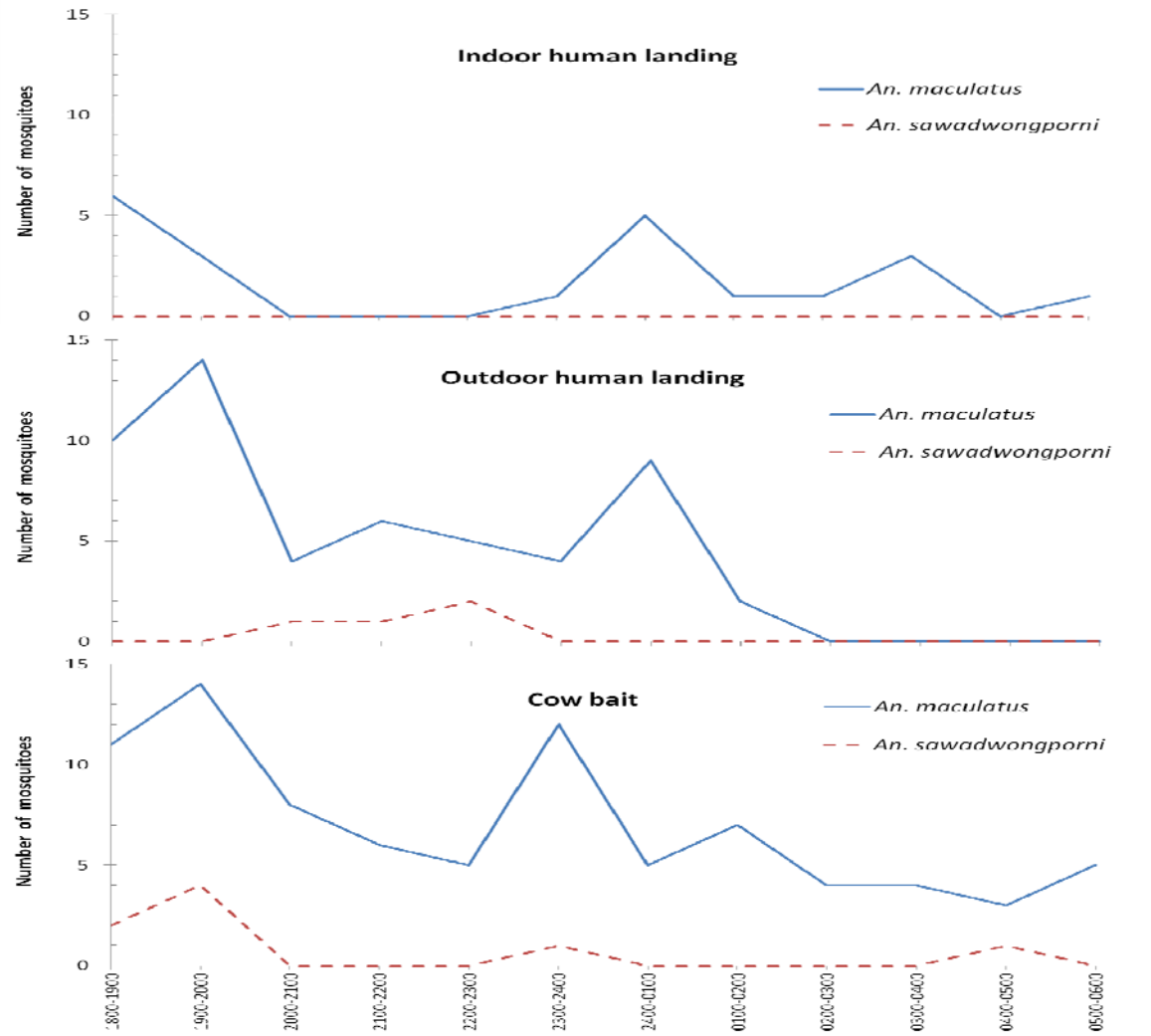


Figure 6 The host biting activity pattern of *Anopheles maculatus* for three collection methods

Biting activities of *An. maculatus* occurred throughout the night with a major peak during the early evening period (18:00-20:00) and the minor peak during before midnight to post-midnight period (23:00-01:00). These two biting peaks of *An. maculatus* was almost similar to the results of a previous study (Muenworn et al., 2009), that also occurred two biting peaks but shift in the time periods, a marked peak between 20:00-23:00 and a minor surge between 01:00-03:00.

Total number of mosquitoes during two-year period was used in a three-way ANOVA, with seasons (hot, wet and dry), collection methods (indoor and outdoor human-landing collections and cow-bait collection) and time intervals (early evening, late evening, post-

midnight and pre-dawn) as discriminating variables. There were significant difference of mosquito densities between seasons for *An. harrisoni*, *An dirus* and *An. maculatus.* The highest number of *An. harrisoni* was occurred in hot season, whereas *An dirus* and *An. maculatus* were highest in the wet season. However, there were no significant difference between collection methods, and also no significant difference between time period of collection.

Subproject 7: Developing *Plasmodium vivax* and *Plasmodium faciparum* cloning system for malaria detection in Anopheles mosquitoes Dr.Waraporn Juntrajumnong

Abstract

Malaria is an infectious disease caused by a parasite, *Plasmodium*, which infects red blood cells. The diseases are abundant and widely spread in the endemic area, including Thailand and neighbor countries. Both *Plasmodium* species are transmitted by *Anopheles* mosquitoes. Usually, Polymerase Chain Reaction is the alternative method to detect *Plasmodium spp.* especially *P. vivax* and *P. falciparum* which are often found in Thailand. Therefore, the detection of *P. vivax* and *P. falciparum* in *Anopheles* mosquitoes can be used to surveillance of malaria transmission in endemic area. However, the detection of *P. vivax* and *P. falciparum* in mosquitoes using PCR method usually required positive control from genomic DNA of both *Plasmodium* species extracted from infected patient blood samples or *Plasmodium* cell culture. The procedures to obtain *Plasmodium* genomic DNA from these specimens are quite complicated. Therefore, development of *P. vivax* and *P. falciparum* cloning system should be more convenient procedure to obtain positive control for *Plasmodium* detection in mosquitoes and also in the patients who are suspected to be infected with malaria. The results from these experiments show that we have success to clone *P. vivax* or *P. falciparum* in pGEM-T Easy vector. These clones can be preserved in glycerol which make it easy to transport under ambient temperature to the place where the detection of *Plasmodium* are performed. Although, malaria has been extensively studied, it still lacks more effective tools to improve its control. The most effective means to prevent malaria infection are protection of human from mosquito biting. Host seeking behavior involving in mosquito biting is regulated by circadian clock which is controlled by genes. One of genes controlled circadian clock is *timeless* (*tim*) which is a crucial element connecting the endogenous clock with the external environment. In addition, the environment, in term of temperature, gradually changes because of the global warming, many researchers attempt to find the relation between global warming and infectious disease. It is interested to investigate the levels of daily *tim* expression under different temperature affected of global warming in *An. dirus* which is one of main malaria vector in Thailand to understand the *tim* expression pattern under the effect of global

warming in the future. However, finding the suitable primers for *tim* expression study is the first step to come across. The results exhibited that new designed primers obtained from this experiment appropriate for detection of *tim* expression by real-time PCR using *rps*7 as constitutive control. In addition, the analysis of PCR product obtained from *tim* amplification in *An. dirus* revealed that DNA sequences of *tim* gene were high consensus to mRNA sequence from *An. gambiae* except an interval of 120 bp which were interpreted to be an intron region. This intron contained microsatellite sequence “CAG” repeats. Since, microsatellites have proved to be versatile molecular markers, particularly for population analysis, they can be developed for particular species can often be applied to closely related species. The representatives of 3 groups of major vectors in Thailand; Dirus, Minimus, Maculatus were studied molecular basis on short sequence of *tim* gene. Phylogenetic analysis of PCR product sequences showed that variation of intron sequence and length were the major factor of clade grouping. The result showed that *tim* gene can be used to assess the genetic variability and divergence among three sympatric cryptic species of this complex.

KEY WORDS *Anopheles*, *Plasmodium vivax*, *Plasmodium falciparum*, *Anopholes dirus*, timeless

Introduction

Malaria is one of the important diseases causing morbidity and mortality in many countries especially in tropical areas (Kazemi et al. 2005). The diseases are abundant and widely spread in the endemic area, especially Thailand and its neighbor countries (Chansiri et al. 2001). Malaria has been extensively studied, however it still lacks more effective tools to improve its control. The standard method for detecting *Plasmodium* infection is the microscopic examination of Giemsa-stained thick blood smears (TBS). This method is often time-consuming and requires experience and its sensitivity drops with the decrease of parasitemia (Scopel et al. 2004). The ELISA technique has been widely used for the diagnosis by binding between antigen and antibody. However, the cost of each test is very high and can become prohibitive (Cunha et al. 2009). It may also produce false positive results in some conditions (Durnez et al. 2011). DNA-based methods such as Polymerase Chain Reaction are sensitive to detect mixed and low level infections of *Plasmodium* especially *P. vivax* and *P. falciparum* which are often found in Thailand. Both *Plasmodium* species are transmitted by *Anopheles* mosquitoes. Therefore, the detection of *P. vivax* and *P. falciparum* in *Anopheles* mosquitoes can be used to surveillance of malaria transmission in endemic area. Usually, small subunit ribosomal RNA (ssRNA) or mitochondrial DNA can be used to detect *Plasmodium* (Chansiri et al. 2001). However, the success of the PCR technique depends on a variety of factors such as high quality DNA obtained from blood samples, good reagents and adequate conditions of amplification (Scopel et al. 2004). Hence, *Plasmodium* positive control need to be used in each time of *Plasmodium* detection to confirm that PCR condition is appropriate. Moreover, the PCR product size of *Plasmodium* positive control can be compared to PCR product size of *Plasmodium* detected in mosquitoes (Swain e al. 2009). Normally, positive control from genomic DNA of both *Plasmodium* species are extracted from infected patient blood samples or *Plasmodium* cell culture (Cunha et al. 2009, Winograd and Sherman 1989). The procedure to obtain these specimens for *Plasmodium* genomic DNA and the extraction method are quite complicate. *Plasmodium* genomic DNA extraction from infected blood requires the agreement from the ethical research committee before drawing venous blood (Aslan et al. 2007). *Plasmodium* cell culture needs to be performed using specific chemical and equipments (Winograd and Sherman 1989). In addition, Scopel (2004) reported that the

whole blood has been shown to be a reliable source of high-quality DNA when it is carefully transported, handled and stored to prevent contamination or degradation of the DNA. Therefore, PCR product of both *Plasmodium* species cloning in *Escherichia coli* should be more convenient procedure to obtain positive control for *Plasmodium* detection in mosquitoes. Since *E. coli* contained plasmid inserted with PCR product of *P. vivax* or *P. falciparum* mitochondrial DNA can grow under normal aerobic incubation to produce more cells (Sambrook et al. 1989). Moreover, these cells can be preserved in glycerol which make it easy to transport under ambient temperature to the place where the detection of *Plasmodium* are performed.

There are approximately 74 *Anopheles* species in Thailand. However, only *An. dirus* complex, *An. minimus* complex and *An. maculatus* group are considered to be main malaria vectors. However, *An. dirus* is consider to be one of major vectors involved in malaria transmission in Thailand and Southeast Asia (Sinka et al. 2011; Suwonkerd et al. 2013). Although, malaria has been extensively studied, it still lacks more effective tools to improve its control. The most effective means to prevent malaria infection are protection of human from mosquito biting. Host seeking behavior involving in mosquito biting is regulated by *timeless* which is a crucial gene to control circadian rhythms.

Circadian rhythms are widespread in living organisms and are controlled by endogenous clocks. Circadian pacemaker is controlled by a number of main genes which are *timeless* (*tim*), *period* (*per*), *clock* (*clk*) and *cycle* (*cyc*). Two of them, *clk* and *cyc*, encode transcription factors that together form the heterodimer CLK-CYC which activates the expression of *tim* and *per* (Gentile et al. 2006). However, *tim* is found to be the central to the control of the circadian pacemaker in *Drosophila melanogaster* (Myers et al. 1995; Sehgal et al. 1994). PER is dependent on its interaction with TIM for cycling and protection against phosphorylation and consequent degradation (Kloss et al. 2001). *Tim* also plays an essential role in resetting the endogenous clock upon light stimulation. Thus *tim* is a crucial element connecting the endogenous clock with the external environment. In addition, the environment, in term of temperature, gradually changes because of the global warming, many researchers attempt to find the relation between global warming and

infectious disease. It should be good to investigate the levels of daily *tim* expression under different temperature affected of global warming in *An. dirus* to understand the *tim* expression pattern of *An. dirus,* vector of malaria in Thailand under the effect of global warming in the future. However, finding the suitable primers for *tim* expression study is the first step to come across.

Usually, constitutive gene *actin* is used as constitutive control in the studies of *tim* expression since this gene can be amplified by universal primers (Staley et al. 2010). From previous study, we can amplify partial *actin* gene about 800 bp using primers obtained from universal primers from Stayley et al. 2010. In addition, partial *tim* gene was obtained in the size of > 1 kb using primers design by alignment of *Aedes*, *Anopheles* and *Culex* species from the previous report. However, the size of these PCR products are not appropriate to use in real-time PCR for detection of *tim* expression since it is larger than 200-300 bp. Therefore, *actin* and *tim* primers were newly designed by alignment of DNA sequences of *Ae. aegypti*, *Ae. albopictus*, *An. gambiae*, *Cu. quinquefasciatus* and *D. melanogaster*. These newly designed primers were used to test the amplification of *actin* and *tim* gene in *An. dirus*, also *An. minimus* and *An. epiroticus* which are major vector of malaria in Southeast Asia.

The appropriate identification of *Anopheles* species has direct medical and practical implications, such as in malaria control. Species identification was performed using either morphological or cytogenetic methods (Swain et al. 2009). The precisely morphological identification takes a long time since the entail holding wild-caught females for egg laying and rearing of larvae to fourth instars. Polytene chromosome identification is much faster than morphology. However, this technique can be used only in half gravid female. Therefore, molecular methods have received great attention in recent year to improve the accuracy of species identification (Collins and Paskwitz 1996). Molecular approach for species identification has mainly used rDNA since it is one of the multigene families frequently distributed in the genome. The specific parts of the gene encoding the ITS2 spacer of ribosomal DNA was permitted the identification of *An. minimus* group for rapid, easy and reliable identification (Garros et al. 2004). Molecular methods for species

identification have been frequently used in recent years. The methods have been applied to important groups of mosquito species complexes. Particularly important are complexes containing vectors of malaria and other vector-borne diseases. Correct and precise identification of the vector species has direct medical and practical implications, such as in vector control.

Behavior, especially circadian genes are also involved in the control of mating rhythms that are potentially important in maintaining sexual isolation between closely related species, involving in malaria transmission in mosquitoes is regulated by circadian rhythm (Rona et al. 2009). The circadian rhythms of malaria vectors belonging to the subgenus Kerteszia were formerly studied by Pittendrigh (1949) and, recently, these rhythms were also studied in *An. cruzii* (Chahad-Ehlers et al. 2007). The *tim* gene was also found to involve in the control of activity rhythms in *Drosophila*, and controls differences in mating rhythms between closely related *Drosophila* species. Therefore, in the study, a fragment of about 400 bp of *tim* gene was investigated whether it can be used as a molecular marker to assess intraspecific variability among populations of *Anopheles* spp., main malaria vector founded in Asia.

Materials and Methods

1. Cloning of *P.vivax* and *P. falciparum* in *E.coli*

***Plasmodium* cell culture.** Red blood cell culture infected with *P. vivax* and *P. falciparum were* kindly provided by Faculty of Tropical medicine, Mahidol University, Thailand

DNA extraction. Infected blood cell culture were extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) to obtain *Plasmodium vivax* and *P. falciparum* genomic DNA.

PCR amplification. Pf1 forward 5'-CCT GCA TTA ACA TCA TTA TAT GGT ACA TCT-3', Pf2 reverse 5'-GAT TAA CAT TCT TGA TGA AGT AAT GAT AAT ACC TT-3' and Pv1 forward 5'-AAG TGT TGT ATG GGC TCA TCA TAT G-3', Pv2 reverse 5'-CAA AAT GGA AAT GAG CGA TTA CAT-3' were used to amplify mitochondrial DNA of *P. falciparum* and *P. vivax*, respectively (Cunha et al. 2009). The PCR was performed in 20 µl PCR reaction mix using 5 µl of DNA template. The samples were heated at 96 °C for 10 min before 30 cycles of amplification at 95 °C 1 min, 60 °C 5 min followed by a final extension step at 60 °C for 1 h. Ten µl of PCR product was loaded to electrophoresis in 2.5% agarose gel with TAE buffer and stain with Gel Star.

PCR product cloning. Both *P. vivax* and *P. falciparum* PCR product were added polyA by Taq DNA polymerase at 72 °C for 10 min using PCR machine. The polyA added PCR products were ligated to pGEM-T Easy Vector System following manufacture protocol (Promega, USA). The pGEM-T Easy Vector containing *P. vivax* or *P. falciparum* PCR product were then transformed into *E. coli* DH5α and grown on LB plate containing X-Gal/IPTG/ampicillin overnight at 37°C.

Colony PCR. A white colony were picked to amplify a region of both *Plasmodium* species which were inserted in pGEM-T Easy Vector by PCR using M13 forward primer (5'-AGGGTTTTCCCAGTCAC-3') and M13 reverse primer (5'-GTCATAGCTGTTTCCTG-3'). The PCR products were analysed by electrophoresis in 0.8% agarose gels.

Glycerol stock. A single colony providing the expected bands from colony PCR was inoculated into LB media containing ampicillin and grown overnight with shaking. The entire culture were added glycerol to make 80% glycerol stock solution and kept in -80 $^{\circ}$C.

2. *Actin* and *tim* amplification

Anopheles dirus*, *An. minimus* and *An. epiroticus were kindly provided by Department of Medical Entomology, Faculty of Tropical medicine, Mahidol University, Thailand.

DNA extraction. DNA of mosquitoes was extracted using FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) to obtain genomic DNA.

PCR amplification. Act31106F (5'-ACTGGGACGATATGGAGAAGATCTGG-3'), Act31106R (5'-GAGCACAATACCGGTGGTACGAC-3'), Tim31101F (5'-TTGATGTA CAAGGACCAGCACATCAG-3'), Tim31101R (5'-GTTTGGTGGTACATTTGGTGGGAT TTG-3'), RPL8T1F (5'-GTGTTATTCGTGCAC AGCGTAAAGG-3'), RPL8T1R (5'-GCCT TACCGATGTGCTGATGGTTAC-3'), GAPDHT2F (5'-CGAAGATCGGAATCAACGGA TTTGGC-3'), GAPDHT2R (5'-CGAACTCGTTGTCGTACCAGGAGAT-3'), 18S31124F (5'-TTAGCTGCGGTCTTCATCGATCCA-3'), 18S31124R (5'-GTCCCTCGGGATTACA TTTGAATCGC-3') were used to amplify *actin*, *tim*, *rps7*, *rpl*8, *GAPDH* and 18sRNA, respectively. The PCR was performed in 25 µl PCR reaction mix using 3 µl of DNA template. The samples were heated at 94 $^{\circ}$C for 1 min before 30 cycles of amplification at 94 $^{\circ}$C 60 sec, 55 $^{\circ}$C 30 sec, 72 $^{\circ}$C 60 sec followed by a final extension step at 72 $^{\circ}$C for 10 min. Twenty µl of PCR product of *timeless* gene was loaded to electrophoresis in 2.0% agarose gel with TAE buffer and stain with Gel Star. The expected bands were cut and sent to sequence.

Sequencing. Agarose gel contained *timeless* PCR product were sent to Macrogen for sequencing.

Primer design. BioEdit Sequence Alignment Editor and AmplifX were used to design primer.

3. Verify the *rps7* and *tim* primers for real-time PCR

Mosquitoes. *Anopheles dirus* female were kindly provided by Department of Medical Entomology, Faculty of Tropical medicine, Mahidol University, Thailand.

DNA extraction. DNA of mosquitoes was extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp.) to obtain genomic DNA.

RNA extraction. RNA of mosquitoes was extracted using FavorPrep™ Tissue RNA Extraction Mini Kit (Favorgen Biotech Corp.).

cDNA synthesis. cDNA synthesis was conducted using ''ReverTra Ace® qPCR RT Master Mix'' (Toyobo). Reverse transcription was carried out at 37 °C for 60 min, 50 °C for 5 min, and 98 °C for 5 min.

PCR amplification. Act31106F (5'-ACTGGGACGATATGGAGAAGATCTGG-3'), Act31106R (5'-GAGCACAATACCGGTGGTACGAC-3'), Tim31101F (5'-TTGATGTAC AAGGACCAGCACATCAG-3'), Tim31101R (5'-GTTTGGTGGTACATTTGGTGGGATT TG-3'), RPS7T1F (5'-TGGAGATGAACTCGGACCTGAAGC-3'), RPS7T1R (5'-GACGT GCTTGCCGGAGAACTT) were used to amplify *actin*, *tim* and *rps*7, respectively. The PCR was performed in 25 µl PCR reaction mix using 3 µl of DNA template. The samples were heated at 94 °C for 1 min before 30 cycles of amplification at 94 °C 60 sec, 55 °C 30 sec, 72 °C 60 sec followed by a final extension step at 72 °C for 10 min. Twenty µl of PCR product of *tim* gene was loaded to electrophoresis in 2.0% agarose gel with TAE buffer and stain with Gel Star. The expected bands were cut and sent to sequence.

Real-time PCR. Real-time PCR was conducted in Bio-Rad using the SYBR Green PCR Master Mix (KAPA Biosystems). Reactions were carried out for *tim* and constitutive gene *rps*7. The amplifying a fragment of *tim* and *rps*7 are 360 and 180 bp, respectively. A reading temperature of hold at 95 °C for 3 min; 40 cycles of three temperatures: 95 °C for 5 sec, 50-60 °C for 20 sec, and 72 °C for 15 s, determined by analysis of the melting

curve of each amplicon. The reading temperature is established in order to certify that the measured fluorescence is from the amplicon without interference from fluorescence generated by primer dimers.

Sequencing. Agarose gel contained *tim* PCR product were sent to Macrogen for sequencing.

Primers design. BioEdit Sequence Alignment Editor and AmplifX were used to design primer.

4. Investigation of *tim* as molecular marker

Mosquitoes. Female *An. minimus* groups were kindly provide by Dr.Krajana Tainchum, Dr.Wanapa Ritthison from Department of Entomology, Faculty of Agriculture, Kasetsart University and Dr.Sungsit Sungvornyothin from Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. All mosquito samples used in this study were characterized by morphology according to Rattanarithikul (1996) and confirmed by molecular biology according to Garros et al. (2004), Walton et al. (1999) and Walton et al. (2007).

DNA extraction. DNA of mosquitoes was extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp.) to obtain genomic DNA.

RNA extraction. RNA of mosquitoes was extracted using FavorPrep™ Tissue RNA Extraction Mini Kit (Favorgen Biotech Corp.).

cDNA synthesis. cDNA synthesis was conducted using ‘‘ReverTra Ace® qPCR RT Master Mix’’ (Toyobo). Reverse transcription was carried out at 37 $^{\circ}$C for 60 min, 50 $^{\circ}$C for 5 min, and 98 $^{\circ}$C for 5 min.

PCR amplification. Tim31101F (5’-TTGATGTACAAGGACCAGCACATCAG-3’), Tim31101R (5’-GTTTGGTGGTACATTT GGTGGGATTTG-3’) were used to amplify *tim*. The PCR was performed in 25 µl PCR reaction mix using 3 µl of DNA template. The samples were heated at 94 $^{\circ}$C for 1 min before 30 cycles of amplification at 94 $^{\circ}$C 60 sec, 55 $^{\circ}$C 30 sec, 72 $^{\circ}$C 60 sec followed by a final extension step at 72 $^{\circ}$C for 10 min. Twenty µl of PCR product of *tim* gene was loaded to electrophoresis in 2.0% agarose gel with TAE buffer and stain with Gel Star. The expected bands were cut and sent to sequence.

Results

Cloning of *P.vivax* and *P. falciparum* in *E.coli*

Plasmodium vivax and *P. falciparum* genomic DNA obtained from blood cell culture were amplified by PCR. The expected 273 bp and 290 bp bands were obtained in PCR product obtained from *P. falciparum* and *P. vivax,* respectively. The results exhibited that both of primers (Cunha et al. 2009) are specific to amplify mitochondrial DNA of *P. vivax* and *P. falciparum* since only single band of both *Plasmodium* species were observed on the gel (Fig. 1). Both PCR products were then add polyA at the end of each DNA strand by PCR reaction but only dATP was added in the reaction. The PCR reaction was incubated at 72 $^{\circ}$C for 10 min similar to the final extension in standard PCR cycle. The polyA added *P. vivax* and *P. falciparum* PCR product were ready to ligate to pGEM-T Easy vector which contain T overhang at insertion site. The polyA of both *Plasmodium* species could bind to T overhang. This could make good efficiency of ligation. The inserted pGEM-T was then transformed in to *E. coli* DH5α and grown on LB plate containing X-Gal/IPTG/ampicillin overnight. Only white colonies were expected to be pGEM-T containing *P. vivax* or *P. falciparum* PCR fragment since the inserted fragment causes inactivation of beta-galactosidase, which otherwise is expressed and produces blue coloration seen clearly in the bacterial colonies. Therefore, successfully cloned colonies can be selected based on the lack of beta-galactosidase activity, which has been inactivated by the insert. This blue/white selection screen, in addition to antibiotic selection adds to the robustness of the pGEM-T vector systems. The selected white colonies can then be picked from the plate, cultured up to required quantities and used as reservoirs of the plasmid carrying the cloned fragment of interest. White colonies were picked to amplify a region of both *Plasmodium* species which were inserted in pGEM-T Easy Vector by PCR to confirm that white colony has pGEM-T containing *P. vivax* or *P. falciparum* PCR fragment. The expectd 477 bp and 494 bp bands were obtained in PCR product obtained from pGEM-T containing *P. falciparum* and pGEM-T containing *P. vivax,* respectively (Fig. 2). Therefore, white colony providing the expected bands from colony PCR was inoculated into LB media containing ampicillin and grown overnight with shaking. The entire culture was added glycerol to make 80% glycerol stock solution and kept in -80 $^{\circ}$C until use.

Actin and *tim* amplification

Timeless primers were newly designed by alignment of DNA sequences of four importance vector species from three genera including *Ae. albopictus*, *Ae. aegypti*, *An. gambiae*, *Cx. quinquefasciatus* and *D. melanogaster* since there is no report of *tim* gene of *An. dirus*. Partial *tim* gene of *An. dirus* was amplified using Tim31101F and Tim31101R primers. The expected about 300 bp was obtained from PCR product of *An. dirus*, *An. minimus* and *An. epiroticus* including male and female. Although several bands were observed in *An. epiroticus* both male and female, about 300 bp bands were still observed on the gel. Therefore, the 300 bp bands were cut from the gel and sent to sequence. The results exhibited that Tim31101F and Tim31101R primers can be used to amplify partial *tim* gene of *An. dirus* and *An. minimus* since only single band were observed on the gel (Fig. 3). However, it seems that *actin* gene in three mosquito species in this experiment cannot be amplified by newly designed actin primer for some reasons even the conditions of PCR was modified (Fig. 4). Therefore, some other housekeeping genes including *rpl*8, *GAPDH* and 18s RNA were tested instead of *actin* gene. The results showed that *rpl*8 primers should be better candidate primers than *GAPDH* and 18s RNA since one major band of *rpl*8 can be observed in *An. minimus* and *An. epiroticus* both male and female whereas one major band of *GAPDH* can be observed only in *An. epiroticus* male and no bands was observed in all three species (Fig. 5).

The sequencing results of partial *tim* gene from gDNA show in Figure 6. The results exhibited that Tim31101F and Tim31101R primers can be used to amplify partial *tim* gene of *An. dirus*. In addition, these *tim* primers were used to amplify cDNA to check whether *tim* RNA can be detected under normal rearing condition. The results exhibited that two bands of PCR product can be observed on agarose gel (Fig. 8). However, major band is most suspected to be the product obtained from *tim* gene amplification whereas minor band is somehow nonspecific band. Therefore the major band was cut and sent to sequence. The sequencing results of partial *tim* gene from cDNA show in Figure 7. This obtained sequence is very similar to the sequence obtained from genomic DNA. The length of PCR product of cDNA template is shorter than PCR product of gDNA template.

The *rps*7 primers were newly designed by alignment of cDNA sequences of *Ae. albopictus*, *Ae. aegypti*, *An. gambiae*, *An. dirus*, *An. stephensi*, *Cx. quinquefasciatus*, *Cx. pipiens* and *Armigeres subalbatus*. Partial *rps*7 gene of *An. dirus* was amplified using RPS7T1F and RPS7T1R primers. The expected about 200 bp was obtained from PCR product of *An. dirus* exhibited that RPS7T1F and RPS7T1R primers can be used to amplify partial *rps*7 gene of *An. dirus* (Fig. 10). In addition, these *rps*7 primers were used to amplify cDNA. The results exhibited that two bands of PCR product can be observed on agarose gel (Fig. 11). However, major band is most suspected to be the product obtained from *rps*7 gene amplification whereas minor band is somehow nonspecific band. Therefore the major band was cut and sent to sequence. The sequencing results of partial *rps*7 gene from cDNA show in Figure 12.

The *tim* and *rps*7 primers were then used in real-time PCR to check whether these primers can provide only one peak of melt peak of each gene to certify that the measured fluorescence is from the amplicon without interference from fluorescence generated by primer dimers. The result of melting curve using various annealing temperature are illustrated in Figure 13 and 14. Only one peak of each amplicon can be observed in any temperature varied between 50-60 $^{\circ}$C.

Investigation of *tim* as molecular marker

Since the obtained sequence of *tim* cDNA is very similar to the sequence obtained from gDNA. The length of PCR product of cDNA template is shorter than PCR product of gDNA template. The sequences of *tim* gene from gDNA and cDNA were then aligned by clustal W. The result exhibited that the PCR product contained intron region which is about 120 bp. In addition, microsatellite contained sequence “CAG” repeats (Fig. 15). Since, microsatellites have proved to be versatile molecular markers, particularly for population analysis, they can be developed for particular species can often be applied to closely related species. Therefore, the 8 species of *Anopheles* mosquitoes were used to verify the capability of *tim* gene to identify species of *Anopheles* mosquitoes which are main malaria vectors in Thailand. The gDNA of *tim* gene from 8 species of Anopheles mosquitoes such

as *An. dirus, An. baimaii, An. sawadwongporni, An. dravidicus, An. minimus, An.aconitus, An. varuna, An. epiroticus* was amplified by Tim31101F and Tim31101R primers and the was sent for sequencing. The sequences of only intron were analyzed by Clustal W using neighbour-joining method to investigate phylogenetic relation of *An. minimus, An. dirus* and *An. maculatus* complex. *Anopheles epiroticus* was used for outgroup. The result showed that *tim* gene can be used to assess the genetic variability and divergence among three sympatric cryptic species of this complex (Fig. 16).

Discussion

Cloning of *P. vivax* and *P. falciparum* in *E.coli*

Previously, molecular techniques for *Plasmodium* detection are nested-PCR (Snounou et al. 1993), real-time PCR (Mens et al. 2006). However, for routine screening a great number of samples are examined, the analysis using nested-PCR is limited since it is laborious and expensive method. Although real-time PCR is very sensitive and fast, this method is very expensive and required the specialist to interpret the result. The simple PCR method which is cheaper, faster and sensitive was therefore applied in this study using mitochondrial DNA amplification to detect *P. vivax* and *P. falciparum*. This method is less laborious and time-consuming when compared to nested PCR. Therefore, the detection of *P. vivax* and *P. falciparum* in *Anopheles* mosquitoes can be used to surveillance of malaria transmission in endemic area. However, positive control from genomic DNA of both *Plasmodium* species are normally extracted from infected patient blood samples or *Plasmodium* cell culture (Cunha et al. 2009, Winograd and Sherman 1989) to confirm that nothing is going wrong in the PCR reaction and PCR cycle. The procedure to obtain these specimens for *Plasmodium* genomic DNA and the extraction method are quite complicate. In addition, Scopel (2004) reported that the whole blood has been shown to be a reliable source of high-quality DNA when it is carefully transported, handled and stored to prevent contamination or degradation of the DNA. Therefore, PCR product of both Plasmodium species cloning in *E. coli* should be more convenient procedure to obtain positive control for *Plasmodium* detection in mosquitoes. Since *E. coli* contained plasmid inserted with PCR product of *P. vivax* or *P. falciparum* mitochondrial DNA can grow under normal aerobic incubation to produce more cells (Sambrook et al.

1989). The results from these experiments show that we have success to clone *P. vivax* or *P. falciparum* in pGEM-T Easy vector. These clones can be preserved in glycerol which make it easy to transport under ambient temperature to the place where the detection of *Plasmodium* are performed.

Actin **and** ***tim*** **amplification**

Molecular marker of major malaria vector is important for basic and applied research for vector biology and modern vector control. *Actin* gene can be a good candidate to be used as house-keeping gene which conserved across genera of mosquito since it is typically constitutive genes which are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions (http://en.wikipedia.org/wiki/Housekeeping_gene). Housekeeping genes are commonly used as endogenous references in quantitative Real-time PCR. A normalization of the target gene data with up-regulated housekeeping genes would equalize or underestimate up-regulated data and overestimate down-regulated data.

In *Drosophila*, *tim* is one of the main genes regulating the endogenous clock, and is involved in the negative feedback control of the circadian clock's central loop (Hardin, 2005) as well as in its resetting by the light stimulus (Ceriani et al., 1999). It seems to be one of the most conserved clock genes in Drosophilidae (Myers et al., 1997; Nishinokubi et al., 2003). For example, when compared to *D. melanogaster*, the identity of amino acid sequence is 76% for *D. virillis* (Ousley et al., 1998). A similar level of conservation (73.9% identity) is observed between the TIM proteins of *Ae. aegypti* and *An. gambiae*. The identity is low (around 50%) when *Ae. aegypti* was compared to *D. melanogaster* (Ousley at al., 1998; Saez and Young 1996). *Tim* has been characterized in a few other insect species, revealing sequence similarity across most of the gene especially in domains known in *D. melanogaster* to be important in keeping the endogenous clock, suggesting functional conservation within insects (Chang et al., 2003; Goto and Denlinger, 2002). However, *tim* gene of *An. dirus* which is a major vector of malaria in Southeast Asia is never studied before. Therefore, the results of Daily expression of the *tim* gene in *An. dirus* will be the first step to understand the molecular regulation of this mosquito endogenous clock, which controls the activity and biting rhythms (Clements, 1999) that are

so important in the dynamics of disease transmission. From the results, it can be suggested that we can find the *tim* primers appropriate for detection of *tim* expression by real time PCR and *rps*7should be the good candidate of housekeeping gene. Since the size of PCR product of both genes are about 200-300 bp.

Investigation of *tim* as molecular marker

The species distribution and biology of Anophelines has been limited due to the absence of reliable diagnostic characteristic (Swain et al. 2009). Morphological method is difficult to apply because members of closely related groups have overlapping morphologic characters (Garros et al. 2004). *Anopheles funestus* and *An. minimus* groups belong to the *Myzomyia* series, subgenus *Cellia*, and are closely related based on various cytogenetic and molecular studies (Green 1982 and Pape 1992). They are probably considered distinct only because of their geographic separation. Vectorial variations found among these species groups or complexes constitute the major reason for the accurate and precise identification (Garros et al. 2004). Therefore, molecular techniques are used to resolve identification issue. The advantages of molecular approach are easy to perform, sensitive, applicable to all stages of mosquitoes (Swain et al. 2009) using molecular marker which is very important for basic and applied research for vector biology and modern vector control.

The rDNA has been used for molecular identification for *Anopheles* species complex. Concerted evolution acting on rDNA arrays maintains sequence homogeneity only within species explaining that the rDNA can be used for species-specific diagnostic (Collins and Paskwitz 1996). This locus has many advantages: the rDNA represents in multiple copies throughout the genome in mosquitoes leading to the high amplification signal. It is also contains variable region including ITS2 which facilitate the selection of primer binding sites for each species to obtain specific amplification product in different sizes (Swain et al. 2009). Therefore, the molecular species identification was performed using specific primer in ITS2 region reported by Garros et al. 2004. However, the circadian clock controls several important biological activities, including mating rhythms, which may be involved in temporal reproductive isolation between closely related species (Sakai & Ishida 2001,

Tauber et al. 2003). The molecular control of circadian rhythms involves several clock genes (Hardin 2005), among them *tim* (Myers et al. 1995) is crucial element. Rona et al. (2009) found genetic variation in the timeless gene that was useful for distinguishing two cryptic species among Brazilian populations in the *An. cruzii* complex of the subgenus Kerteszia. Additionally, their results suggested that populations of *An. cruzii* from South and Southeast Brazil also constitute distinct incipient species of this complex.

In *Drosophila*, *tim* is one of the main genes regulating the endogenous clock, and is involved in the negative feedback control of the circadian clock's central loop (Hardin, 2005) as well as in its resetting by the light stimulus (Ceriani et al., 1999). It seems to be one of the most conserved clock genes in Drosophilidae (Myers et al., 1997; Nishinokubi et al., 2003). For example, when compared to *D. melanogaster*, the identity of amino acid sequence is 76% for *D. virillis* (Ousley et al., 1998). A similar level of conservation (73.9% identity) is observed between the TIM proteins of *Ae. aegypti* and *An. gambiae*. The identity is low (around 50%) when *Ae. aegypti* was compared to *D. melanogaster* (Ousley at al., 1998; Saez and Young 1996). *Tim* has been characterized in a few other insect species, revealing sequence similarity across most of the gene especially in domains known in *D. melanogaster* to be important in keeping the endogenous clock, suggesting functional conservation within insects (Chang et al., 2003; Goto and Denlinger, 2002). However, *tim* gene of *Anopheles* which is a major vector of malaria in Asia is never studied before. Therefore, in the study, a fragment of ~400 bp of *tim* gene was investigated whether it can be used as a molecular marker to assess intraspecific variability among populations of *Anopheles* spp., main malaria vector founded in Asia. The result exhibited that DNA sequences of *tim* gene were high consensus to mRNA sequence from *An. gambiae* except an interval of 120 bp which were interpreted to be an intron region. In addition, microsatellite contained sequence "CAG" repeats. Since, microsatellites have proved to be versatile molecular markers, particularly for population analysis, they can be developed for particular species can often be applied to closely related species. The result showed that timeless gene can be used to assess the genetic variability and divergence among three sympatric cryptic species of this complex.

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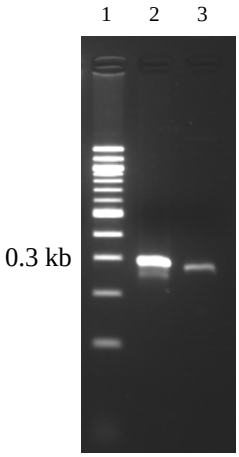


Figure 1 Amplification of mitochondrial DNA of *P*. *falciparum* and *P*. *vivax*. Lane 1: molecular marker; lane 2-3: amplified fragments obtained from *P. vivax* and *P. falciparum* from red blood cell culture, respectively.

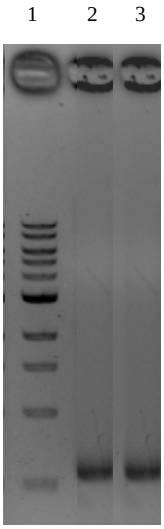


Figure 2 Colony PCR of mitochondrial DNA of *P*. *falciparum* and *P*. *vivax* PCR product inserted in pGEM-T Easy vector. Lane 1: molecular marker; lane 2-3: PCR product amplified by PCR with M13 primers from pGEM-T Easy vector containing *P. falciparum* and *P. vivax*, respectively.

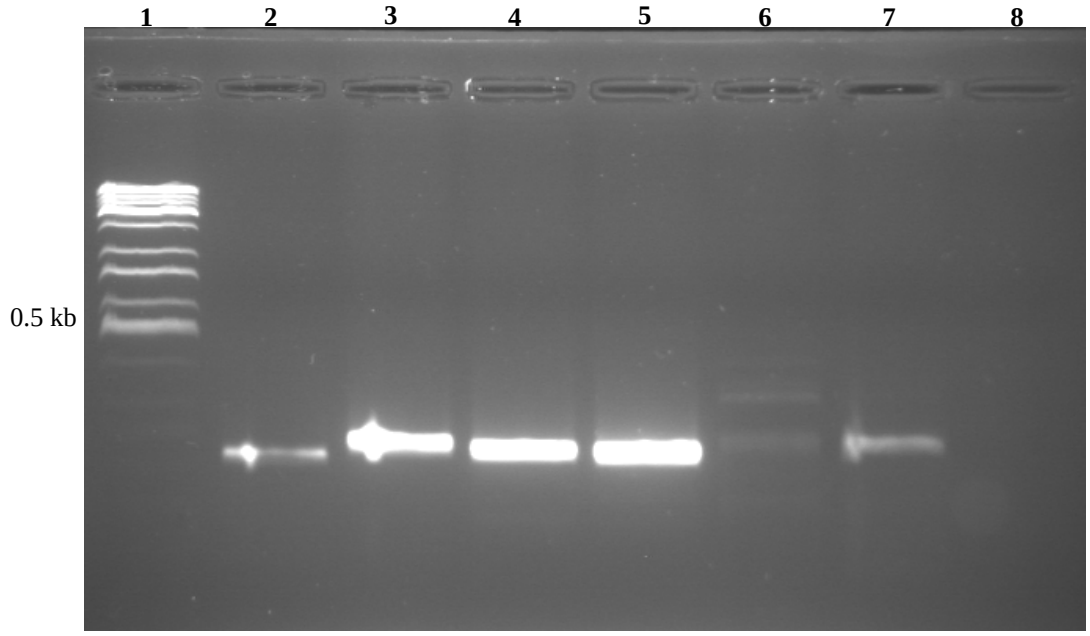


Figure 3 Amplification of partial *timeless* gene of *Anopheles dirus*, *An.minimus* and *An. epiroticus*. lane 1: molecular marker; lane 2: amplified fragments obtained from partial *timeless gene* from *An. dirus* male; lane 3: amplified fragments obtained from partial *timeless* gene from *An. dirus* female; lane 4: amplified fragments obtained from partial *timeless* gene from *An. minimus* male; lane 5: amplified fragments obtained from partial *timeless* gene from *An. minimus* female; lane 6: amplified fragments obtained from partial *timeless* gene from *An. epiroticus* male; lane 7: amplified fragments obtained from partial *timeless* gene from *An. epiroticis* female and lane 8: negative control, respectively.

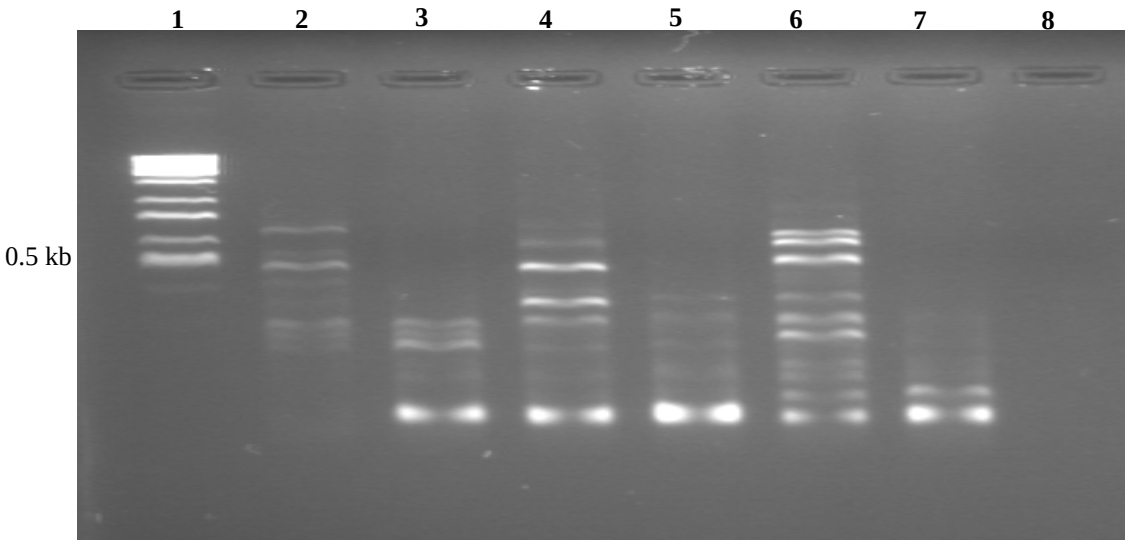


Figure 4 Amplification of partial *actin* gene of *Anopheles dirus*, *An.minimus* and *An. epiroticus*. lane 1: molecular marker; lane 2: amplified fragments obtained from partial *actin gene* from *An. dirus* male; lane 3: amplified fragments obtained from partial *actin* gene from *An. dirus* female; lane 4: amplified fragments obtained from partial *actin* gene from *An. minimus* male; lane 5: amplified fragments obtained from partial *actin* gene from *An. minimus* female; lane 6: amplified fragments obtained from partial *actin* gene from *An. epiroticus* male; lane 7: amplified fragments obtained from partial *actin* gene from *An. epiroticis* female and lane 8: negative control, respectively.

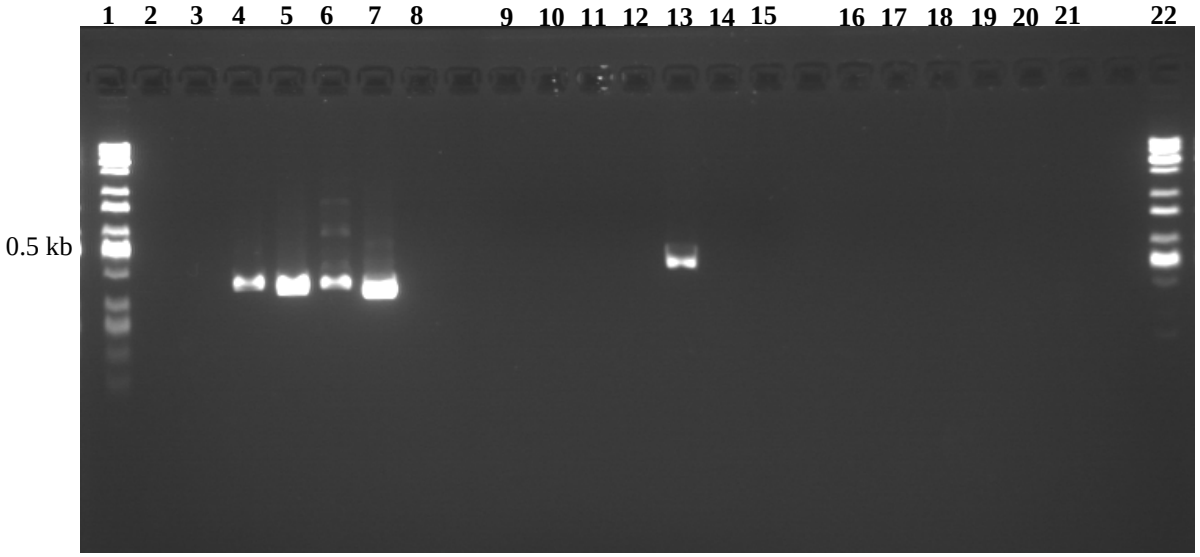


Figure 5 Amplification of partial *rpl*8, *GAPDH* and 18s RNA gene of *Anopheles dirus*, *An.minimus* and *An. epiroticus*. lane 1: molecular marker; lane 2-8: amplified fragments obtained from partial *rpl*8 gene from *An. dirus, An.minimus*, *An. epiroticus* (male and female) and negative control, respectively; lane 9-15: amplified fragments obtained from partial *GAPDH* gene from *An. dirus, An.minimus*, *An. epiroticus* (male and female) and negative control, respectively; lane 16-21: amplified fragments obtained from partial 18s RNA gene from *An. dirus, An.minimus*, *An. epiroticus* (male and female) and negative control, respectively; lane 22: molecular marker.

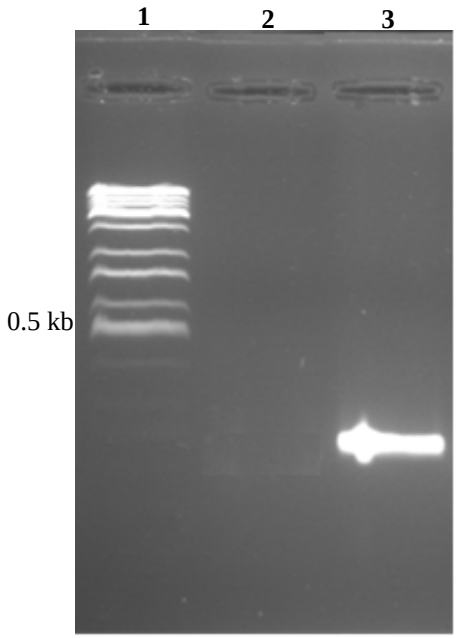


Figure 6 Amplification of partial *timeless* gene of *An. dirus.* lane 1: molecular marker; lane 2: negative control; lane 3: amplified fragments obtained from partial *timeless gene* from *An. dirus*, respectively.

```
         10         20         30         40         50         60         70         80         90        100
TGTGGTTCGAGTCGACGCTGTCGGAGAGCTCGGAAGACAACGAGAGCAATACGTCGCCGCCGAAGCAGTGCAGCGGCGACTCCAGTCCCATGCTCACCTC
ACACCAAGCTCAGCTGCGACAGCCTCTCGAGCCTTCTGTTGCTCTCGTTATGCAGCGGCGGCTTCGTCACGTCGCCGCTGAGGTCAGGGTACGAGTGGAG

        110        120        130        140        150        160        170        180        190        200
CGATCCGACGTCCGATTCGTCCGACAATGGTGAATGTCTCGGTCGGGAACGCAACGTTCCCGGACGGCAGCCGCAGCAGCAGCAGCAGCAGCAGCAG
GCTAGGCTGCAGGCTAAGCAGGCTGTTACCACTTACAGAGCCAGCCCTTGCGTTGCAAGGGCCTGCCGTCGGCGTCGTCGTCGTCGTCGTCGTC

        210        220        230        240        250        260        270        280        290        300
CACCAAACGCACCACAACATAGAACAAGCCGGACTTATGAACGCTGTTTGCATTTTAGGCAGTGGCAAAATGCCATCGTCCATGAGCAAGGAATGTTCGT
GTGGTTTGCGTGGTGTTGTATCTTGTTCGGCCTGAATACTTGCGACAAACGTAAAATCCGTCACCGTTTTACGGTAGCAGGTACTCGTTCCTTACAAGCA

        310        320        330
CCGAGGCGCCGCAGGTCACGCGCATGATTGCCGACT
GGCTCCGCGGCGTCCAGTGCGCGTACTAACGGCTGA
```

Figure 7 Partial sequence of *timeless* gene from gDNA from *An. dirus*.

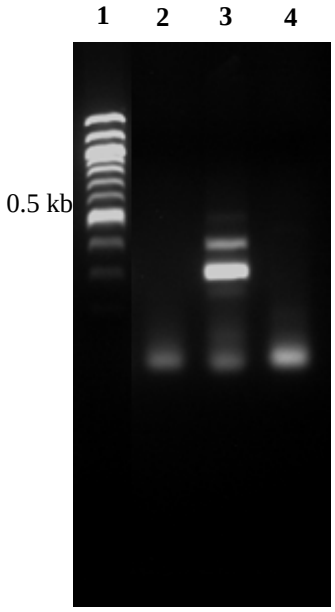


Figure 8 Amplification of partial *timeless* gene of *An. dirus* lane 1: molecular marker; lane 2: negative control of reverse transcription (no reverse transcriptase); lane 3: amplified fragments obtained from partial *timeless* gene; lane 4 negative control of PCR, respectively.

```
          10        20        30        40        50        60        70        80        90       100       110
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
ACCTGTGGTTCGAGTCGACGCTGTCGGAGAGCTCGGAAGA-AACGAGAGCAATACGTCGCCGCCGAAGCAGTGCAGCGGCGACTCCAGTCCCATGCTCACCTCCGATCCG
TGGACACCAAGCTCAGCTGCGACAGCCTCTCGAGCCTTCTGTTGCTCTCGTTATGCAGCGGCGGCTTCGTCACGTCGCCGCTGAGGTCAGGGTACGAGTGGAGGCTAGGC

          120       130       140       150       160       170       180       190       200
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|--
ACGTCCGATTCGTCCGACAATGGCAGTGGCAAAATGCCATCGTCCATGAGCAAGGAATGTTCGTCCGAGGCGCCGCAGGTCACGCGCATGAT
TGCAGGCTAAGCAGGCTGTTACCGTCACCGTTTTACGGTAGCAGGTACTCGTTCCTTACAAGCAGGCTCCGCGGCGTCCAGTGCGCGTACTA
```

Figure 9 Partial sequence of *timeless* gene from cDNA from *An. dirus*.

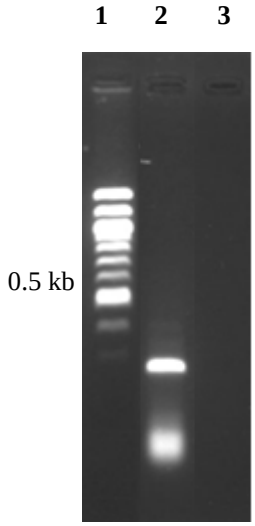


Figure 10 Amplification of partial *rps*7 gene of *An. dirus.* lane 1: molecular marker; lane 2: negative control; lane 3: amplified fragments obtained from partial *rps*7 *gene* from *An. dirus*, respectively.

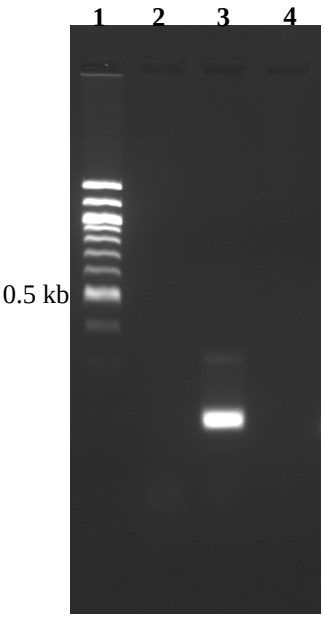


Figure 11 Amplification of partial *rps*7 gene of *An. dirus* lane 1: molecular marker; lane 2: negative control of reverse transcription (no reverse transcriptase); lane 3: amplified fragments obtained from partial *rps*7 gene; lane 4 negative control of PCR, respectively.

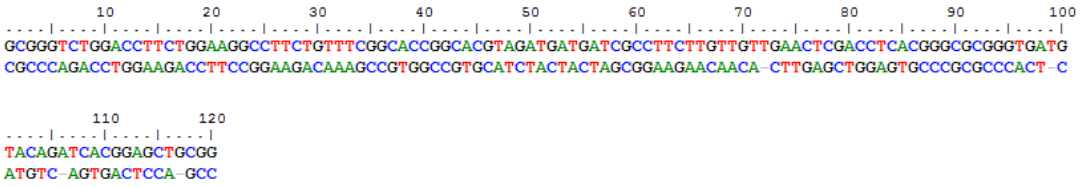


Figure 12 Partial sequence of *rps*7 gene from cDNA from *An. dirus*.

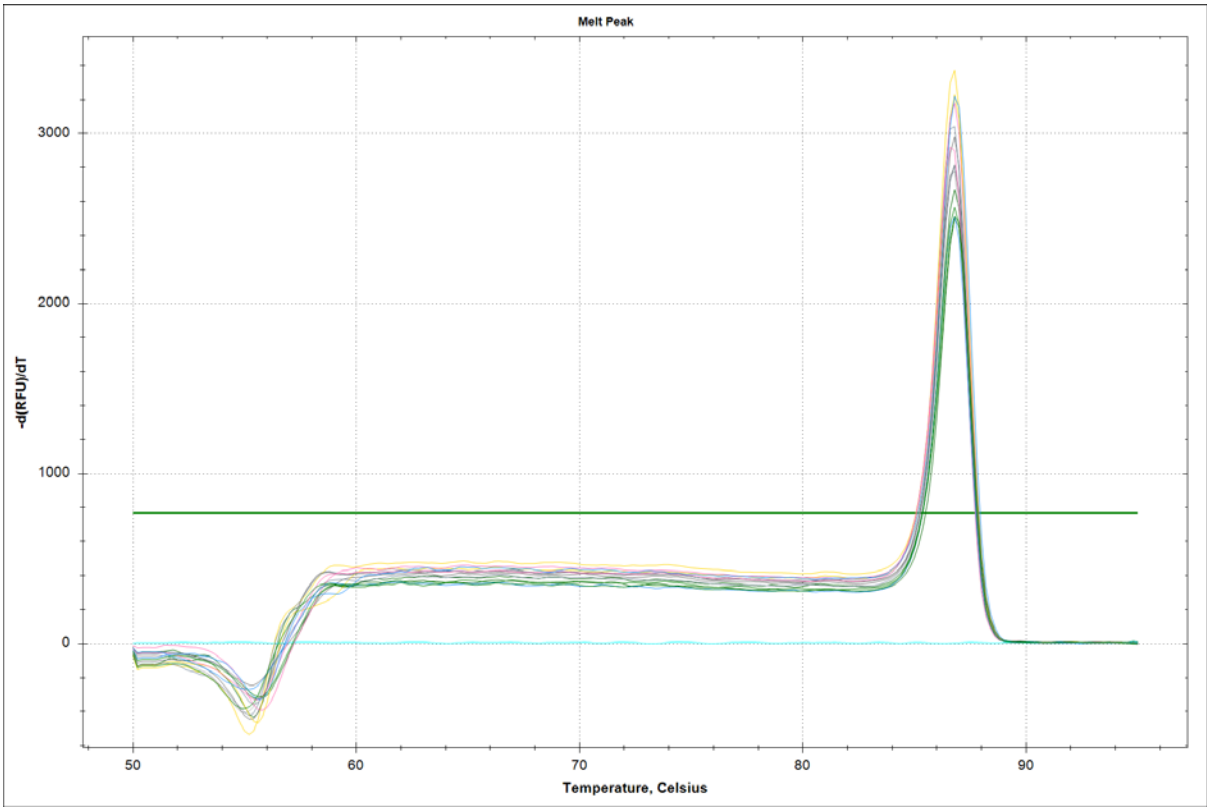


Figure 13 Melt peak of *timeless* gene of *An. dirus* by real-time PCR using SYBR Green. The annealing temperature was varied between 50-60 ^{o}C indicated by different colour.

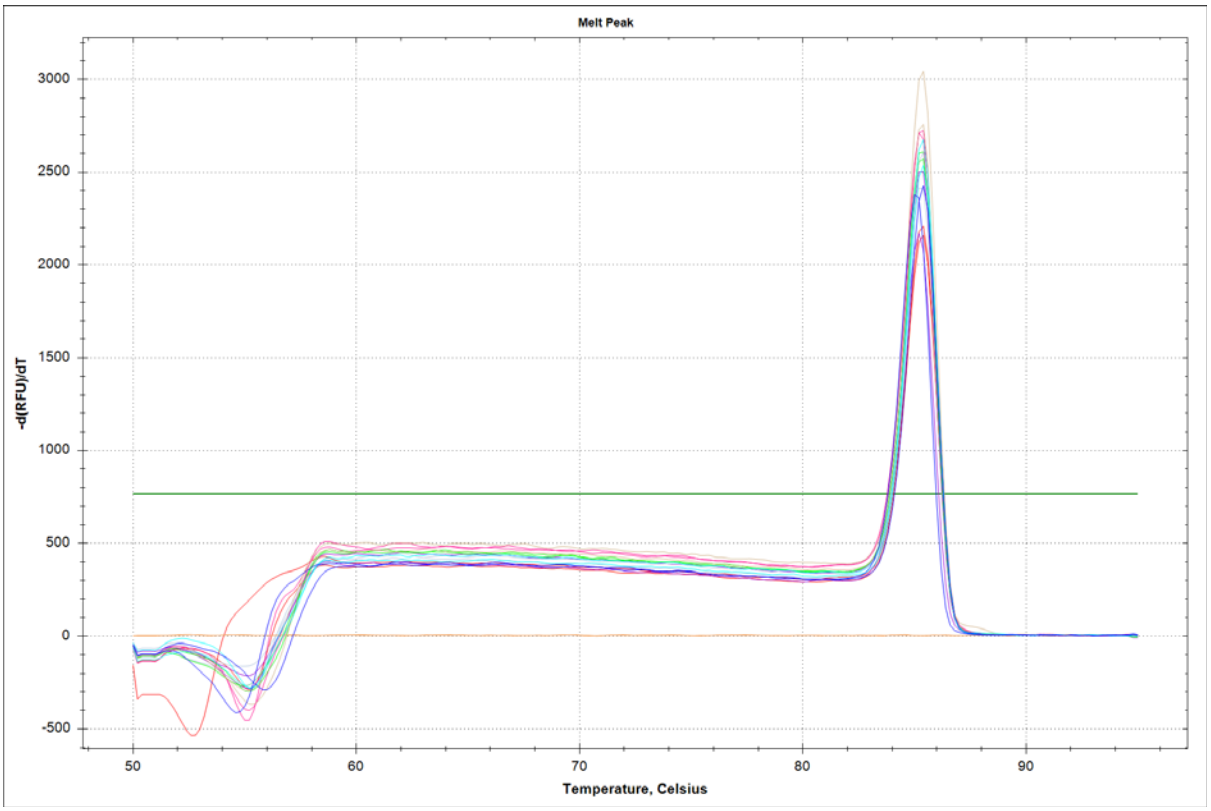


Figure 14 Melt peak of *rps*7 gene of *An*. *dirus* by real-time PCR using SYBR Green

The annealing temperature was varied between 50-60 °C indicated by different colour.

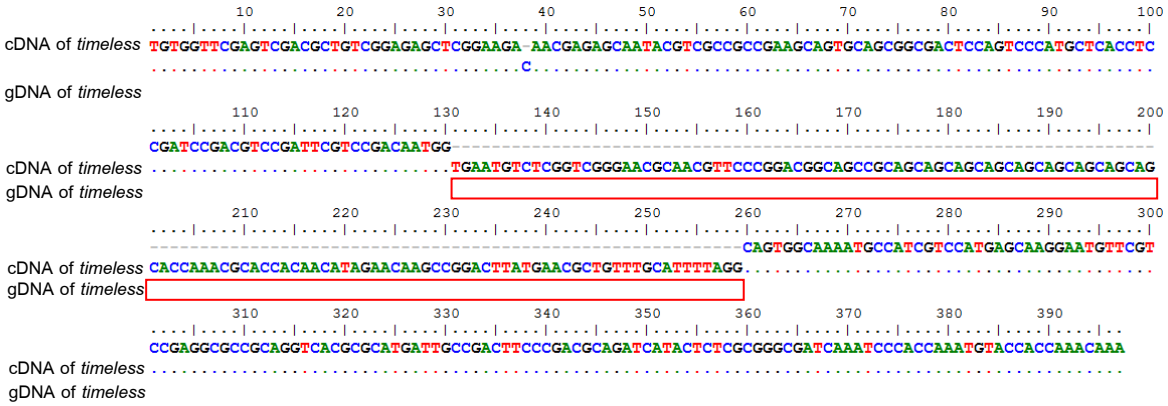


Figure 15 Intron analysis of *timeless* gene of *An. dirus*. Sequence of *timeless* gene using cDNA as template was aligned to sequence obtained from amplification of gDNA. Dot symbol indicated similarity nucleotide of both sequence from gDNA and cDNA whereas dash symbol indicated the gap of the sequence implying intron position in red box.

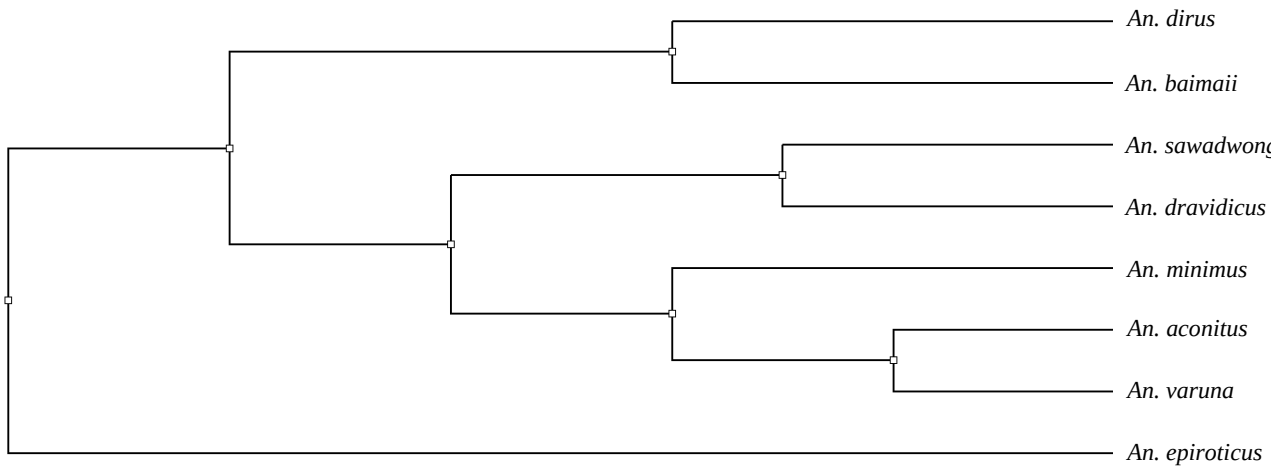


Figure 16 Phylogenetic analysis of the timeless gene of Anopheles mosquitoes which are the species founded in Thailand using the neighbour-joining method.

Subproject 8: Studies on Population Dynamic and Diversity of Mosquitoes Susceptible to Dengue Viruses and Filarial Parasites in Rubber plantation at Kanchanaburi Province, Western Thailand. (Jinrapa Phothikasikorn)

Abstract

The research was aimed to survey the diversity of mosquitoes which are vectors of dengue viruses and filarial parasites of human in Hui Ka-yeing subdistrict, Thong Pha Phoom district, Kanchanaburi province, Thailand. Between 2012 and 2014 (2 years). 62 species of various mosquitoes were found, among the total number of wild caught mosquitoes of 13,206 mosquitoes divided into wet and dry season period. For the wet season period, between July and September, there were 9,065 mosquitoes, compost with Culicine about 8,367 (92.30%) and Anopheline 698 (7.70%). For the dry season period, between January and March, were found 4,141 mosquitoes, compost with Culicine 4,063 (98.11%) and Anopheline 78 (1.89%). Detection of filarial worm by dissected on about 13,000 mosquitoes found that 6 species of mosquitoes including *Armigeres* (*Armigeres*) subalbatus, *Ar.* (*Arm.*) *kesselli*, *Coquilletedia crassipes, Aedes* (*Stegomyia*) *immitator*, *Ae.* (*St.*) *annandalei* and *Ae.* (*St.*) *malikuli* were positive with infective of filarial worm. These infective filarial worms composted with *Wuchereria bancrofti, Brugia pahangi, Dirofilaria immitis*, *Dipetalonema reconditum* and unknown species. Detection of dengue viruses by polymerase chain reaction (PCR) was performed on 3,500 mosquitoes of 4 abundance mosquito species in that area including 110 *Aedes* (*St.*) *aegypti,* 1,500 *Ae.* (*St.*) *albopicta,* 100 *Ae* (*St.*) *desmotes* and 100 *Ae.* (*St.*) *annadalei* and 3 *Armigeres* species, 1,500 *Armigeres.*(*Arm.*) *subalbatus*, 140 *Ar.*(*Arm.*) *kesselli* and 50 *Ar.* (*Lei*) *dolichocephalus* and found that there were negative detecting.

KEYWORDS: Filarial, Dengue viruses, Mosquitoes, Vectors

Introduction

The prevalence of disease caused by dengue viruses and *Wuchereria bancrofti*, has never been eradicated from Thailand because of the illegal immigrants from Myanmar into the border of Thailand. The main endemic areas of the filarial parasites in Thailand include Mae Hong Sorn, Tak and Kanchanaburi province. Regarding to Kanchanaburi, Thong Pha Phum district is considered as one of the most important endemic area. The knowledge about the species specific mosquitoes that are abundant in most urban, semi-urban and forest areas for the bancroftian filariasis of Thailand-Myanmar border is limited. These are the risk factors that would favor the complications and increase incidence of filariasis for Thai people. This research is conducted to survey the diversity of mosquitoes which are vectors of filarial parasites of both human and animal in sub-urban and forest areas at sub unit of Ban Mea Num Noi in rubber forest plantation, Huy Kayieng sub district, Thong Pha Phum district, Kanchanaburi province, and because of this area is dengue occurred throughout the year, the population dynamics and biting peak of various mosquito vectors of the dengue and filarial parasites of both disease in the area will be investigated. All of the dengue and filarial parasites from the collected mosquitoes were identified by the PCR technique.

Materials and Methods

Collection site

The study sites are located in an endemic area with both dengue and *Wuchereria bancrofti* infections occurring. The village selected as having low pesticide agricultural use was located in Huay Ka Yeng sub-district, Thong Pha Phum district, Kanchanaburi province, near the western border of Thailand. The study site is situated at latitude 14° 35' and longitude 98° 36', approximately 200 km from Muang district of Kanchanaburi Province. The area is surrounded by rubber plantations and hills.

Adult sampling

Wild adult females various mosquitoes were collected 3 months/ season between January, February and March in dry season and in July, August and September in wet season by human bait catches in rubber plantation collecting. The rubber forest is about 300 m apart

from the village. Mosquito collections were undertaken for 24 hours by two persons at each period. The first period was collected mosquitoes from 06.05 to 12.00 h, the second period collected from 12.05-18.00 h, the third period was 18.05-24.00 h and the forth period was 24.05-06.00 h, the humidity and temperature were recorded each hour. Torches were used to observe mosquitoes at night and aspirators for collecting them. Collections were made two consecutive days each month at the same sites. Collected mosquitoes were placed in separate marked plastic cups, one for each hour, and covered with netting material. All live mosquito specimens were provided with 10% sugar solution and transported to the laboratory for morphological identification. During transport and storage mosquitoes were kept in larger containers covered with damp cotton towels to avoid desiccation.

Parasite identifications

Filarial observations

The identified mosquito body was separated into 3 parts, head, thorax and abdomen, under a dissecting microscope for parasite observation. The filarial parasites found in each body part of wild caught female mosquitoes were collected and stored in 0.85 % Normal Saline Solution in 1.5 micro centrifuge tubes and frozen at -20°C until identify into the species by the technique of PCR.

DNA extraction

Filaria genomic DNA was extracted from third stage larva using DNeasy Mini Kit. Briefly, individual third stage larva in 0.85 % Normal Saline Solution 200 µl was added with 10% lysozyme 20 µl and incubated for 15 minute at 37□C. Third stage larva was digested with 20 µl of proteinase K and 200 µl of AL buffer in tissue grinder, crush sample 30-40 times and incubate for 10 minute at 56□C. Absolute ethanol 200 µl were added for DNA precipitation. Transfer sample from tissue grinder to DNeasy Mini Spin Column and centrifuge for 1 minute at 8,000 rpm and discarded supernatant. AW1 buffer 500 µl were added and centrifuge for1 minute at 8,000 rpm and discarded supernatant.AW2 buffer 500 µl were added and centrifuge for 5 minutes at 12,000 rpm and discarded supernatant.

Transfer DNeasy Mini Spin Column into 1.5 ml centrifuge tube, added AE buffer 200 µl to dissolved DNA, incubated for 10 minutes at room temperature and centrifuge for 3 minute at 12,000 rpm (2 times). Supernatant was collected for next experiment. Quantification and purity of The DNA was measured using a spectrophotometer (Nanodrop1000, Thermo Scientific). Supernatant was store at -20 □C until use.

Primer design for semi-nested PCR amplification

The nematode specific primer designs were based on alignments of the 18s rRNA and 5.8s rRNA gene of nematodes including filarial parasites for first PCR reaction. Uni-F1 (5'GGTGAACCTGCGGAAGGATCATTA3') and Uni-R1 (5'TTCGCACTATTTATCGCAGCTAG3') were used as forward and reverse primer, respectively. Product sequence from first PCR reaction used for designed species specific primer based on alignments of the 18s and ITS 1 rRNA gene.

Specific forward primer for *B. malayi* is Bm-F1 (5'TATTTATGC TACTACCAGGATCC3'),
B. pahangi is Bp-F1 (5'TTACTTATGCTACTAC CAGGATCC3'),
W. bancrofti is Wb-F1 (5'CATATTATTTACTATTACCAG GATCC3'),
D. immitis is Di-F1 (5'AACTACTAACTACTACTAACTATTC ATC3'),
D. repens is Dr-F1 (5'GACTAACTACTAACTAACTAACTATCC3'),
D. reconditum is Dre-F1 (5'TCTTTCTCACACCACACCAATC3').

Reverse primer is Uni-R1 in all six species. Filaria sequences were obtained from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). The specificity of primer and variation in target DNA were check by the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST). Investigation of annealing temperature, GC content and the chance of primer secondary structure formation were done by Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu).

Semi-nested PCR amplification

The first round PCR amplifications were performed to screen the filarial existing, All PCR amplifications were carried out in a 25 µl reaction mixture contain 2 µl genomic template DNA, 2.5 µl 10 X Coll PCR buffer, 12.5 µl Toptag Polymerqase 2X, 7.5 µl distill water, 0.25 µl nematode specific primer pairs for all filarial parasites (forward primer; Uni-F1 and reverse primer; Uni-R1). The second round PCR amplifications used to identify the filarial species, *B. malayi, B.pahangi, W. bancrofti, D. immitis, D. repensis and D. reconditum*. The investigation were carried out in a 25 µl reaction mixture contain 2 µl first round PCR product, 2.5 µl 10 X Coll PCR buffer, 12.5 µl Toptag Polymerqase 2X, 7.5 µl distill water, 0.25 µl genus specific primer pairs for each filarial parasites (forward primer for *B. malayi* is Bm-F1, *B. pahangi* is Bp-F1, *W. bancrofti* is Wb-F1, *D. immitis* is Di-F1, *D. repens*is Dr-F1, *D. reconditum* is Dre-F1. Reverse primer is Uni-R1 in all six species.

Sensitivity test of PCR detection

DNA of Filarial worms will 10-fold serially diluted (1 ng–0.001 fg) and amplified. The PCR products from the semi-nested PCR amplification will finally analyzed on 1.5% (w/v) agarose gel and visualized under UV save gel documentation.

Specificity test of PCR detection

The purified DNA of human, vector mosguitoes (*Aedes*, *Culex*, *Anopheles*), parasites (*Plasmodium*, *Trypanosoma*) and possibility host (*Bovine, Feline* and *Canine*) will used as the negative controls. The PCR products obtained from the semi-nested PCR amplification will finally analyze on 1.5% (w/v) agarose gel and visualize under UV save gel documentation.

Gel electrophoresis

The PCR products will analyze by gel electrophoresis in 1.2% (w/v) agarose gel with 100 bp ladder marker. The PCR product will electrophorese at 100V for 50 minutes and staine with ethidium bromide for 1 minute. The target DNA will document on UV save gel documentation.

Virus detections

Dengue viruses from the culicine mosquitoes especially *Aedes* or *Stegomyia* genus and *Armigeres* genus group, will be detect after dissect for filarial observation, head of their body will be preserved at -20^{o}C until virus identifications by PCR technique.

For the sample size of mosquitoes, is use of the Krejcie & Morgan formula in their 1970 article “Determining Sample Size for Research Activities” (*Educational and Psychological Measurement*, #30, pp. 607-610). Detection of dengue virus by polymerase chain reaction (PCR) and typing the serotype of the virus by the first nested primer including D1 and D2. Serotype typing is required the specific primers which TS1, TS2, TS3 and TS4 as reward primers for detect the serotype 1,2,3 and 4 respectively, and D1 as a forward primer that show in table 4. All viruses were propagated in C6/36 cells prior to extraction of RNA the isolation of virus on C6/36 cells led to successful isolation of Dengue type 1, 2, 3 and 4 viruses. RNA was extracted from culture supernatant by TRIZOL reagent following the manual book. Moreover, preparing the cDNA by AMV reverse transcriptase enzyme with antisense D2 primer and doing PCR with *Taq* polymerase, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM$MgCl_2$, 200μM dNTP, primer D1 and D2. The correctly sized DNA product (511 bp) was obtained for each of the dengue viruses after amplification with consensus primers D1 and D2 (Fig.1). Each DNA product was correctly typed by a second round of amplification with the type-specific primers TS1, TS2, TS3 and TS4. Expected product sizes are as follows: dengue-1, 482 bp; dengue-2, 119 bp; dengue-3, 290 bp; dengue-4, 392 bp (Fig. 2).

Results

Mosquito captured in the rubber plantation area at Thong Pha Phoom district, Kanchanaburi province, Thailand, made on 2012 - 2014 divided into wet and dry season period, the distribution and diversity of the mosquitoes were studied. During this period representing 62 species of various mosquitoes were found, among the total number of 13,206 mosquitoes from wet and dry season period. For the wet season period, between July and September, there were 9,065 mosquitoes, composted with Culicine 8,367

(92.30%) and Anopheline 698 (7.70%). For the dry season period, between January and March, were found 4,141 mosquitoes, composted with Culicine 4,063 (98.11%) and Anopheline 78 (1.89%) (Table and Figure1)

For filarial detection, about 13,000 of wild caught mosquitoes were dissected (table 2-13) and summarize in table 14, found that 7 species of mosquitoes including *Aedes* (*St.*) *annandalei*, *Aedes* (*St.*) *desmotes*, *Ae.* (*St.*) *imitato*r. *Ae.* (*St.*) *malikuli, Armigeres* (*Armigeres*) *subalbatus*, *Ar.* (*Arm.*) *kesselli* and *Coquilletedia crassipes* were positive with infective stage of filarial worm in their bodies (figure2). Almost of these worms were animal filarialsis such as *Dirofilaria immitis*, *Dipetalonema reconditum* and *Blugia pahangi*. There are only two worms from *Ae* (*St.*) *desmotes* were similarly as *Whuchereria bancrofti* when identified by the pictorial key and the semi-nested PCR technique. 68 infective stages of filarial worms that found from wild caught mosquitoes including 14 *Brugia pahangi*, divided into 12 worms from *Armigreres kesselli* and 2 worms from *Aedes malikuli*, 14 *Dirofilaria immitis* composted with 3 worms from *Ar. subalbatus,* 8 worms from *Aedes immitator* and 3 worms from *Coquilletedia crassipes*, for 10 *D. reconditum* divided into 6 worms from *Ar. subalbatus* and 4 worms from *Ae. annandalei* respectively, for 2 *Wuchereria bancrofti* infective worms found only in a species of *Ae. desmotes* (table 14). Moreover there were 31 unknown species of the infective filarial worms from *Ar. subalbatus* and *Ae. annandalei* which collected on February, August and September were found (table 14) .

Detected of dengue viruses by polymerase chain reaction (PCR) were performed on 3,500 mosquitoes of 4 abundance mosquito species in the area including 110 *Aedes aegypti,* 1,500 *Ae. albopicta,* 100 *Ae desmotes,* 100 *Ae. annadalei*, 1,500 *Armigeres.*(*Arm*.) *subalbatus*, 140 *Ar.*(*Arm*.) *kesselli* and 50 *Ar.* (*Lei*) *dolichocephalus*, with 10 head of each mosquito species per 1 PCR reaction but the results still negative detecting (Table 16 and Figure7) .

Discussion

There are very low populations of mosquito from the area in both of the seasons, especially in the dry season, January, February and March, because of the weather at that time is very hot and dry. But interestingly, in February of the first year of collections (2013), there were very high population of the mosquitoes with 1,676 culicine mosquitoes

(20 species) were found in this month (table and figure 1). For this phenomenon there is not normal for Thai season, may be because of influence from the La Niña phenomenon that extend from the last year, with the last heavy rain at the end of January and 2 week after that we went to collected the mosquitoes as in annually of the research plan and found that there were very high population of mosquitoes as shown in table and figure1. The diversity of the mosquito species in this area is very high especially for the genera of *Armigeres* and *Aedes*, there were 13 and 7 species were found respectively. *Armigeres* and *Aedes* genera group such as *Ar. subalbatus* and *Ae. albopictus*, were dominant mosquito species that found very abundance with high population throughout the year. For the parasite detection, about 13,000 of wild caught mosquitoes were dissected and found that 7 species from these collected mosquitoes including *Aedes* (*Stegomyia*) *annandalei*, *Ae.* (*St.*) *desmotes*, *Ae.* (*St.*) *imitator*, *Ae.* (*St.*) *malikuli*, *Armigeres* (*Armigeres*) *subalbatus*, *Ar.* (*Arm.*) *kesselli* and *Coquilletedia crassipes* were positive. 68 infective of filarial worms were found in their body parts such as head, thorax and abdomen (Table14 and Figure 2). Almost of these worms were animal filarialsis including *Dirofilaria immitis*, *Dipetalonema reconditum* and *Brugia pahangi*. More interestingly we found two worms from *Ae* (*St.*) *desmotes* that similarly as *Whuchereria bancrofti* when identified by the pictorial key and the semi-nested PCR technique, whereas at decade years there has been no patient case reported in this area. A wide range of mosquitoes can transmit the bancroftian parasite, depending on the geographic area. In Africa, the most common vector is *Anopheles* and in the Americas, it is *Culex quinquefasciatus*. *Aedes* and *Mansonia* can transmit the infection in the Pacific and in Asia. However, *Ae.*(*St.*) *desmotes* is play as natural vector of bancroftian filariasis in Thailand ((Sucharit, et. Al., 1975, Gould et al, 1982 and Pothikasikorn et. Al., 2008) and we can detected them as showed in our results. Moreover there were 31 others or unidentified worms from *Armigeres* (*Arm.*) *subalbatus* and *Ae.* (*St.*) *annandalei* (Figure 3 and 4) because there was no specific primer for detected them.

For the filarial worms that detected in this research, *Dirfilaria immitis* and *Dipetalonema reconditum* are an animal filariasis cause of heartworm infected dogs and cats. Especially heartworm infected with *Dirofilaria immitis*, it is called a "heartworm" because the parasite, in the final reproductive stage of its life cycle, resides most often in the heart of its host

where it can live for many years, potentially eventually killing its host that go untreated may die. Both of *Dirofilaria immiti* and *Dipetalonema reconditum* may be found in blood of infected dogs but it is not easy to distinguish *D. immitis* from *D. reconditum* in morphology.

Dirofilaria immiti influence on human, it is an infrequently reported cause of lung disease in humans. Approximately 80 cases have been reported in the United States, with increasing numbers reported in recent years (Watson, et. al. 1991). For the vectors, found that they are at least 70 species of mosquitoes can serve as intermediate hosts of *Dirofilaria immitis*, the most common vectors are *Aedes, Anopheles*, *Armigeres* and *Culex* (Cullens, L., 2008). In this researched found that the abundance culicine mosquitoes in rubber plantation area of Thailand such as *Aedes* (*St.*) *annandalei, Ae.* (*St.*) *imitator*, *Armigeres* (*Armigeres*) *subalbatus* and *Coquilletedia crassipes* were positive of *D. immitis* and *Dipetalonema reconditum.*

Dipetalonema reconditum is a parasite you will not hear much about since it rarely causes significant disease. Its importance lies in the fact that its immature form (slender larvae called microfilariae) can be easily confused with those of *Dirofilaria immitis* (heartworm). *D. reconditum* lives in the body cavity and subcutaneous (just below the skin) tissues of dogs and is found throughout the United States and tropical countries and had the same vectors as *Dirofilaria immitis* as found in this research.

For *Brugia pahangi*, it has been reported from cats in Malaysia; Mak et al. (1980) reported that about 11% of cats sampled in Peninsular Malaysia were infected. In Thailand, Chungpivat and Sucharit (1993) reported that about 25% of blood samples from 83 cats living in Buddhist temples were found to be infected with *Brugia pahangi* in the area of Lat Krabang. About 61 of 325 cats (18.8%) in South Kalimantan, Indonesia, were found to have circulating microfilariae of Brugiapahangi (Palmieri et al., 1985). The vectors of *Brugia pahangi* are mosquitos of the genus *Mansonia, Anopheles*, and *Armigeres* (Edeson et al., 1960). In our results showed that *Armigeres* (*Armigeres*) *kesselli* and *Aedes* (*Stegomyia*) *malikuli* were play as the vectors for the zoonotic *Brugia pahangi* infections. Thus these mosquito species should now be categorised as a medically important

mosquito species in Malaysia. Its role in the transmission of zoonotic *B. pahangi* must therefore be considered in future studies on filarial infections.

For dengue viruses detection by polymerase chain reaction (PCR) and typing the serotype of the viruses by the first nested primer including D1 and D2. Serotype typing is required the specific primers which TS1, TS2, TS3 and TS4 as reward primers for detect the serotype 1,2, 3 and 4 respectively, and D1 as a forward primer. The results after performed on 3,500 mosquitoes composted with 8 abundance mosquito species included *Aedes* (*St.*) *aegypti, Ae.* (*St.*) *albopicta, Ae* (*St.*) *desmotes, Ae.* (*St.*) *annadalei*, *Armigeres.*(*Arm.*) *subalbatus*, *Ar.*(*Arm.*) *kesselli* and *Ar.* (*Lei*) *dolichocephalus*, with 10 head of each mosquito species per 1 PCR reaction but the results still negative detecting with the result of positive control expression (Table 16 and Figure7).

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Table1 Total numbers of mosquitoes were collected between dry and wet season (2012-2014).

Season	Months	Year	Number of mosquitoes		Total
			Culicine	Anopheline	
Dry	January	2013	360	28	658
		2014	261	9	
	February	2013	1662	14	2262
		2014	575	11	
	March	2013	351	2	1221
		2014	854	14	
Wet	July	2013	979	117	2523
		2014	1242	185	
	August	2012	1153	153	3047
		2013	1615	126	
	September	2012	1676	32	3495
		2013	1702	85	
Totol					**13206**

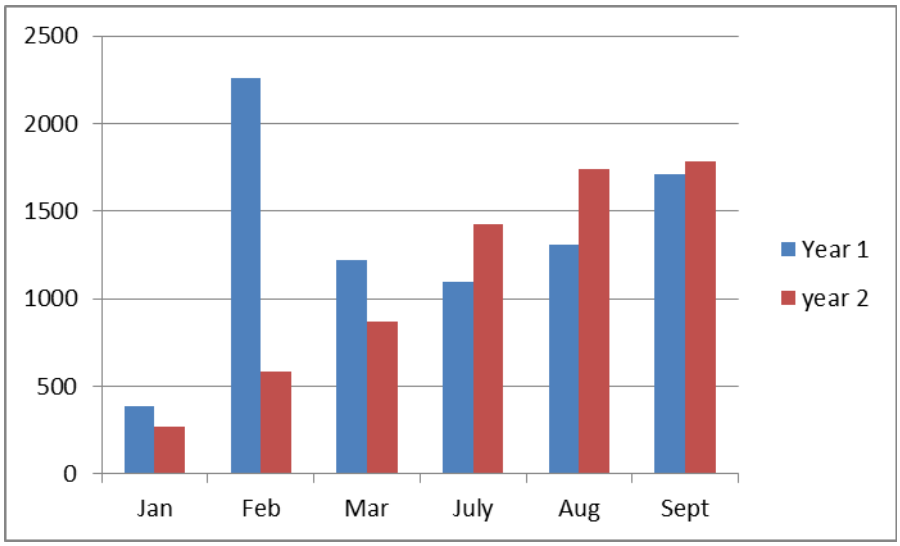


Figure1 Population dynamic of wild caught mosquito comparison between dry season and wet season of the first and the second year of collection

Table 2 Showing filarial infective stage from dissected wild caught mosquitoes that collected in August 2012

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquito No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae. Aegypti*	13	-				
2	*Ae. Albopictus*	242	-				
3	*Ae. Desmotes*	11	-				
4	*Ae. Imitator*	8	-				
5	*Ae. Annandalei*	2	-				
	Anopheles						
No.							
6	*An. aconitus*	2	-				
7	*An. barbirostris*	3	-				
8	*An. baimai*	102	-				
9	*An. minimus*	39	-				
10	*An. maculatus*	7	-				
11	*An. philippinensis*	1	-				
	Armigeres						
12	*Ar. Subalbatus*	817	3	1	0	0	1
				2	0	2	0
				3	0	8	1
13	*Ar. Mouton*	4	-				
14	*Ar. Kesseli*	18	-				
15	*Ar. Flavus*	4	-				

	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquito No	head	thoracic	abdomen
16	*Ar.* (Lei) *inchoatus*	1	-				
17	*Ar.* (Lei) *annulipalpis*	2	-				
18	*Ar.* (Lei) *dolichocephalus*	3	-				
	Culex						
19	*Cx. quinquefasciatus*	10	-				
20	*Cx. Vishnui*	6	-				
21	*Cx. nigropunctatus*	1	-				
	Heizmania						
22	*Hz. Chengi*	2	-				
23	*Hz. Macdonaldi*	1	-				
24	*Hz. Covelli*	1	-				
25	*Hz. Mattinglyi*	1	-				
	Others						
26	*Ochlerotatus vigilax*	2	-				
27	*Finlaya poicillia*	2	-				
28	*Mansonia dives*	2	-				
	Total	**1,306**	**3**			**10**	**2**

Remark The collected worms preserved in 0.85% NaCl at 20°C identify by PCR technique

Table 3 Showing filarial infective stage from dissected wild caught mosquitoes that collected in September 2012

No.	Mosquitoes			No. of filarial L3 from mosquito parts		
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**					
1	*Ae. Aegypti*	8				
2	*Ae. Albopictus*	365	-			
3	*Ae. Annandalei*	19	-			
4	*Ae. Desmotes*	121	1	1	1	0
5	*Ae. Imitator*	14	-			
6	*Ae. Malikuli*	11	-			
	Anopheles					
7	*An. barbirostris*	3	-			
8	*An dirus*	16	-			
9	*An. maculatus*	5	-			
10	*An. minimus*	8	-			
	Armigeres					
11	*Ar.* (*Arm.*) *subalbatus*	886	1	6	0	0
12	*Ar* (*Lei.*) *annulitarsis*	33	-			
13	*Ar* (*Lei.*) *dolichocephalus*	21	-			
14	*Ar* (*Lei.*) *inchoatus*	13	-			
15	*Ar* (*Lei.*) *annulipalpis*	2	-			
	Culex					
16	*Cx. Gelidus*	11	-			
17	*Cx quinquefasciatus*	56				

No.	Mosquitoes		No. of filarial L3 from mosquito parts			
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
	Mansonia					
19	*Mn. Annulifera*	37	-			
20	*Mn. Dives*	13	-			
	Others					
21	*Coquilletidia crassipes*	2	-			
22	*Downsiomyia harisoni*	16	-			
23	*Heizmannia macdonaldi*	14	-			
24	*Heizmannia communis*	11	-			
25	*Hulecoeteomyia reinerti*	2	-			
	Total	**1,708**	**2**	**7**	**1**	**0**

Remark No positive mosquitoes

Table 4 Showing filarial infective stage from dissected wild caught mosquitoes that collected in January 2013

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae. aegypti*	5					
2	*Ae. albopictus*	135	-				
3	*Ae. desmotes*	21	-				
4	***Anopheles***						
5	*An. barbirostris*	2	-				
6	*An. minimus*	25	-				
7	*An. philippinensis*	2	-				
	Armigeres						
8	*Ar. subalbatus*	144	-				
9	*Ar* (Lei.) *dolichocephalus*	7	-				
	Culex						
10	*Cx. quinquefasciatus*	6	-				
11	*Cx. vishnui*	23	-				
	Heizmania						
12	*Heizmannia communis*	9	-				
	Hodgesia						
13	*Hodgesia bailyi*	1	-				
	Mansonia						
14	*Mn. annulifera*	8	-				
	Total	**388**	**-**				

Remark: No positive mosquitoes

Table 5 Showing filarial infective stage from dissected wild caught mosquitoes that collected in February 2013

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae. Aegypti*	64	-				
2	*Ae. Albopictus*	1046	-				
3	*Ae. Desmotes*	58	-				
4	*Ae. Imitator*	123	1	1	-	8	8
5	*Ae. Annandalei*	15	1	1	-	-	8
6	*Ae. Malikuli*	5	-				
	Armigeres						
7	*Ar. Subalbatus*	261	-				
8	*Ar. Kesseli*	7	-				
9	*Ar.* (Lei) *annulipalpis*	1	-				
	Anopheles						
10	*An. dirus*	2	-				
11	*An. barbirostris*	2	-				
12	*An. Philippinensis*	2	-				
13	*An. maculatus*	1	-				
14	*An. minimus*	7	-				
	Culex						
15	*Cx. quinquefasciatus*	45	-				
16	*Cx. Vishnui*	23	-				
17	*Cx. Infantulus*	6	-				
	Others						

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
18	*Aediomyia catasticta*	2	-				
19	*Coquillettidia crassipes*	2	-				
20	*Finlaya poicillia*	4	-				
	Total	**1,676**	**2**	**2**		**8**	**16**

Remark The collected worms preserved in 0.85% NaCl at – 20^{o}C until identify by PCR technique

Table 6 Showing filarial infective stage from dissected wild caught mosquitoes that collected in March 2013

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Stegomyia* (*Aedes*)**						
1	*Ae. Aegypti*	7	-				
2	*Ae. Albopictus*	196	-				
3	*Ae. Desmotes*	1	-				
4	*Ae. Imitator*	7	-				
5	*Ae. annandalei*	2	-				
	Armigeres						
6	*Ar. Subalbatus*	76	-				
7	*Ar. Flavus*	1	-				
8	*Ar. (Lei) annulipalpis*	1	-				
9	*Ar. (Lei) annulitarsis*	1					
	Culex						
10	*Cx. quinquefasciatus*	53	-				
11	*Cx. Infantulus*	2	-				
	Anopheles						
12	*An. minimus*	2	-				
	Others						
13	*Downsiomyia vana*	1	-				
14	*Lorrainea amesii*	1	-				
	Total	**351**	**-**				

Table 7 Showing filarial infective stage from dissected wild caught mosquitoes that collected in July 2013

No.	Mosquitoes			No. of filarial L3 from mosquito parts		
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**					
1	*Ae.aegypti*	21	-			
2	*Ae. albopictus*	189	-			
3	*Ae. annadalei*	76	1	2	1	1
4	*Ae. desmotes*	54	-			
5	*Ae. imitator*	11	-			
6	*Ae. malikuli*	26	1	2	0	0
	Anopheles					
7	*An. dirus*	67	-			
8	*An. maculatus*	5	-			
9	*An. minimus*	43	-			
10	*An sawadwongporni*	2	-			
	Armigeres					
11	*Ar.* (*Arm.*) *subalbatus*	277	-			
12	*Ar* (*Lei.*) *annulitarsis*	14	-			
13	*Ar* (*Lei.*) *dolichocephalus*	13	-			
14	*Ar* (*Lei.*) *inchoatus*	3	-			
	Culex					
15	*Cx. sitiens*	8	-			
16	*Cx. withmorei*	1	-			
17	*Cx. vishnui*	13	-			

No.	Mosquitoes			No. of filarial L3 from mosquito parts		
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
19	*Heizmannia communis*	48	-			
	Mansonia					
20	*Mansonia annulifera*	54	-			
21	*Mn. dives*	39	-			
	Others					
22	*Coquilletedia crassipes*	2	-			
	Downsiomyia harinasutei.	87	-			
23	*Orthopodomyia wilsoni*	1	-			
24	*Hodgesia lampangnensis*	1	-			
25	*Mimomyia fusca*	1	-			
26	*Hulecoeteomyia reinerti*	6	-			
27	*Finlaya flavipennis*	2	-			
28	*Edwardaedes imprimens*	4	-			
	Total	**1,096**	**2**	**4**	**1**	**1**

Remark The collected worms preserved in 0.85% NaCl at – 20°C until identify by PCR technique

Table8 Showing filarial infective stage from dissected wild caught mosquitoes that collected in August 2013

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae.aegypti*	11	-				
1	*Ae. albopictus*	273	-				
2	*Ae. desmotes*	26	-				
3	*Ae. immitator*	7	-				
4	*Ae. annandalei*	12	-				
	Anopheles						
5	*An. barbirostris*	3	-				
6	*An. baimai*	34	-				
7	*An. minimus*	75	-				
8	*An. maculatus*	11	-				
9	*An. philippinensis*	3	-				
	Armigeres						
10	*Ar. subalbatus*	642	-				
11	*Ar. moutoni*	6	-				
12	*Ar. kesseli*	13	1	1	0	6	1
13	*Ar. flavus*	8	-				
14	*Ar.* (*Lei*) *inchoatus*	2	-				
15	*Ar.* (*Lei*) *annulipalpis*	3	-				
16	*Ar.* (*Lei*) *dolichocephalus*	4	-				
	Culex						
17	*Cx. quinquefasciatus*	7	-				

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
18	*Cx. vishnui*	11	-				
	Heizmania						
19	*Hz. communis*	2	**-**				
20	*Hz. macdonaldi*	5	-				
21	*Hz. mattinglyi*	1	-				
	Others						
22	*Ochlerotatus vigilax*	6	-				
23	*Finlaya poicillia*	2	-				
24	*Mansonia dives*	4	-				
	Total	**1,171**	**1**		**0**	**6**	**1**

Table 9 Showing filarial infective stage from dissected wild caught mosquitoes that collected in September 2013

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae.aegypti*	21	-				
2	*Ae. albopictus*	426					
3	*Ae. desmotes*	25	-				
4	*Ae. immitator*	11	-				
5	*Ae. annandalei*	3	-				
	Anopheles		-				
6	*An barbirostris*	2	-				
7	*An. dirus*	20	-				
8	*An. maculatus*	6	-				
9	*An. minimus*	54	-				
10	*An. philippinensis*	2	-				
	Armigeres						
11	*Ar. subalbatus*	1042	2	1	0	0	2
				2	2	1	3
12	*Ar. moutoni*	3	-				
13	*Ar. kesseli*	12	1	1	2	2	1
14	*Ar. flavus*	16	-				
15	*Ar.* (Lei) *inchoatus*	6	-				
16	*Ar.* (Lei) *annulipalpis*	2	-				
17	*Ar.* (Lei) *dolichocephalus*	24	-				
	Culex						

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
18	*Cx. quinquefasciatus*	18	-				
19	*Cx. vishnui*	20	-				
	Heizmania						
20	*Hz. Chengi*	2	-				
21	*Hz. maedenaldi*	25	-				
22	*Hz. communis*	14	-				
23	*Hz. mattinglyi*	1	-				
24	*Hulecoeteomyia. reinerti*	14	-				
	Others						
25	*Coquilletedia crassipes*	6	-				
26	*Ochlerotatus vigilax*	2	-				
27	*Finlaya poicillia*	13	-				
28	*Mansonia dives*	16	-				
29	*Phagomyia assamensis*	2	-				
	Total	**1,787**	**3**		**4**	**3**	**6**

Remark The collected worms preserved in 0.85% NaCl at – 20°C until identify by PCR technique

Table10 Showing filarial infective stage from dissected wild caught mosquitoes that collected in January 2014

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae. aegypti*	-	-				
2	*Ae. albopictus*	75	-				
3	*Ae. desmotes*	13					
	Armigeres						
4	*Ar. subalbatus*	47	-				
5	*Ar. moutoni*	1	-				
6	*Ar. Flavus*	1	-				
7	*Ar.* (Lei) *dolichocephalus*	1	-				
	Culex						
8	*Cx. quinquefasciatus*	41	-				
9	*Cx. vishnui*	13	-				
10	*Cx. infantulus*	58	-				
	Anopheles						
11	*An. barbirostris*	4	-				
12	*An. maculatus*	2	-				
13	*An. minimus*	2	-				
14	*An. philippinnensis*	1	-				
	Others						
15	*Ochlerotatus vigilax*	1	-				
16	*Finlaya poicillia*	1	-				
17	*Mansonia dives*	7	-				

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
18	*Phagomyia assamensis*	2	-				
	Total	**270**	**-**				

Table11 Showing filarial infective stage from dissected wild caught mosquitoes that collected in February 2014

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae.aegypti*	12	-				
2	*Ae. albopictus*	208	-				
3	*Ae. desmotes*	38	-				
4	*Ae. immitator*	7	-				
	Armigeres						
5	*Ar. subalbatus*	266	-				
6	*Ar. kesseli*	7	-				
7	*Ar.* (Lei) *annulipalpis*	1	-				
	Anopheles						
8	*An. barbirostris*	2	-				
9	*An. philippinensis*	2	-				
10	*An. maculatus*	1	-				
11	*An. minimus*	6	-				
	Culex						
12	*Cx. quinquefasciatus*	12	-				
13	*Cx. vishnui*	9	-				
14	*Cx. infantulus*	5	-				
	Others						
15	*Coquilletedia crassipes*	6	1	1	1	2	0
16	*Finlaya poicillia*	4	-				
17	*Ochlerotatus vigilax*	1	-				

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
18	*Finlaya poicillia*	3	-				
19	*Mansonia dives*	1	-				
	Total	**586**	**1**		**1**	**2**	**0**

Remark No positive mosquitoes

Table12 Showing filarial infective stage from dissected wild caught mosquitoes that collected in March 2014

No.	Mosquitoes				No. of filarial L3 from mosquito parts		
	Genus Species	No. dissected	No. positive	Mosquites No	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**						
1	*Ae. aegypti*	11	-				
2	*Ae. albopictus*	163	-				
3	*Ae. desmotes*	21	-				
4	*Ae. immitator*	2	-				
5	*Ae. annandalei*	11	-				
	Anopheles						
6	*An. minimus*	13	-				
7	*An philippinensis*	1	-				
	Armigeres						
8	*Ar. subalbatus*	614	1	1	0	2	1
9	*Ar. Flavus*	2	-				
10	*Ar. (Lei) annulipalpis*	4	-				
11	*Ar. (Lei) annulitarsis*	1					
	Culex						
12	*Cx. quinquefasciatus*	13	-				
13	*Cx. infantulus*	7	-				
	Others						
14	*Downsiomyia harinasutai*	1	-				
15	*Finlaya poicillia*	2	-				
16	*Hulecoeteomyia reinerti*	2	-				
	Total	**868**	**1**		**0**	**2**	**1**

Table13 Showing filarial infective stage from dissected wild caught mosquitoes that collected in July 2014

No.	Mosquitoes			No. of filarial L3 from mosquito parts		
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
	***Aedes* (*Stegomyia*)**					
1	*Ae.aegypti*	10	-			
2	*Ae. albopictus*	275	-			
3	*Ae. annadalei*	23	-			
4	*Ae. desmotes*	36	-			
5	*Ae. imitator*	7	-			
6	*Ae. malikuli*	4	-			
	Anopheles					
7	*An. dirus*	71	-			
8	*An. maculatus*	10	-			
9	*An. minimus*	102	-			
10	*An philippinensis*	2	-			
	Armigeres					
11	*Ar. subalbatus*	715	-			
	Ar. Flavus	9	-			
12	*Ar* (Lei.) *annulitarsis*	3	-			
13	*Ar* (Lei.) *dolichocephalus*	16	-			
14	*Ar* (Lei.) *inchoatus*	1	-			
	Culex					

No.	Mosquitoes			No. of filarial L3 from mosquito parts		
	Genus Species	No. of mosquitoes	No. positive	head	thoracic	abdomen
15	*Cx. quinquefasciatus*	14	-			
16	*Cx. sitiens*	5	-			
17	*Cx. vishnui*	36	-			
	Heizmannia					
18	*Heizmannia macdonaldi*	8	-			
19	*Heizmannia communis*	12	-			
	Mansonia					
20	*Mansonia annulifera*	24	-			
21	*Mansonia dives*	3	-			
	Others					
22	*Coquilletedia crassipes*	4	-			
	Downsiomyia harinasutei.	21	-			
23	*Hulecoeteomyia reinerti*	11	-			
24	*Edwardaedes imprimens*	5	-			
	Total	**1,427**	**-**			

Remark No positive mosquitoes



Figure 2 Showing the infective stage of the filarial worm (white arrow) that found in the abdomen of collected wild caught *Armigeres subalbatus* collected in August 2012

PCR technique for filarial identification

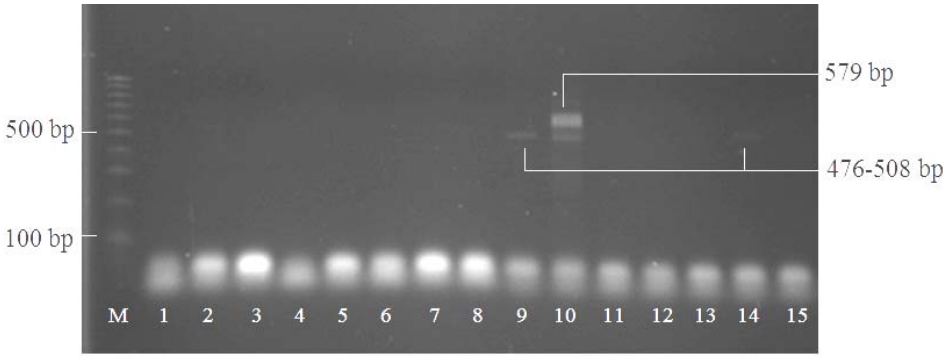


Figure 3 Filarial identification result. Lane 14 is *Brugia* spp. with 476-508 bp of product size. Lane 9 and 10 are positive control of *B. pahang*i and *D. immitis* with 476-508 bp and 579 bp of product size, respectively.

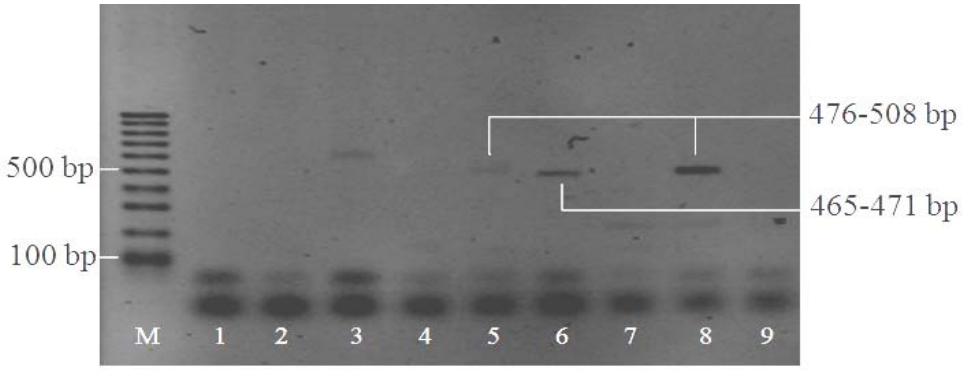


Figure 4 Filarial identification result, Lane 5 and 8 are *Brugia* spp. with 476-508 bp, Lane 6 is *W. bancrofti* with 465-471 bp of product size. Lane 1, 2 and 3 are positive control of *B. pahan*gi, *W. bancrofti* and *D. immitis* with 476-508 bp, 465-471 bp and 579 bp of product size, respectively.

Table 14 Summary of filarial worm identification obtained from wild caught mosquito collected during August, 2012 to July, 2014 by PCR technique

Month	Mosquitoes				Filarial infective form				
	Genus – species	No. of dissected	No. of positive	% positive	*Whuchereria* spp.	*Brugia* spp.	*Dirofilaria* spp.	Others	PCR results
Jan.	-	-	-	-	-	-	-	-	-
Feb.	*Ae. Imitator*	123	1	0.81	-	-	8	-	***Dirofilaria immitis***
	Ae. annandalei	15	1	6.67	-	-	-	8	**X**
	Coquilletedia crassipes	6	1	16.67	-	-	3	-	***Dirofilaria immitis***
Mar.	*Ar. Subalbatus*	614	1	0.16	-	-	3	-	***Dirofilaria immitis***
Jul.	*Ae. Malikuli*	26	1	3.81	-	2	-	-	***Brugia pahangi***
	Ae. annandalei	76	1	1.32	-	-	4	-	***Dipetalonema reconditum***
Aug.	*Ar. Subalbatus*	817	3	0.24	-	-	-	12	***X***
	Ar. Kesseli	13	1	7.69	-	7	-	-	***Brugia pahangi***
Sep.	*Ae. Desmotes*	121	1	0.83	2	-	-	-	***Wuchereria bancrofti***
	Ar. subalbatus (2012)	886	3	0.11	-	-	6	-	***Dipetalonema reconditum***
	Ar. Subalbatus (2013)	1,042	2	0.19	-	-	-	8	**X**
	Ar. Kesseli	12	1	8.33	-	5	-	-	***Brugia pahangi***
Total	**7 Positive mosquito species**	**3,751**	**17**	**0.45**	**2**	**14**	**21**	**31**	

X = unknown species of the worms.

PCR technique for dengue virus detection

Table15 Sequences of primer (ROBERT S. LANCIOTTI & GWONG-JEN CHANG, 1992)

Primer	Sequence	Position	Size of PCR product (bp)
D1	5’ TCAATATGCTGAAACGCGCGAGAAACCG	134-161	511 (D1 and D2)
D2	5’ TTGCACCAACAGTCAATGTCTTCAGGTTC	616-644	511 (D1 and D2)
TS1	5’ CGTCTCAGTGATCCGGGGG	568-586	482 (D1 and TS1)
TS2	5’ CGCCACAAGGGCCATGAACAG	232-252	119 (D1 and TS2)
TS3	5’ TAACATCATCATGAGACAGAGC	400-421	290 (D1 and TS3)
TS4	5’CTCTGTTGTCTTAAACAAGAGA	506-527	392 (D1 and TS4)

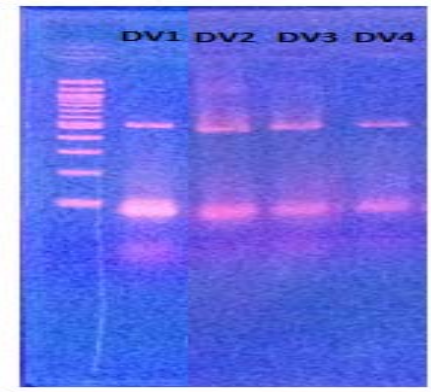


Figure5 After amplification with consensus primers D1 and D2 (511 bp)

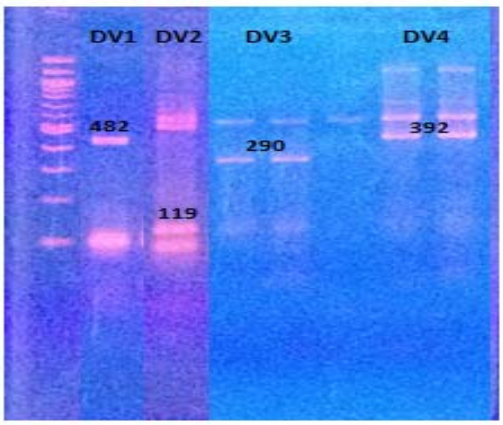


Figure6 After second-round amplification with type-specific primers TS1, TS2, TS3 and TS4. Virus products: dengue-1, 482 bp; dengue-2, 119 bp; dengue-3, 290 bp; dengue-4, 392 bp

Table16 Viruses detection from the wild caught mosquitoes.

No.	Mosquito species	No. mosquitoes (head)	Viruses detection by PCR		Dengue viruses			
			positive	Negative	DV1	DV2	DV3	4
1	*Armigires subalbatus*	1,500						
	Ar. (*Arm.*) *keselli*	140						
	Ar. (Lei) *dolichocephalus*	50						
2	*Ae. Aegypti*	110						
3	*Ae. Albopictus*	1,500						
4	*Ae. Desmotes*	100						
5	*Ae. Annadalei*	100						
	Total	**3,500**						

** 10 heads/ PCR reaction

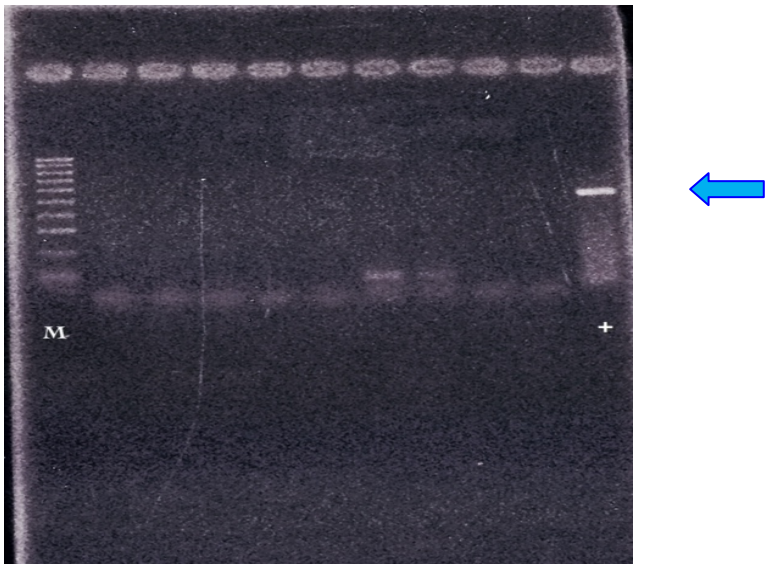


Figure7 Showing the negative detection of dengue viruses from wild caught mosquitoes and expression only positive control

Output ที่ได้จากโครงการ

1.ผลงานตีพิมพ์รดับนานาชาติ 39 เรื่อง (2012-2013, Yr 2 :2013-2014, Yr: 3 2014-2015)

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8.Suwannasilp K, Visetson S, Prabaripai A, Tanasilchayakul S, Grieco J, Bangs M, **Chareonviriyaphap T.** 2012. Behavioral responses of *Aedes aegypti* and *Culex*

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39. Surat W., Mhuangtong W., Sangsrakru D., **Charoenviriyaphap T.**, Arunyawat U., Kubera A., Sittivicharpinyo T., Siripan O and Pootakham W. 2015. Gut bacterial diversity in *Plasmodium*-infected and *Plasmodium*-uninfected *Anopheles minimus.* Chiang Mai J. Sci 42: 1-22.

Manuscripts for international journals (Pendings)

1. Arunyawat U., P. Phunngam, M.J. Bangs, and **T. Chareonviriyaphap**. Evolutionary genetics of the partial immune genes (TOLL6 and CLIPA6) in *Anopheles minimus* populations. In preparation

2. Arunyawat U., P. Phunngam, W. Kongkachana, and **T. Chareonviriyaphap**. Nucleotide variation of the partial TOLL immune genes in *Anopheles dirus* complex mosquitoes. In preparation

3. Phunngam P, U. Boonkue, **T. Chareonviriyaphap**, and U. Arunyawat. Molecular identification of four members of the *Anopheles dirus* (Diptera: Culicidae) complex based on the mitochondrial cytochrome c oxidase subunit I (COI) gene. In preparation

Book chapters

1. Manguin S, Thuy NC, Tainchum K, Juntarajumnong W, **Chareonviriyaphap T**, Michon AN and E Jumas-Bilakin E. 2013. Bacterial Biodiversity in Midguts of *Anopheles* Mosquitoes, Malaria Vectors in Southeast Asia by the book "*Anopheles* mosquitoes, New insights into malaria vectors" edited by Sylvie Manguin, ISBN 978-953-51-1188-7, In Tech, 549-551, July 7, 2013.

2. Suwonkerd W, Ritthison W, Thuy NC, Tainchum K, Bangs MJ and **Chareonviriyaphap T.** 2013. Vector Biology and Malaria Transmission in Southeast Asia by the book "*Anopheles* mosquitoes, New insights into malaria vectors" edited by Sylvie Manguin, ISBN 978-953-51-1188-7, In Tech 273-325, July 7, 2013

3. **Chareonviriyaphap T**. 2012. INSECTICIDES; PEST ENGINEERING. Chapter Title Behavioral Responses of Mosquitoes to Insecticides 201-225: In Tech Publishing Company: 538 pp.in the book "Insecticides - Pest Engineering" edited by Farzana Perveen, ISBN 978-953-307-895-3, ISBN 978 953 307 895 3.

2.ผลงาน Oral และ Poster Presentations

1. **Chareonviriyaphap T**. 2012 Recent developments in the management of vector mosquitoes in Thailand. Invited Lecture, 2012 Pest Control Association of the Phiippines 24926 Boracay Island, October 2012.

2.**Chareonviriyaphap T**. 2013. Behavioral responses of mosquito to test chemicals. Invited Lecture at Department of Entomology, Iowa State University of Science and Technology, March 27 2013, Iowa, U.S.A.

3. Boonyuan W., S. Tiawsirisup, W. Jantarachumnong, J.P. Grieco and **T. Chareonviriyaphap** 2013. Mosquito investigation in two pig farms (Japanese encephalitis endemic or non endemic), Chon Buri Province, Easthern, Thailand. The Asian Society of Vector Ecology and Mosquito Control (ASVEMC), The 3rd International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (IFSCMMD) at Lakeside Hotel, Suzhou, China. May 27-31, 2013.

4. Prabaripai A, Tainchum K, **Chareonviriyaphap T.** 2013 Behavioral response of *Aedes aegypti* and *Culex quinquefasciatus* to DEET during the day and night times. 79th Annual Meeting Febuary 24-28 2013, Atlantic City, NJ, U.S.A.

5. Suwonkerd W, Sumlmatr S, Hengtrakulvenich N, Todphet T, **Chareonviriyaphap T.** 2013 Innovative rearing technique to establish wild caught anopheline colonies in Thailand. 79th Annual Meeting Febuary 24-28 2013, Atlantic City, NJ, U.S.A.

6. Kongmee M, Achee NL, Masuoka P, Aimpun P, Lerdthusnee K, **Chareonviriyaphap T.** 2013 Characterization of the *Anopheles minimus* Complex in Thailand 79th Annual Meeting Febuary 24-28 2013, Atlantic City, NJ, U.S.A.

7.Chuaycharoensuk T., **T. Chareonviriyaphap**, S. Manguin, J. Grieco, and N. Achee. 2014. Using GIS to assess rish of JE base on Entomological and Environmental Parameters. The GIS meeting in University of Notre Dame. Poster Presentation. 19 November 2014. Indiana, USA.

8. Kongmee M, Sathantriphop S, Bangs M, **Chareonviriyaphap T.** 2014. A comparision of field and laboratory tests of escape responses in *Aedes aegypti* dengue mosquito. 45th Annual SOVE Conference, San Antonio Texas, U.S.A. September 28-2 October-2014.

9. Phothikasikorn J, Boonplueang R, Noonpakdee W, **Chareonviriyaphap T.** 2014. A comparision of four anti coagulants for developing of Bancroftain microfilaria infected in *Aedes aegypt*i. 45th Annual SOVE Conference, San Antonio Texas, U.S.A. September 28-2 October-2014.

10.Sathantriphop S, Bangs M, **Chareonviriyaphap T.** 2014. Evaluation os spatial repellent, irritant and toxic properties of plant essential oils against dengue vector *Aedes aegypti*. 45th Annual SOVE Conference, San Antonio Texas, U.S.A. September 28-2 October-2014.

11. **Chareonviriyaphap T.** 2014. Malaria and vector control intervention in Greater Makong Region: Thailand as a case study. Invied lecture. Amercican Society of Tropical Medicine Hygeine, 5 November 2014, News Oleans, Louisiana, U.S.A.

12. **Chareonviriyaphap T.** 2014. Excito repellency responses of plant derived compounds on mosquitoes. Society of Vector Ecology, 27 Septem-3 October 2014, San Antonio, Texas, U.S.A.

13. **Chareonviriyaphap T**. 2015. Species complexes and vector control in Thailand. Invited Lecture. The Asian Society of Vector Ecology and Mosquito Control, 23-28 May 2015, China

14. Arunyawat U., U. Boonkue, and **T. Chareonviriyaphap.** DNA barcodes for species identification of the malaria mosquitoes (*Anopheles dirus* complex). The 5th International Barcode of Life Conference. 27 – 31 October. 2013. Kunming, China

15. Arunyawat U., P. Phunngam, and **T. Chareonviriyaphap**. Evolutionary genetics of the partial immune genes (*TOLL6* and *CLIPA6*) in *Anopheles minimus* mosquito. American Mosquito Control Association (AMCA) 81st Annual Meeting. 29 March – 2 April 2015. New Orleans, USA

16. Arunyawat U., P. Phunngam, W. Kongkachana, and **T. Chareonviriyaphap.** Molecular evolution of *TOLL* immune genes in *Anopheles dirus* complex mosquitoes. Society of Molecular Biology and Evolution (SMBE2015) Annual meeting. 12–16 July 2015. Vienna, Austria

17. **Chareonviriyaphap T**. 2015. Species Complex and vector control in Thailand Joint International Tropical Medicine Meeting 2015 (JITMM 2015) "Emergence of Tropical Diseases: trans-disciplinary strategies" and "The 10 thSeminar on Food- and Water-Borne Parasitic Zoonoses (FBPZ7)" at Centara Grand & Bangkok Convention Centre at Central World Bangkok Thailand. December 7, 2015.

18. Arunyawat U., U. Boonkue, and **T. Chareonviriyaphap**. DNA barcodes for species identification of the malaria mosquitoes (Anopheles dirus complex). The 5th International Barcode of LifeConference. 27 – 31 October. 2013. Kunming, China

19. Arunyawat U., P. Phunngam, and **T. Chareonviriyaphap**. Evolutionary genetics of the partial immune genes (TOLL6 and CLIPA6) in *Anopheles minimus* mosquito. American Mosquito Control Association (AMCA) 81st Annual Meeting. 29 March – 2 April 2015. New Orleans, USA

20. Arunyawat U., P. Phunngam, W. Kongkachana, and **T. Chareonviriyaphap.** Molecular evolution of TOLL immune genes in *Anopheles dirus* complex mosquitoes. Society of Molecular Biology and Evolution (SMBE2015) Annual meeting. 12–16 July 2015. Vienna, Austria

3.การส่งนิสิตในโครงการไปต่างประเทศ

3.1 ฝึกอบรมในมหาวิทยาลัยต่างประเทศ

1. นางสาวกราญ์จนา ถาอินชุม นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on Parasite susceptibility and vector implications within the two complexes, *Anopheles dirus* and *Anopheles minimus* at Comparative Molecular Immuno-Physiopathology Lab, UM1, and Institute of Research for Development (IRD), January 2012. Montpellier, France สนับสนุนโดยมหาวิทยาลัยเกษตรศาสตร์ และ IRD France

2. นางสาวทิพย์วรา ช่วยเจริญสุข นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on JE virus detection and mosquito identification and molecular level at Comparative Molecular Immuno-Physiopathology Lab, UM1, and Institute of Research for Development (IRD), June 2012. Montpellier, France สนับสนุนโดย มหาวิทยาลัยเกษตรศาสตร์ และ IRD France

3. นางสาวกราญ์จนา ถาอินชุม นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on Behaviour and Vectorial Capacity of Species Complexes, *Anopheles dirus* and *Anopheles minimus*, in the Transmission of *Plasmodium* spp. and *Wuchereria bancrofti* in Western Thailand at Comparative Molecular Immuno-Physiopathology Lab, UM1, and Institute of Research for Development (IRD), 9 March 2012 – 18 January 2013. Montpellier, France, สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว) และรัฐบาลฝรั่งเศส

4. นายจิโรจ นระรักษ์ นิสิตระดับปริญญโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: BioVectrol project (Bio-Asia program), French Ministry of Foreign Affairs. 2 months (May-June 2013): Training on “Ethnobotanical study in Cambodia” Faculty of Pharmacy, Heath and Science University, Phnom Penh, Cambodia สนับสนุนโดย IRD, France

5. นางสาวกราญ์จนา ถาอินชุม นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on Team management for Jeunes Equipes AIRD (JEAI) project by Institute of Research for Development (IRD), France at Can Tho University, 17-22 February 2014, Can Tho, Vietnam, สนับสนุนมหาวิทยาลัยเกษตรศาสตร์ และรัฐบาลฝรั่งเศส

6. นายจิโรจ นระรักษ์ นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Meeting in the framework of the BioVectrol project (Nov 28-Dec 2, 2014) Lab. Pharmacognosy, Faculty of Pharmacy, Aix-Marseille University, Marseille, France สนับสนุนโดยมหาวิทยาลัยเกษตรศาสตร์ และรัฐบาลฝรั่งเศส

7. นายจิโรจ นระรักษ์ นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: 2 months (December, 2013-January, 2014): Training on “PCR technique to identify *Anopheles* mosquitoes” IRD, Faculty of Pharmacy, Montpellier University, Montpellier, France สนับสนุนโดยรัฐบาลฝรั่งเศส

8. นายจิโรจ นระรักษ์ นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: 4 months (February, 2014-May, 2014): Training on “ Phytochemistry of Southeast Asian plants to control malaria and dengue vectors” Lab. Pharmacognosy, Faculty of Pharmacy, Aix-Marseille University, Marseille, France สนับสนุนโดยรัฐบาลฝรั่งเศส

9. นายณัฐพล มารศรี นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on ‘Phytochemical studied of Capparis micracantha, a plant used as a bio-insecticides in Southeast Asia” On 8 April- 8 June 2014, Under BIO-ASIE Project by Laboratory of Pharmacognosy-Ethnopharmacology-Homeopathy, UMR-MD3, Faculty of Pharmacy, 27 Boulevard Jean Moulin-13385 Marseille Cedex 5, France Marseille. สนับสนุนโดยรัฐบาลฝรั่งเศส

10. นายณัฐพล มารศรี นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on Molecular assay for Anopheles mosquitoes species identifications, on 9 June- 31 July 2014. Under project MALVEC Project by Laboratory of Immuno-Physiopathologie Moléculaire Comparée (LIPMC), UMR-MD3, Institute of Research for Development Faculty of Pharmacy, University of Montpellier 2. 15, Ave Charles Flahault, 34093 Montpellier Cedex 5, France สนับสนุนโดยรัฐบาลฝรั่งเศส

11. นางสาววาสนา บุญญวน นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on the Bloodmeal analysis technique, September 2014-July 2015 at the Laboratory of Immuno-Physiopathologie Moléculaire Comparée (LIPMC), UMR-MD3, Institute of Research for Development Faculty of Pharmacy, University of

Montpellier 2. 15, Ave Charles Flahault, 34093 Montpellier Cedex 5, France สนับสนุนโดย มหาวิทยาลัยเกษตรศาสตร์และรัฐบาลฝรั่งเศส

12. นางสาวทิพย์วรา ช่วยเจริญสุข นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on the GIS to assess rish of JE base on Entomological and Environmental Parameters. The GIS meeting in University of Notre Dame. Poster Presentation. 19 November 2014. Indiana, USA.สนับสนุนโดย Bills Millinda Gate Foundation, USA.

13. นายเด่นภูมิ วงศ์ทังสิริ นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on House fly control in the Department of Entomology and Nematology, University of Florida, U.S.A. September 2014-February 2015. สนับสนุนโดย มหาวิทยาลัยเกษตรศาสตร์ และ BASF

14. นางสาวกฤษวรรณ พลจัตุรัส นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: 2015 Training on High-throughput screening system at ECK Institute Department of Biology University of Notre Dame, Indiana, USA 30 July – 30 November 2015. สนับสนุนโดย มหาวิทยาลัยเกษตรศาสตร์

15. นางสาวมนัสวี พัฒนกุล นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: Training on the anlysis of biopesticide technique, September-December 2015 at the Laboratory of Immuno-Physiopathologie Moléculaire Comparée (LIPMC), UMR-MD3, Institute of Research for Development Faculty of Pharmacy, University of Montpellier 2. 15, Ave Charles Flahault, 34093 Montpellier Cedex 5, France สนับสนุนโดย รัฐบาลฝรั่งเศส

3.2 นำเสนอผลงานวิจัยระดับนานาชาติ

1.นางสาววรรณภา ฤทธิสนธ์ นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: ไปเสนอผลงานแบบปากเปล่า Diversity of Anopheles mosquito in Koh Change, Trat Province. The Asian Society of Vector Ecology and Mosquito Control (ASVEMC), The 3rd International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (IFSCMMD) at Lakeside Hotel, Suzhou, China. May 27-31, 2013. สนับสนุนโดย Bills Millinda Gate Foundation, USA.

2.นางสาวสุนัยนา สะท้านไตรภพ นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: ไปเสนอผลงานแบบปากเปล่า. Plants with repellent activity on *Aedes aegypti*. The Asian Society of Vector Ecology and Mosquito Control (ASVEMC), The 3rd International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (IFSCMMD) at Lakeside Hotel, Suzhou, China. May 27-31, 2013. สนับสนุนโดยมหาวิทยาลัยเกษตรศาสตร์และ Bills Millinda Gate Foundation, USA.

3.นางสาววาสนา บุญญวน นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: ไปเสนอผลงานแบบปากเปล่า Mosquito investigation in two pig farms (Japanese encephalitis endemic or non endemic), Chon Buri Province, Easthern, Thailand. The Asian Society of Vector Ecology and Mosquito Control (ASVEMC), The 3rd International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (IFSCMMD) at Lakeside Hotel, Suzhou, China. May 27-31, 2013. สนับสนุนโดย Bills Millinda Gate Foundation, USA.

4.นางสาวรุ่งอรุณ ทิศกระโทก นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ เสนอผลงานแบบปากเปล่าเรื่อง Chemical induced behavioral responses in *Anopheles epiroticus* in Thailand. 6th International Congress Society for Vector Ecology September 22-27, 2013 La Quinta (Palm Springs), California, USA. สนับสนุนโดย CIRAD, France และ Bills Millinda Gate Foundation, USA.

5.นางสาวพัชราวรรณ สิริโสภา นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์: ได้รับการคัดเลือกจาก The SOVE, USA. ให้ไปเสนอผลงานแบบปากเปล่าเรื่อง Insectcide resistance in Aedes aegypti in Thailand งาน 6th International Congress Society for Vector Ecology September 22-27, 2013 La Quinta (Palm Springs), California, USA. สนับสนุนโดย SOVE, U.S.A.

6.นายวิถี เหมือนวอน นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ เสนอผลงานแบบปากเปล่าเรื่อง Genetic diversity of stable fly in Thailand 6th International Congress Society for Vector Ecology September 22-27, 2013 La Quinta (Palm Springs), California, USA. สนับสนุนโดย CIRAD, France และ Bills Millinda Gate Foundation, USA. สนับสนุนโดย SOVE, U.S.A. และ สำนักงานกองทุนสนับสนุนการวิจัย (สกว)

7. นางสาวกราญ์จนา ถาอินชุม นิสิตระดับปริญญาเอก ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ เสนอผลงานแบบปากเปล่าเรื่อง Bacterial biodiversity in abdomens of *Anopheles* mosquitoes in western Thailand. The 4th International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (IFSCMMD). May 25-28, 2015. Guangzhou, China. (Oral presentation) สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว)

8.นางสาวกฤษวรรณ พลจัตุรัส นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ เสนอผลงานแบบปากเปล่าเรื่อง Evaluation of LLINs for Mosquito Control in Malaria Endemic Area of Thailand. The 4th International Forum for Surveillance and Control of Mosquito and Mosquito-borne Diseases (IFSCMMD) May 25-28, 2015 Guangzhou China (Oral presentation) สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว)

9. นางสาวกฤษวรรณ พลจัตุรัส นิสิตระดับปริญญาโท ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ เสนอผลงานแบบปากเปล่าเรื่อง Evaluation of LLINs for Mosquito Control for Malaria Endemic Area of Thailand. The 64th Annual Meeting American Society of Tropical Medicine & Hygiene (ASTMH). October 25-29, 2015. Philadelphia, Pennsylvania, USA. (Poster presentation) สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว) มหาวิทยาลัยเกษตรศาสตร์

4.นักศึกษาและนักวิจัยต่างประเทศ

1. Dr.Amanda Eiden นักวิจัยหลังปริญญาเอก จาก University of Florida ประเทศสหรัฐอเมริกา (Mar 2014-Jan 2015)

2. Dr. Camelle Drouguse นักวิจัยหลังปริญญาเอก จาก University of Marselle ประเทศฝรั่งเศส (April 2014-Sept 2014)

3. Dr.Alaxandra นักวิจัยหลังปริญญาเอก จาก University of Marselle ประเทศฝรั่งเศส (Aug 2015-Aug 2016)

4. Dr.Dominique Cerqueira นักวิจัยหลังปริญญาเอก จาก University of Marselle ประเทศฝรั่งเศส (Aug 2015-Aug 2016)

5. Mr. Victor Chaumeau นักศึกษาปริญญาเอก จาก University of Montpellier ประเทศฝรั่งเศส (Feb 2014-Feb 2015)

6. Mr. Phubeth Ya-Umphan นักศึกษาปริญญาเอก จาก University of Montpellier ประเทศฝรั่งเศส (Feb 2014-Feb 2015)

7. Ms. Celine Montazeau นักศึกษาปริญญาโท จาก University of Montpellier ประเทศฝรั่งเศส (Feb 2014-Feb 2015)

8. Ms. Sella Defonne นักศึกษาปริญญาโท จาก University of Montpellier ประเทศฝรั่งเศส (Jan 2015-June 2015)

9.Ms. Macy Brusich นักศึกษาปริญญาโท จาก Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame ประเทศสหรัฐอเมริกา (July-Sep 2014)

10. Mr.Atthicus L.นักศึกษาปริญญาโท จาก Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame ประเทศสหรัฐอเมริกา (July-Sep 2015)

11.Trang H. นักศึกษาปริญญาโท จาก Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame ประเทศสหรัฐอเมริกา (July-Sep 2015)

5.การเชื่อมโยงกับต่างประเทศ

1.นักวิจัยจากประเทศฝรั่งเศสจากโครงการ STOPVEC ภายใต้การสนับสนุนของสำนักงานความร่วมมือเพื่อการพัฒนาระหว่างประเทศ (สพร.) ประจำที่ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ (2012-2014, 2014-2016)

1.1 Dr. Vincent Corbel, Senior scientist IRD/France

1.2 Ms. Benedict Fustec, Junior scientist IRD/France

1.3 Mr. Stephane Duchon, Junior scientist IRD/France

1.4 Mr. John Zadrozny, Junior scientist IRD/France

2.นักวิจัยจากประเทศสหรัฐอเมริกาจากโครงการการควบคุมแมลงพาหะนำโรค สัมมนาและสอนที่ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์

2.1 Dr. Nicole Achee, Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame

2.2 Dr. John Grieco, Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame

2.3 Dr. Michael Bangs, NAVY, Bethesda, Maryland, USA

2.4 Prof. Joe Coats, Department of Entomology, Iowa State University, USA

2.5 Prof.Dr.Philipp Koelher, Department of Entomology and Nematology, University of Florida, USA

2.6 Dr. Pereira Roberto, Department of Entomology and Nematology, University of Florida, USA

3.นักวิจัยจากประเทศนอรเวย์ จากโครงการการควบคุมแมลงพาหะนำโรคสอนที่ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์

3.1 Dr. Hans Overgaads, Senior scientist

4.นักวิจัยจากประเทศฝรั่งเศสจากโครงการการควบคุมแมลงพาหะนำโรคสัมมนาและสอนที่ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์

4.1 Prof.Dr.Sylvie Manguin, University of Montpellier, France

4.2 Prof. Dr.Garard Duvallet, University of Montpellier, France

5.นักวิจัยจากประเทศอังกฤษจากโครงการการควบคุมแมลงพาหะนำโรคร่วมทำวิจัยสร้างเครือข่ายที่ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์

5.1 Dr.Mariance Sinka, Senior scientist, Deparment of Zoology, Oxford University

5.2 Prof.Simon Hay, Deparment of Zoology, Oxford University, UK

6.นิสิตที่สำเร็จการศึกษา

6.1 นิสิตระดับปริญญาเอก ที่จบการศึกษาระหว่างงานวิจัยฯ

1. ดร.สังสิทธิ์ สังวรโยธิน	คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล
2. ดร.มณฑาทิพย์ คงมี	คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ กำแพงแสน
3. ดร.วิถี เหมือนวอน	คณะเกษตรศาสตร์ มหาวิทยาลัยขอนแก่น
4. ดร.กราญ์จนา ถาอินชุม	คณะทรัพยากรธรรมชาติมหาวิทยาลัยสงขลานครินทร์
5. ดร.จินรภา โพธิกสิกร	คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
6. ดร.ววรภา ฤทธิสนธิ์	กรมควบคุมโรค กระทรวงสาธารณสุข
7. ดร.ปิติ มงคลางกูร	กรมควบคุมโรค กระทรวงสาธารณสุข
8. ดร.คนัจฉรีย์ ถานิสพงษ์	กรมควบคุมโรค กระทรวงสาธารณสุข
9. ดร.สุนัยนา สะท้านไตรภพ	กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
10. ดร.สุภารดา สุคนธาภิรมณ์	กรมวิชาการเกษตร กระทรวงเกษตรและสหกรณ์
11. Dr.Ferdinan Salazar	Director of Medical Entomology Department, Ministry of Health, the Philippines

6.2 นิสิตระดับปริญญาโท ที่จบการศึกษาระหว่างงานวิจัยฯ

1. นางสาวพัชราวรรณ ศิริโสภา	กระทรวงสาธารณสุข
2. นางสาวกรวิกา สุวรรณศิริศิลป์	Pest Control Company
3. นายสุนทร พิมพ์นนท์	คณะสาธารณสุขศาสตร์ ม.มหิดล
4. นางสาวสุพัตรา เขียวระยับ	ระหว่างการเตรียมตัวเพื่อเรียนต่อ USA
5. นางสาวนันทวรรณ สุวรรณโชติ	กระทรวงสาธารณสุข
6. นางสาวศุภลักษณ์ ผลสมบูรณ์	ศึกษาปริญญาเอกที่ USUSH/USA
7. นางสาววาสนา บุญญวน	ศึกษาปริญญาเอกที่ ม.เกษตรศาสตร์
8. นางสาวนริศรา มาลัยทอง	ศึกษาปริญญาเอกที่ ม.เกษตรศาสตร์
9. นางสาวรุ่งอรุณ ทิดกระโทก	ศึกษาปริญญาเอกที่ ม.เกษตรศาสตร์
10. นางสาวทิพย์วรา ช่วยเจริญสุข	ศึกษาปริญญาเอกที่ ม.เกษตรศาสตร์
11. นายฉัตรชัย ทะนันไชย	ศึกษาปริญญาเอกที่ UM, France
12. นางสาวอมรรัตน์ ปันต๊ะวงศ์	ศึกษาปริญญาเอกที่ UM, France

7. การนำผลจากโครงการไปใช้ประโยชน์

1. ด้านการเรียนสอน นิสิตปริญญาโท เอก

2. เป็นข้อมูลการเฝ้าระวังโรคที่ติดต่อโดยแมลงแก่หน่วยงานสาธารณสุขและปศุสัตว์

3. กระทรวงสาธารณสุขนำข้อมูลยุงพาหะนำโรคไปใช้ในการพัฒนานโยบายการคุมโรคที่มีแมลงเป็นพาหะ

4. เป็นข้อมูลพื้นฐานฝนการสร้างและพัฒนางานวิจัยต่อไปในอนาคต

8. การจัดการประชุมประจำปี

1. การจัดการประชุมประจำปี 2557 ครั้งที่ 1 ภายใต้โครงการวิจัยเรื่อง “นิเวศวิทยาของยุงและแมลงวันคอกสัตว์และนัยยะความเป็นไปได้ในการใช้สารสกัดพืชเพื่อควบคุมแมลง พาหะนำโรค” เมื่อวันพฤหัสบดี ที่ 24 กรกฎาคม 2557 ณ ห้องธีระสูตบุตร อาคารสารนิเทศ 50 ปี มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน

2. การจัดการประชุมประจำปี 2558 ครั้งที่ 2 ภายใต้โครงการวิจัยเรื่อง “นิเวศวิทยาของยุงและแมลงวันคอกสัตว์และนัยยะความเป็นไปได้ในการใช้สารสกัดพืชเพื่อควบคุมแมลง พาหะนำโรค” เมื่อวันพฤหัสบดี ที่ 22 พฤษภาคม 2558 ณ ห้องรวงข้าว อาคารวชิรานุสรณ์ คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน

3. การจัดการประชุมประจำปี 2559 ครั้งที่ ภายใต้โครงการวิจัยเรื่อง “นิเวศวิทยาของยุงและแมลงวันคอกสัตว์และนัยยะความเป็นไปได้ในการใช้สารสกัดพืชเพื่อควบคุมแมลง พาหะนำโรค” เมื่อวันพฤหัสบดี ที่ 25 กุมภาพันธ์ 2559 ณ ห้องกำพลอดุลวิทย์ อาคารสารนิเทศ 50 ปี มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน

9. ผลงานที่สำคัญ 6 เรื่องที่เป็นประโยชน์ต่อองค์กรที่จัดทำนโยบาย กรมปศุสัตว์ กระทรวงเกษตรฯ และควบคุมโรค กระทรวงสาธารณสุข

1. การแบ่งสารเคมีตามแนวคิดใหม่ในวารสาร Parasites and Vectors ในปี 2013 (ค่า IMPACT FACTOR 3.2)

2. แผนที่ภูมิศาสตร์การต้านทานสารเคมีในยุงนำโรคต่างๆ ในวารสาร Parasites and Vectors ในปี 2013 (ค่า IMPACT FACTOR 3.2)

3. แผนที่ภูมิศาสตร์ของยุงพาหะในประเทศไทยที่จำแนกด้วยเทคนิคทางด้านโมเลกุลเป็นครั้งแรกในวารสาร Trends in Parasitology 2015 (ค่า IMPACT FACTOR 6.24)

4. การศึกษาการดื้อยาฆ่าแมลงของยุงลายด้วยเทคนิคใหม่ Unravelling genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. Genome Res. 2015 23. (ค่า IMPACT FACTOR 16.2)

5. ทีมวิจัยได้พบว่าพืชสมุนไพรไทย 4 ชนิด มีศักยภาพพัฒนาเป็นสมุนไพรไล่ยุง และอยู่ระหว่างการดำเนินการพัฒนาในเชิงพานิชย์กับสำนักงานกองทุนสนับสนุนการวิจัย

6. แผนที่ภูมิศาสตร์ของชนิดของแมลงวันคอกสัตว์ที่พบในประเทศไทย (อยู่ระหว่างการตีพิมพ์)

10.รางวัลที่ได้รับ

ชื่อรางวัล	หน่วยงานทีให้	ปี
1. โล่ห์ประกาศเกียรติคุณ นิสิตเก่าดีเด่น	คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์	2555
2.รางวัลนักวิจัยผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ระดับนานาชาติ ปี 2554	มหาวิทยาลัยเกษตรศาสตร์	2556
3.รางวัลนักวิจัยผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ระดับนานาชาติ ปี 2555	มหาวิทยาลัยเกษตรศาสตร์	2556
4.รางวัลเชิดชูเกียรติ ปราชญ์วิจัย มหาวิทยาลัยเกษตรศาสตร์ ปี 2555	มหาวิทยาลัยเกษตรศาสตร์	2556
5.รางวัลนักวิจัยผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ระดับนานาชาติ ปี 2556	มหาวิทยาลัยเกษตรศาสตร์	2557
6.โล่ห์ประกาศเกียรติคุณอาจารย์ดีเด่นแห่งชาติ	ที่ประชุมประธานสภาอาจารย์แห่ง ปทท.	2558
7. รางวัลวิทยาศาสตร์และเทคโนโลยีดีเด่น กลุ่มวิจัย บทบาทของแมลงและสัตว์ขาปล้องที่เป็นปัญหาในชุมชน ปศุสัตว์และสาธารณสุข	มูลนิธิโทเรเพื่อการส่งเสริมวิทยาศาสตร์ประเทศไทย	2558

11. กิจกรรมอื่น ๆ

1. Appointed to be “Regional Director of Thailand, Laos, Vietnam of Asian Society of Vector Ecology and Mosquito Control from 2012-2017

2. ร่วมจัดแสดงผลงานนวัตกรรมในงานเพื่อนพึ่ง (ภา) เฉลิมพระเกียรติมหาราชินี ปี 2555

3.คณะทำงานโครงการพัฒนาหลักสูตรผู้ช่วยนักกีฏวิทยา ภายใต้ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ ในโครงการพระราชทานความช่วยเหลือแก่ราชอาณาจักรกัมพูชาด้านสาธารณสุข (Malaria) ขอสมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารี เป็นเวลา 2 ปี (2555-2556)

4. สนับสนุนสมุนไพรไล่ยุงพื้นบ้าน ผลงานวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัยแก่โรงเรียนอนุบาล มหาวิทยาลัยธรรมศาสตร์ ในอุปถัมภ์ของสมเด็จพระพี่นางเธอเจ้าฟ้ากัลยานิวัฒนา กรมหลวงนราธิวาสราชนครินทร์ ที่ ศธ.04086 อ.20/0101 ลงวันที่ 5 มิถุนายน 2558

5. สนับสนุนสมุนไพรไล่ยุงพื้นบ้าน ผลงานวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัยแก่โรงเรียนอนุบาล ปรางทิพย์ เขตจตุจักร กทม. ที่ ปท. มิย. 014/2558 ลงวันที่ 12 มิถุนายน 2558

6. สนับสนุนสมุนไพรไล่ยุงพื้นบ้านเข้าร่วมในงาน “เมืองสุขภาพ วิถีชุมชน” ระหว่างวันที่ 7-26 กรกฎาคม 2558 ณ ตลาดคลองผดุงกรุงเกษม โดยกรมควบคุมโรค กระทรวงสาธารณสุข

7. ที่ปรึกษาโครงการฯ ด้านกีฏวิทยาระหว่างประเทศของสถาบัน CIRAD (France), IRD (France), Notre Dame U (U.S.A.), Norwigian University of the Life Science (Norway), Mont U (France), USUHS (U.S.A.), NNMC (U.S.A.)

Activities and Events Obtained

Different mosquito repellent products containing essential oils scientifically proven to repel mosquitoes by this subproject



BALM



LOTION



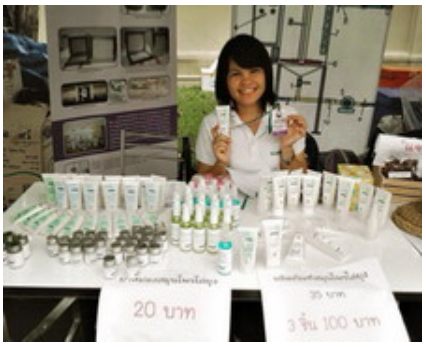
SPRAY



LIQUID BALM



Public demonstrations



Demonstrating and selling various mosquito repellent products at the Friends In Need (of 'PA') event. The event took place at Suan Kularb Palace on December 17-26, 2013



Demonstrating and selling mosquito repellent products at “Kaset Fair 2015” at Kasetsart University. The event was held on January 30-February 7, 2015







Demonstrating and selling mosquito repellent products by joining with Bureau of Vector Borne Diseases, Department of Disease Control at “Healthy City, Community Way of Life Fair” at Khlong Phadung Krung Kasem Market held on July 7-26, 2015. Prime Minister Gen Prayut Chan-o-cha attended opening the event.



Distributing mosquito repellent products to the TRF meeting's participants prior to the meeting

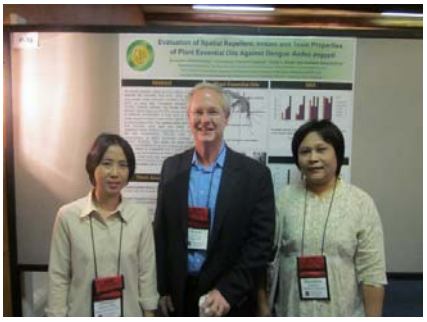


Demonstrating the excito-repellent chambers and the high throughput screening system at the TRF meeting

Presentations



Poster presentation related to this subproject held at Kasetsart University on July 1, 2015



Presenting the poster at 45th Annual Conference Society for Vector Ecology (SOVE), Texas, USA on September 28-October 2, 2014

Research to people



Teaching people how to make mosquito repellent balm at “Kaset Fair” at Kasetsart University in 2014

Research to communities



Giving the mosquito repellent products to communities in Tambon Rai Khing for their protection against mosquito bites accepted by vice chairman of the Rai khing Town municipality. He will encourage and serve as anti-mosquito product models for local community projects.

Evaluation of a peridomestic mosquito trap for integration into an *Aedes aegypti* (Diptera: Culicidae) push-pull control strategy

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Received 18 April 2011; Accepted 19 September 2011

ABSTRACT: We determined the feasibility of using the BG-Sentinel™ mosquito trap (BGS) as the pull component in a push-pull strategy to reduce indoor biting by *Aedes aegypti*. This included evaluating varying numbers of traps (1-4) and mosquito release numbers (10, 25, 50, 100, 150, 200, and 250) on recapture rates under screen house conditions. Based on these variations in trap and mosquito numbers, release intervals were rotated through a completely randomized design with environmental factors (temperature, relative humidity, and light intensity) and monitored throughout each experiment. Data from four sampling time points (05:30, 09:30, 13:30, and 17:30) indicate a recapture range among treatments of 66-98%. Furthermore, 2-3 traps were as effective in recapturing mosquitoes as 4 traps for all mosquito release numbers. Time trends indicate Day 1 (the day the mosquitoes were released) as the "impact period" for recapture with peak numbers of marked mosquitoes collected at 09:30 or 4 h post-release. Information from this study will be used to guide the configuration of the BGS trap component of a push-pull vector control strategy currently in the proof-of-concept stage of development in Thailand and Peru. ***Journal of Vector Ecology* 37 (1): 8-19. 2012.**

Keyword Index: *Aedes aegypti*, screen house, BG-Sentinel™ trap, push-pull strategy, Diptera, Thailand.

INTRODUCTION

Dengue and dengue haemorrhagic fever occur in the tropics and subtropics with an estimated 2.5 billion people residing in areas where dengue is endemic (WHO 2009). Dengue viruses are transmitted primarily by *Aedes aegypti*, a day-biting mosquito that feeds and rests indoors and preferentially bites humans (Gubler 1998, Harrington et al. 2005). Despite years of public health efforts and research progress, an effective vaccine against dengue virus is not yet available. For this reason, disease prevention remains dependent on vector management and control strategies (Reiter and Gubler 1997, WHO 2009). However, controlling *Ae. aegypti* has proven difficult due to its strong association with domestic and peridomestic human environments which harbor and sustain development sites for the immatures (i.e., artificial containers). Human-vector contact in and around human dwellings highlights the need for an intervention that is effective against the target mosquito while reducing potential hazards to humans.

Historically, indices for measuring the abundance of the immature stages of the mosquito (e.g., Breteau Index and more recently, pupae per person) have guided when and where control operations should be implemented (Reiter and Gubler 1997, Focks 2003, WHO 2009). Although these indices can provide useful information, they are not consistently predictive of the abundance of adult mosquitoes or dengue incidence (Tun-Lin et al. 1996, Morrison et al. 2004). Thus, the development of new, improved traps for adults, such as the BG-Sentinel™ (BGS) and Zumba™ traps, provides an opportunity for improved entomological surveillance and possibly also control of *Ae. aegypti* (Krockel et al. 2006, Maciel-de-Freitas et al. 2006, Williams et al. 2006, 2007, Ball and Ritchie 2010a, b, Bhalala and Arias 2009) and *Ae. albopictus* (Ritchie et al. 2006, Farajollahi et al. 2009).

The combination of attractant baits and insecticide-treated traps have been used to effectively create "infestation barriers" for nuisance mosquito populations (Kline 2006). Furthermore, trap and lure combinations have been successful in the control of several insects, including tsetse flies, the vector of African trypanosomiasis (Vale 1993, Torr 1994). Traps are important tools for surveying the abundance of vectors (Rupp and Jobbins 1969, Kline 2006) but most traps are relatively ineffective, especially against a day-biting mosquito such as *Ae. aegypti* (Service 1993, Scott and Morrison 2003, Facchinelli et al. 2008). New traps such as the BGS may be effective enough for incorporation as tools for the control of *Ae. aegypti*. The BGS trap incorporates in its design the most important elements of *Ae. aegypti* host-seeking behavior by combining an olfactory cue (BG Lure) with visual cues (black and white contrast) to attract the

mosquito. This trap has proven to be an effective tool for surveillance of *Ae. aegypti* adults, out-performing other collection devices and traps such as the CDC backpack aspirator, the Fay-Prince trap, the Encephalitis Virus Surveillance trap, and the commercially available Mosquito Magnet Liberty™ trap (Maciel-de-Freitas et al. 2006, Krockel et al. 2006, Williams et al. 2006). It has also been suggested that the BGS could be a possible replacement for human-landing catches of *Ae. aegypti* (Krockel et al. 2006).

Based on these findings, the BGS was selected as the trapping device for integration into a push-pull control strategy for *Ae. aegypti* currently in the proof-of-concept stage. The Push component focuses on using spatial repellent (SR) and/or contact irritant (CI) chemicals in sublethal doses (thus rendering them safer for human exposure) and reduced treatment coverages to reduce indoor densities of host-seeking *Ae. aegypti*. The BGS is to serve as the Pull component to remove chemically repelled or irritated *Ae. aegypti* from the peridomestic environment, thereby reducing human-vector contact. As an added benefit, the trap will facilitate the monitoring of mosquito movement among huts allowing for an evaluation of any potential diversion of mosquitoes to untreated locations.

Push-pull control strategies have been proven effective in the control of agricultural pests (Miller and Cowles 1990, Midega et al. 2006). The general concept of a push-pull system involves behavioral manipulation of the target pest population to repel or deter them away from a source (push component) using a stimulus that renders the source unsuitable or unattractive. The pests are simultaneously lured to an attractive source (pull component), such as a trap, through which they are removed from the location (Nielsen 2001, Amudavi et al. 2007, Cook et al. 2007). The same strategy may prove to be effective in the control of pathogen-transmitting mosquitoes through the manipulation of naturally occurring differences in the attractiveness of host species (Hallem et al. 2004, Constantini et al. 2001) or through the use of repellents (Barnard and Xue 2004, Fradin and Day 2002) as push stimuli in combination with attracticides derived from host odors (Bhasin et al. 2001) or attractive pheromones (Blackwell et al. 1994) as pull stimuli.

As an application to the control of dengue, this study was conducted to determine the effect of varying BGS trap numbers and *Ae. aegypti* population densities on recapture rates under semi-field conditions. This information will be used to guide data interpretation from push-pull experimental hut trials performed in a natural setting.

MATERIALS AND METHODS

Study design

A mark-release-recapture experiment in a completely randomized design (CRD) using varied numbers of BGS traps (1-4) and released *Ae. aegypti* female adults (10, 25, 50, 100, 150, 200, and 250) was conducted in a screen house located in Pu Teuy Village, Sai Yok District, Kanchanaburi Province, Thailand (14°20′N 98 °59′E). The study was conducted from August, 2009 to March, 2010.

Screen house

A screen house, measuring 4 m (width) x 3.5 m (height) x 40 m (length), was built to create a semi-field condition and facilitate the recapture of released *Ae. aegypti* (Figure 1). The screen house was subdivided into four 10 m long cubicles, each separated by folding metal screen partitions. The space volume per cubicle section was 140 m^3 (4 x 10 x 3.5 m). This is similar to the area of the experimental huts used in push-pull trials (Chareonviriyaphap et al. 2010) and the expected space volume that *Ae. aegypti* primarily would use in and around a typical home as observed from smaller dengue endemic villages (mean dispersal 28-93 m) in Thailand (Harrington et al. 2005). This area also took into consideration reports that *Ae. aegypti* responds directly to visual cues at a 10 m distance (Clements 1999).

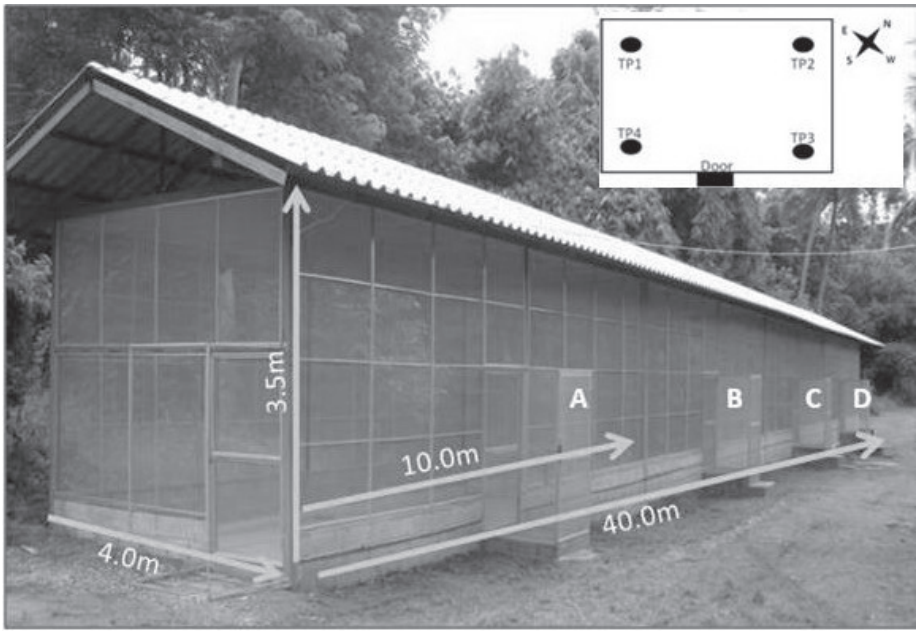


Figure 1. The screen house facility at Pu Teuy, Kanchanaburi, Thailand. All sides are screened and the cement floor is covered with white plastic to detect knocked-down mosquitoes. Collapsible walls are used to partition the screen house into four separate 10m long cubicles (A-D). Top insert: designated BGS trap position within a single cubicle.

The screen house floors were lined with white plastic sheeting to facilitate observation and recovery of knocked-down mosquitoes. The screen house and BGS traps were cleaned regularly to remove predators that otherwise would consume knocked-down or trapped mosquitoes. Environmental parameters (temperature, relative humidity, and light intensity) were measured for each cubicle section using HOBO Data Loggers (Onset Computer Corporation, MA, 1997-2003).

BG Sentinel™ (BGS) traps

The traps used in the study were BGS traps baited with the BG-Lure (Biogents AG, Regensburg, Germany). The trap consists of a collapsible container made of white plastic sack material. The contrasting black and white colors of the trap provide visual attraction. The accompanying lure (BG-Lure) consists of lactic acid, ammonia, and caproic acid compounds, all of which are components of human sweat. The BG-Lures were used within four months after opening as stated in the product label.

Trap placement

Traps were placed in designated positions (i.e., position 1-4 depending on trap number being evaluated) in the four corners of each of the 140 m^3 cubicles (Figure 1, top). Specifically, traps were placed at the 1 m intersection from the adjacent corner walls as per the manufacturer's suggestion to place the trap at least 1 m away from walls. The insert in Figure 1 shows the relative positions of BGS traps in each cubicle of the screen house. Upon entry into a specific cubicle, the first corner to the left was designated Trap Position 4 (TP4), traveling clockwise, the other corners were designated Trap Position 1 (TP1), Trap Position 2 (TP2), and Trap Position 3 (TP3). When only one trap was evaluated, the TP1 position was used. During the evaluation of two traps, both TP1 and TP2 were used while TP1, TP2, and TP3 were used to evaluate three traps and all four designated positions were used when evaluating four traps. Potential positional bias among all four traps (TP1-TP4) within individual cubicles and among cubicles was evaluated in separate trials in which one trap was rotated through all four trap positions over the course of 16 days (four replicates per trap position; data not shown).

Mosquitoes

Ae. aegypti mosquitoes were reared at the Pu Teuy field insectary, Sai Yok District, Kanchanaburi Province, following previously described standard methodology (Kongmee et al. 2004). Adults were from the F_2-F_5 generations and all originated from immatures collected monthly in Pu Teuy village. Test populations were non-blood fed, three to five-day-old nulliparous females. Mosquito Release Numbers (RN) were grouped into three categories: low (10, 25, 50), medium (100, 150), and high (200, 250). The low RNs were based on reported numbers of *Ae. aegypti* from homes in dengue-endemic areas (Jeffery et al. 2009, Sheppard et al. 1969, Perich et al. 2003, Maciel de-Freitas et al. 2008) and represent the most common densities of *Aedes aegypti* occurring in real-life situations. Medium and high RNs were used to assess the feasibility of BGS applications if high *Ae. aegypti* populations were encountered in the field. Mosquitoes were marked with fluorescent dust (BioQuip Products, Rancho Dominquez, CA) 12 h prior to release, following the method of Achee et al. (2005), to facilitate detection of knocked-down individuals and distinguish them from wild mosquitoes that may have entered the screen house during trials. Marked specimens were sugar-starved approximately 24 h prior to testing to encourage and elicit host-seeking behavior but provided water-soaked cotton pads until time of release at 05:30. A new release population was used for each treatment replicate.

An assessment of the dusting application method was performed to validate 100% coverage of mosquitoes and confirm that no negative effects of the marking procedure was seen for BGS recapture rates.

Recapture monitoring

Individual mosquito test populations were released inside screen house cubicles on day 1 at 05:30. Initial baseline trials were conducted using one trap and 100 *Ae. aegypti* RN to determine the length of the monitoring period needed to observe maximum cumulative recapture. This time period was considered the "Impact Period" or time of peak recapture and was used to guide future sampling in the study. Based on these initial trials, monitoring of trap recapture in subsequent experiments was conducted at 09:30, 13:30, and 17:30 on day 1 and at 05:30, 09:30, 13:30, and 17:30 on day two. At each sampling interval, the BGS collection bags were removed and replaced with a clean bag. In addition, the knock-down response, defined as the insect lying on its side and not being able to right itself after gentle prodding (Grieco et al. 2007), were recorded by systematic observation of the flooring of each cubicle. Collection bags were immediately placed at -20° C to kill captured mosquitoes. Recaptured mosquitoes were recorded according to marking color, time of collection, trap number, and the cubicle from which they were collected. Four replicates were performed for each treatment trial.

Data analysis

Percentage of recaptured mosquitoes for each trial was corrected by adjusting for the number of knocked-down mosquitoes. The percentage of recaptured mosquitoes was quantified according to recapture time points (day 1 – 09:30, 13:30, 17:30, and day 2- 05:30). Cumulative recapture rate was transformed using arcsine square root values for analysis of variance (PROC ANOVA). One way ANOVA was performed on transformed values of cumulative recapture rate to determine the effect of number of BGS traps (1, 2, 3, or 4) and density of releasing number (low, medium, or high) at different recapture times, and multiple comparison was done using Scheffe's test at $P = 0.05$ (SAS institute Inc. 2002-2008). One-way ANOVA (using PROC ANOVA in SAS 9.2) was performed on the transformed value of the cumulative recapture rate at different recapture times to determine the effect of number of BGS traps at

varying mosquito release numbers. Multiple comparison of means was done using Scheffe's test ($\alpha = 0.05$). The mean cumulative percentages recapture +/- SE of untransformed data are reported.

RESULTS

Baseline experiments

Results from baseline experiments indicate that the "Impact Period" occurred on day 1 with an overall recapture of 84% and peak recaptures (28-35%) occurring between the time points 09:30 and 13:30. Total recapture on day 2 during these trials was 11.3%. Experiments evaluating positional bias of trap placement indicated no significant difference in trap recapture among the T1-T4 cubicle positions (data not shown).

Baseline trials also indicated no significant difference among cubicles for mean temperature (A=24.8, B=24.7, C=24.3, and D=24.8° C) or relative humidity (A=75.4, B=75.4, C=77.0, and D=76.0%). Baseline light intensities were: A=83.33, B=70.93, C=21.11, and D=33.03 lx/ft^2 with average light intensity of cubicle A being significantly higher than that of cubicle C ($F_{(3,92)}$ =4.11, *p<0.01*). Despite inter-trial variability among cubicles, these values were all greater than previously reported for thresholds of *Ae. aegypti* host-seeking activity of 0.1 lx (0.01 foot candle = 1 lumex (lx)/ft^2(Kawada et al. 2005). Combined, these results justified the use of a completely randomized study design (CRD).

Recapture success in relation to number of traps used

Cumulative percentage recapture on Day 1 for all RNs evaluated ranged from 41-92% for one trap, 66-92% for two traps, 69-95% for three traps, and 72-98% for four traps (Tables 1-3). When including Day 2, the cumulative percentage recapture increased to 66-92%, 80-94%, 86-95%, and 82-98% for 1-4 traps, respectively (Tables 1-3). Going from one trap to two to four traps added a range of 10-18% increase in the total recapture rate across RNs.

Analysis of grouped RN categorical data (low, medium, high) revealed an overall trap and RN relationship (Table 4). Significantly lower BGS recapture success of *Ae. aegypti* resulted when a single trap ($F_{(3,\ 108)}$=5.96, *p<0.0008*) was used (82.7%), whereas there were no significant differences between recapture percentages when using two to four traps; with means of 86.3, 90.2, and 91.3%, respectively, at the end of day 2 (Table 4). The cumulative percent recaptures observed at 13:30 ($F_{=(3.108)}$=4.46, p<0.0054) and 17:30 hours ($F_{(3,\ 108)}$=6.51, p<0.0004) on day 1 showed a statistically similar trend compared to the combined totals from days 1-2. (Table 4). Overall, the highest mean cumulative recapture (91.30%) was recorded from the use of four traps.

Recapture success in relation to number of mosquitoes released

Overall, analyses from combined data for BGS trap density showed that except for the first collection period (09:30), significantly fewer mosquitoes were recaptured on Day 1 from the low RN category when compared to the medium and high RN categories at 13:30 ($F_{(3,109)}$=7.11, p<0.001), 17:30 ($F_{(3,109)}$=8.90, p<0.0003), and at the end of Day 2 ($F_{(3,109)}$=7.30, p<0.001) (Table 5). However, no significant difference was observed between percentages of mosquitoes collected from medium and high RNs when comparing all trap densities (Table 5).

Cumulative recapture trends between RNs and BGS trap density

Combined Day 1 and Day 2 cumulative percentage recapture rates when four BGS were used was >91% for RNs of 100, 150, 200, and 250 and >80% for RNs of 10, 25, and 50 (Tables 1-3). Using two or three traps resulted in cumulative percentage recapture rates of >80% for all RNs. The use of one trap at the RN of 25 resulted in a combined Day 1 and Day 2 percentage recapture rate below 80% (Table 1).

The low RN category consistently resulted in fewer recaptured mosquitoes when using one to four traps (Figures 2A-D) as compared to medium and high RNs. As expected, with the use of one (Figure 2A) and two (Figure 2B) traps, recapture rates increased as the RNs increased. When three (Figure 2C) and four (Figure 2D) traps were used, higher recapture rates were observed from medium RNs compared to the high category RNs but the differences were not statistically significant (Tables 1-3, 5).

Comparison of recapture rates from the low RN category

Overall, cumulative recapture rates at the end of Day 2 were not significantly different when using one to four traps and the low RNs (10, 25, 50) (Table 1). However, significant differences were observed with the release of ten mosquitoes at 09:30 ($F_{(3,12)}$=4.11, p<0.0320), when using one (67.5%) vs four traps (95.0%). With the RN of 25, significant differences were indicated from recaptures made at 13:30 ($F_{(3,12)}$=3.94, p<0.0360) and at the end of day 1 ($F_{(3,15)}$=4.94, p<0.0184). The highest recapture rates for the release of 25 mosquitoes occurred at 17:30 (71.5%). At the RN of 50, no significant differences were observed based on cumulative percent recapture rates (Table 1) at the end of days 1 and 2.

Comparison of recaptures from the medium RN category

The highest overall cumulative recapture rates for Day 2 was recorded when using four BGS in combination with RNs of 100 and 150 (Table 2). At the RN of 100, the use of three (94.8%) or four (97.0%) traps resulted in a significantly higher recapture of females compared to when one (85.5%) or two traps (83.2%) were used ($F_{(3,12)}$=13.80, p<0.0003). Significant differences were also observed at 17:30 on day 1 with the use of four traps resulting in higher numbers of marked females being recapturing than when one to three traps were used (Table 2). Perhaps more importantly, the use of two traps did not significantly increase the recapture rates over what was found when using one trap. The use of two traps was also no different from the use of three traps and the use of three was no different from the use of four traps ($F_{(3,12\ )}$=11.58, p<0.0007). However, by day 2, the use of three and four traps did result in significantly higher recapture rates as compared to when two traps were used.

Table 1. Cumulative mean percentage recapture of *Ae. aegypti*[1] low Release Number (RN) category by mosquito release density, number of BGS traps performing and monitoring interval.

Release Density	Number of traps	Cumulative % recapture by recovery time point during the first day (Day 1)[3]			Cumulative % recapture for days 1-2	Mean Day-Time (12 h) environmental data during the releases		
		09:30	13:30	17:30		Temperature (° C)	Relative Humidity (%)	Light Intensity (lx/ft²)
10	1	67.5b	70.0a	70.0 a **(28/40)**	72.5a	32.5	52.5	208.6
	2	87.5ab	87.5a	87.5a **(35/40)**	87.5a	35.4	51.4	259.8
	3	82.5ab	87.5a	87.5a **(35/40)**	87.5a	31.5	39.2	246.1
	4	95.0a	92.5a	92.5a **(37/40)**	92.5a	30.3	42.0	231.9
25	1	3.0a	24.0b	41.0b **(41/100)**	66.0a	25.5	64.3	145.6
	2	7.0a	40.0ab	66.0ab **(66/100)**	80.0a	25.1	63.9	179.9
	3	7.2a	48.0ab	69ab **(66/96)**	88.5a	23.6	64.8	131.1
	4	8.1a	55.0a	71.5a **(71/99)**	81.7a	26.7	63.5	175.9
50	1	22.0bc	90.4a	92.0a **(183/199)**	92.5a	23.2	77.6	175.4
	2	41.0ab	72.8bc	79.0a **(156/200)**	80.8a	28.8	79.7	263.1
	3	60.5a	84.4ab	86.0a **(171/199)**	85.9a	28.0	74.4	235.1
	4	8.0c	61.8c	75.0a **(149/199)**	81.9a	25.2	68.4	157.6

[1]Three to five-day-old starved females.

[2] Different lowercase letters in the same column within the same release number indicate significant differences between mean recapture percentages (ANOVA at 95% confidence limit).

[3]Values in parentheses are total recaptures/total release-knockdown.

Table 2. Cumulative mean percentage recapture of *Ae. aegypti*[1] medium Release Number (RN) category by mosquito release density, number of BGS traps performing and monitoring interval.

Medium Release Numbers	Number of traps	Cumulative % recapture by recovery time point during the first day (day 1)			Cumulative % recapture for days 1-2	Mean Day-Time (12 h) environmental data during the conduct of the releases		
		09:30	13:30	17:30		Temperature (° C)	Relative Humidity (%)	Light Intensity (lx/ft^2)
100	1	43.3a	68.8a	77.0c **(308/400)**	85.5b	27.2	58.4	285.8
	2	66.3a	81.9a	83.0bc **(331/400)**	83.2b	28.5	80.3	249.4
	3	59.3a	88.5a	93.5ab **(374/400)**	94.8a	27.4	73.8	205.5
	4	51.5a	89.3a	96.0a **(388/400)**	97.0a	26.2	67.9	143.9
150	1	27.6b	60.2b	75.8c **(454/600)**	89.1b	27.2	79.8	186.2
	2	74.1a	85.6a	86.8bc **(517/598)**	88.8b	28.3	81.8	188.2
	3	80.3a	90.5a	91.8ab **(549/599)**	91.8b	28.8	73.9	250.8
	4	64.8a	97.2a	97.8a **(585/599)**	97.8a	26.2	71.4	129.4

[1]Three to five-day-old starved females.
[2] Different lowercase letters in the same column within the same release number indicate significant differences between mean recapture percentages (ANOVA at 95% confidence limit).
[3]Values in parentheses are total recaptures/total release-knockdown.

With the RN of 150, the use of four traps (97.8%) showed the highest cumulative mean percent recapture, significantly higher than when using one (89.1%), two (88.8%), or three traps (91.8%) ($F_{(3,12)}$=16.49, p<0.0001). Consistently lower numbers of females from the 150 RN were caught on day 1 from the use of only one trap compared to the use of two to four traps at 09:30 ($F_{(3,12)}$=9.15, p<0.0020) and 13:30 ($F_{(3,12)}$=19.09, p<0.0001) (Table 2).

Comparison of recaptures from the high RN category

With the RN of 200, consistently lower recapture rates were recorded on Day 1 with the use of one trap compared to the use of two-four traps at 13:30 ($F_{(3,12)}$=7.73, p<0.0039), and day 1 ($F_{(3,12)}$=9.97, p<0.0014) sampling intervals (Table 3). This was true through Day 2 ($F_{(3,12)}$ =9.41, p<0.0018). The greatest overall recapture from the high RN category occurred from the use of four traps though rates were not significantly different from those recorded when using two or three traps (Table 3). Analysis of overall percent cumulative recaptures with the RN of 250 did not indicate significant differences with the use of one to four traps. No correlations were found between the environmental variables measured and recapture rates for any of the RN and BGS trap density combinations.

Recapture from BGS traps based on monitoring interval

The highest recapture collections were made from the 09:30 sampling period with an overall mean recapture of marked *Ae. aegypti* of 43% (Table 5). The 13:30 sampling point resulted in the next highest overall mean recapture (32%) across all RNs and another 12% recaptured from the last collection period at 17:30.

DISCUSSION

Previous studies established that the BG-Sentinel™ mosquito trap (BGS) is, compared to other traps or active collection methods, an effective tool for capturing adult *Ae. aegypti* in the outdoor environment (Krockel et al. 2006, Maciel-de-Freitas et al. 2006, Williams et al. 2006).

Table 3. Cumulative mean percentage recapture of *Ae. aegypti*[1] high Release Number (RN) category by mosquito release density, number of BGS traps performing, and monitoring interval.

High Release Numbers	Number of traps	Cumulative % recapture by recovery time point during the first day (Day 1)			Cumulative % recapture for Days 1-2	Mean Day-Time (12 h) climatic data during the conduct of the releases		
		09:30	13:30	17:30		Temperature (° C)	Relative Humidity (%)	Light Intensity (lx/ft^2)
200	1	26.3b	64.1b	77.0b (627/800)	82.0b	26.7	81.2	138.3
	2	72.1a	87.4a	89.5a (713/796)	90.3ab	28.8	77.6	222.6
	3	73.8a	87.7a	88.8a (707/799)	88.5ab	29.3	73.7	265.2
	4	43.6b	87.0a	94.8a (756/800)	96.4a	26.8	70.5	132.3
250	1	39.4bc	83.2ab	89.0a (886/1000)	91.2a	28.8	70.7	131.3
	2	76.1a	91.5a	92.5a (922/997)	93.6a	28.9	77.8	242.9
	3	54.2ab	89.8a	94.8a (945/998)	94.7a	26.6	77.6	208.2
	4	23.8c	71.2b	84.5b (844/1000)	91.6a	26.8	69.2	120.1

[1]Three to five-day-old starved females.
[2] Different lowercase letters in the same column within the same release number indicate significant differences between mean recapture percentages (ANOVA at 95% confidence limit).
[3]Values in parentheses are total recaptures/total release-knockdown.

Based on this information, the BGS is being evaluated as a component in a spatial repellent push-pull strategy to reduce the abundance of host-seeking *Ae. aegypti* inside homes and in the peridomestic environment, thereby reducing the probability of human-vector contact and dengue virus transmission. The current study was designed to quantify recapture success under controlled screen house conditions and based on varying *Ae. aegypti* release numbers and BGS trap densities in order to guide data interpretation from push-pull experimental trials. The efficacy of the BGS trap was quantified in previous studies (see references in the Introduction); the objective of this study was rather to determine *changes* in BGS recapture rates of *Ae. aegypti* under varying mosquito population densities to identify optimum trap numbers to use in a natural setting. Such information was not previously generated and represents new scientific knowledge important for mosquito surveillance and control implementations.

Overall, the results of this screen house study agree with previously published reports that the BGS trap is effective in recapturing three to five-day-old *Ae. aegypti* (Maciel-de-Freitas et al. 2006, Krockel et al. 2006, Williams et al. 2006). As expected, recapture rates varied based on BGS trap density and mosquito RN, with the highest cumulative percentage recapture over a 24 h period reaching 98%. The "Impact Period" during which the highest recapture success was observed was the first day of release with about 84% of the released mosquitoes recaptured by 17:30 or 12 h post-release. The peak collection time for all trials occurred during the 05:30 to 09:30 interval indicating that test specimens were more likely to be host-seeking (i.e., actively searching for a blood source) in the hours shortly after release, which is consistent with the peak time for landing collections recorded (Thavara et al. 2001) and experimental hut studies done in Thailand (Suwannachote et al. 2009, Chareonviriyaphap et al. 2010). Statistical analysis of grouped *Ae. aegypti* RN consistently showed that recapture rates using two, three, or four traps did not differ significantly at day 1 (13:30 and 17:30) and from cumulative percentage recapture for days 1-2. The use of only one trap, however, consistently showed significantly lower recapture rates. When examining the data by individual RN category,

Table 4. Cumulative BGS recapture rates for combined *Ae. aegypti*[1] release numbers (RNs)[2] by number of traps in performance.

BGS trap	Mean (S.E.) percentage of *Ae. aegypti* recaptured[3]			
	Cumulative % recapture by recovery time point during the first day (day 1)			Cumulative % recapture for days 1-2
	09:30	13:30	17:30	
1	32.7b (0.04)	65.8b (4.25)	74.5b (4.49)	82.7b (2.46)
2	59.8a (5.07)	78.1ab (3.45)	83.5ab (0.14)	86.3ab (1.32)
3	60.4a (4.99)	82.3a (2.99)	87.3a (0.14)	90.2a (1.45)
4	42.1ab (5.82)	79.1a (3.28)	87.4a (0.14)	91.3a (1.51)

[1]Three to five-day-old starved females.
[2]Release numbers categories: Low (10, 25, 50), medium (100, 150), high (200, 250).
[3] Different lowercase letters in the same column indicate significant differences between mean recapture percentages (ANOVA, 95% confidence limit).

Table 5. Cumulative BGS recapture rates by individual *Ae. aegypti*[1] release numbers (RNs)[2].

RN	Mean (S.E.) percentage of *Ae. aegypti* recaptured[3]			
	Cumulative % recapture by recovery time point during the first day (day 1)			Cumulative % recapture for days 1-2
	09:30	13:30	17:30	
Low (10, 25, 50)	40.8a (5.16)	67.8b (3.39)	76.4b (0.14)	83.1b (1.73)
Medium (100, 150)	58.4a (3.63)	82.7a (2.47)	87.8a (0.14)	90.9a (1.02)
High (200, 250)	51.2a (3.97)	82.7a (1.98)	88.8a (0.14)	91.0a (0.99)

[1]Three to five-day-old starved females.
[2]Release numbers categories: Low (10, 25, 50), medium (100, 150), high (200, 250).
[3] Different lowercase letters in the same column indicate significant differences between mean recapture percentages (ANOVA 95% confidence limit).

the use of a single BGS trap at the RN of 10, an *Ae. aegypti* adult density that better reflects what occurs naturally in the field, resulted in a cumulative recapture rate of 72.5% from day 1 and day 2, with 68% being recaptured by the end of the peak (05:30-09:30). The above recapture success was higher than any of the other RN categories using one BGS. Understandably, this experiment performed under controlled conditions and may not represent what is occurring in a natural field setting. The impact this result may have on dengue transmission is unknown but at 72.5% recapture it is likely to contribute to reduced human-vector contact should the trap perform equally well outside of the restricted screen house setting. The fact that two BGS traps collected similar densities as four traps in a space volume of 140 m^3, and that one BGS trap can recapture more than 50% of the *Ae. aegypti* females in this same given space and time may have operational significance for both *Ae. aegypti* surveillance and control activities.

Interestingly, the low *Ae. aegypti* RN categories (10, 25, 50 mosquitoes) showed reduced Day 1 and 2 cumulative percentage recapture compared to both medium (100 and 150) and high *Ae. aegypti* RN (200 and 250) categories, while the percentage recapture among medium and high RN did not differ significantly across all BGS trap densities evaluated. Theoretically, higher recapture was expected at lower RN categories due to mosquito to trap ratio; our results do not support this. This could be a factor of "observation loss" such that mosquitoes may have escaped the screen house when trap monitoring was being conducted and/or that knocked-down mosquitoes in the non-captured test population

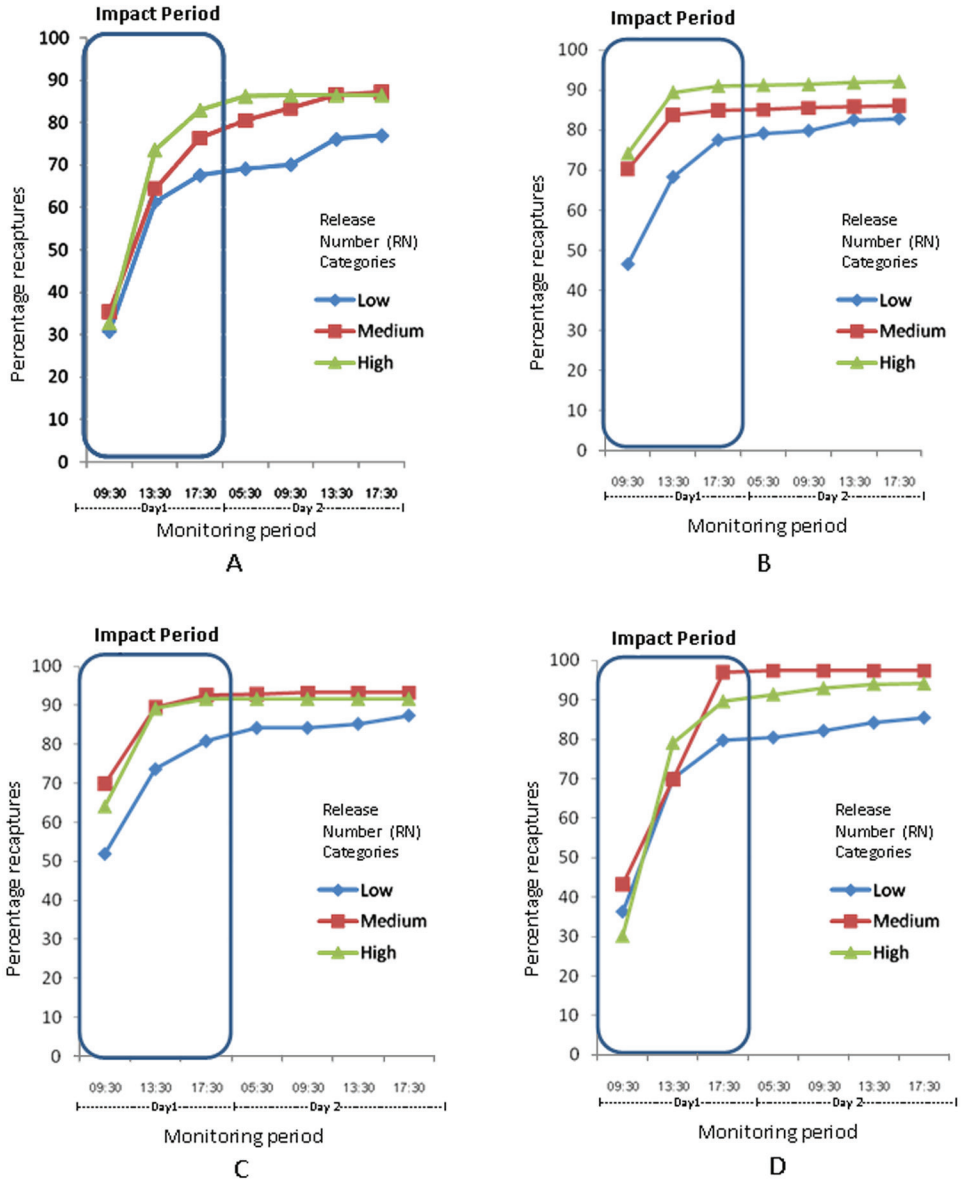


Figure 2. Cumulative percentage recpature of marked *Ae. aegypti* females using: A) one, B) two, C) three, and D) four BGS traps against low, medium, and high mosquito release densities . The Impact Period designates the monitoring interval when the greatest recapture occurred.

were not completely recovered. Additionally, because the mosquito test population in the lower RN categories was small relative to the medium and high RN categories, this could have reduced the overall percent total recapture. The opposite scenario may be expected using one BGS trap vs four traps, i.e., the lower the density of BGS traps, the lower the recapture. However, recapture percentages using the 250 *Ae. aegypti* RN, for example, did not differ significantly among the varying BGS trap densities; in other words, one trap recaptured similar numbers as compared to four traps. This might be the result of one BGS being the only "host" available in the immediate environment. Another possible explanation could be that the collection of females in the one trap increased the attractiveness of that trap due to an increased level of aggregation pheromone/s when female *Ae. aegypti* are in groups, thereby causing attraction of more females. Therefore, individual female mosquito behaviors of flight and response to host cues may not be independent under the study design and setting presented herein. Pheromone-mediated swarming has been reported in *Ae. aegypti* for both males and females (Cabrera and Jaffe 2007). This phenomenon could be further explored in future BGS studies.

We recognize that the screen house does not match a real-life situation. However, it should be noted that this study represents the first component in optimizing the BGS for a push-pull strategy. This requires quantifying BGS recapture rates against varying mosquito numbers prior to performing outdoor trials in which total population densities are not known. Such controlled experiments provide the ability to model efficacy rates based on known mosquito denominators in the screen house releases. The

screen house experiments provide critical information before moving to a more realistic setting, as to how many traps to use per house for maximum recapture given certain expected mosquito population densities. Results from this experiment will also be used to interpret potential changes in BGS trap recapture rates when used in conjunction with experimental huts during outdoor push-pull trials.

Some inherent limiting factors in the current study design may have contributed to artificially high recapture success as compared to a natural setting. For example, in Queensland, Australia, BGS traps collected a mean of 1.92 $\pm$ 0.39 female *Ae. aegypti* per continuous 24-h period (Williams et al. 2006). In Brazil, a study reported a mean *Ae. aegypti* collection of 1.5 female/trap/3 h (Krockel et al. 2006). The high recapture values from our experiments are likely a result of the confined space volume of the screen house cubicle under which evaluations were performed. By restricting full free movement and choice, female mosquitoes may in effect have been "forced" into contact with the BGS trap more frequently than in a natural setting containing alternative and competing stimuli and cues. It should be noted, however, that the overall "available" natural population size in the studies from Brazil and Australia were unknown. Furthermore, the screen house did not present competing (confounding) host sources or resting places for the mosquito, which again may have led to artificially elevated levels of mosquito contact with the trap. Future screen house tests could be modified to include items typically found in the peridomestic environment that could compete with or alter trap attractiveness. For example, a recent study by Ball and Ritchie (2010a) indicate that presence of low-reflectance (black) containers significantly reduces recapture rates by BGS traps of male and female *Ae. aegypti*. Another potential bias of the study is the exclusive use of non-blood fed, three to five-day-old, assumed mated and nulliparous females. Ball and Ritchie (2010b) found no difference in recapture rate for *Ae. aegypti* females with respect to age (10-12 vs 24-26 and 32-34 d) but reported that physiologically, nulliparous females were recaptured at lower rates compared to parous or gravid ones. However, we purposefully selected the physiological conditions for the release population to match those of the *Ae. aegypti* test populations used in evaluating the "Push" component in experimental huts located in the same study locality; therefore allowing use of the screen house data to help interpret findings during outdoor field trials. In addition, because the screen house studies were conducted at the same locality where the push-pull trials are being performed, the climatic conditions during this study were expected to be similar to when the BGS are used in the outdoors.

Data from the current study has revealed several potential field-applicable pieces of information. The use of the individual screen house cubicles with an approximated space (140 m^3) equal to the area of experimental huts used for related field trials and the immediate peridomestic area typically surrounding local households (i.e., backyard, gardens etc.) may help bridge the gap between semi-field and field studies to ascertain expected recaptures from BGS traps using varying *Ae. aegypti* release densities. The current study also revealed a peak BGS recapture interval between 05:30-13:30, which is similar to the time period when *Ae. aegypti* most commonly enters experimental huts under field conditions (Grieco et al. 2007, Suwannachote et al. 2009). Although releases were made at 05:30 alone, this time trend still reflects the normal host-seeking activity pattern of *Ae. aegypti* females and has potential operational significance to when BGS traps would be most effective in removing flight-active host(blood)-seeking mosquitoes in a push-pull strategy. Considering that the BGS trap requires an external power source (i.e., battery pack or direct electricity), this information is specifically important for informing when the trap may be most effective, how long it should run, and the possibility of cost-effective operations if the traps can be turned off to conserve battery/electricity power supplies. Being able to maximize trapping efficiency and minimize cost is just one important consideration to provide a cost-effective and sustainable control method. If the push-pull strategy proves successful, it could be integrated into a consumer-implemented control method to augment organized vector control measures. Knowledge as to when homeowners should operate the peridomestic trap would be important for ensuring maximum benefit. Further studies are necessary to substantiate such claims, and datasets from studies similar to the current report could prove useful in modeling these outcomes.

In conclusion, the results from this study have provided useful information on the expected range of BGS recapture rates and timing efficiency under semi-field conditions against varying *Ae. aegypti* release and trap densities. Such findings can help guide the implementation and interpretation of the impact of the BGS trap in experimental push-pull field trials using outdoor mosquito releases and can provide insight for the operational refinement of the BGS trap as a monitoring and surveillance device, in addition to being a potential control tool.

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Discriminating Lethal Concentrations and Efficacy of Six Pyrethroids for Control of *Aedes aegypti* in Thailand

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DISCRIMINATING LETHAL CONCENTRATIONS AND EFFICACY OF SIX PYRETHROIDS FOR CONTROL OF *AEDES AEGYPTI* IN THAILAND

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ABSTRACT. Establishing baseline insecticide discriminating doses is crucial in accurately determining susceptibility status and changing temporal patterns of physiological response in mosquito populations. Pyrethroids are the predominant chemicals used for controlling adult *Aedes aegypti* and *Ae. albopictus*, both vectors of dengue viruses, in Thailand. Presently, only 2 pyrethroids, permethrin and λ-cyhalothrin, have published diagnostic dose rates for monitoring *Ae. aegypti*. This study established the diagnostic lethal concentrations for 6 different pyrethroids available in Thailand for dengue vector control. United States Department of Agriculture insecticide-susceptible strain of *Ae. aegypti* was used to establish the baseline concentrations for subsequent susceptibility testing of field populations. Our findings showed lower discriminating concentrations for λ-cyhalothrin and permethrin than those recommended by the World Health Organization (WHO), at 2.5- and 1.7-fold lower dosing, respectively. The susceptibility status of 3 different geographical populations of field-collected *Ae. aegypti* were tested using the standard WHO procedures. All 3 field strains demonstrated varying levels of physiological resistance to each compound. We conclude that establishing the baseline diagnostic concentration of an insecticide is of paramount importance in accurately determining the susceptibility status in field-collected mosquitoes. If possible, discriminating doses should be established for all insecticides and test assays run concurrently with a known susceptible strain for more accurate monitoring of resistance in mosquito populations in Thailand.

KEY WORDS *Aedes aegypti*, pyrethroids, diagnostic concentration, Thailand

INTRODUCTION

Many tropical and subtropical countries around the world present risk for dengue fever and dengue hemorrhagic fever. Between 2.5 and 3 billion people (two-fifths of the world's population) are at risk of contracting dengue, many of whom live in the Southeast Asian region (WHO 2002). With an estimated 50–100 million people having symptomatic dengue infection each year, the majority of cases occur primarily in crowded, impoverished urban regions of the world (Gubler 1998, Gibbons and Vaughn 2002). In Southeast Asia, dengue hemorrhagic fever, a severe manifestation of dengue, has shown a disturbing increase from an annual rate of <10,000 in the 1960s to >200,000 in the 1990s (Gibbons and Vaughn 2002). In Thailand, there were 115,845 reported dengue cases and 141 deaths in 2010, which represented a small fraction of the actual number of mild and asymptomatic infections that same period (MOPH 2010). The 4 different virus serotypes (DEN-1, -2, -3, -4) are transmitted by mosquitoes, primarily *Aedes aegypti* (L.), a highly efficient vector mosquito because of its close association with humans and exploitation of domestic and peri-domestic environments, most notably in dense urban areas. As yet, no commercial multivalent dengue vaccine is available; therefore, prevention of this disease remains almost entirely dependent on using methods of control that attack both adult and immature stages of the mosquito. Vector control remains the most effective means of reducing risk of virus transmission (Reiter and Gubler 1997, WHO 1999). Unfortunately, *Ae. aegypti* has confounded most organized control efforts to bring vector population densities below sustainable thresholds to eliminate transmission.

In Thailand, the standard vector control techniques are based on use of chemicals and source reduction of larval habitats. Many chemical compounds, including organophosphates, carbamates, pyrethroids, and so-called bio-rational pesticides (bacterial toxins and insect growth regulators) have been used in national public health vector control programs (Reiter and Gubler 1997, WHO 1999). In Thailand, pyrethroids, e.g., deltamethrin, cyfluthrin, and permethrin, are common AIs in many commercial products designed for controlling household adult *Ae. aegypti*. However, control efforts have been hampered by the development of resistance to many of these insecticides by *Ae. aegypti* throughout Thailand (Chareonviriyaphap et al. 1999; Somboon et al. 2003; Sathantriphop et al. 2006; Thanispong et al. 2008, 2010). The selection pressure for developing resistance to pyrethroids has largely been attributed to the frequent and pervasive use of the same chemical class of

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compounds and is believed to have a direct bearing on the effective management and prevention of vector-borne diseases in general (Hemingway and Ranson 2000). Although there are a number of reports that describe the status of pyrethroid resistance in *Ae. aegypti* populations in Thailand (Chadwick et al. 1977, Chareonviriyaphap et al. 1999, Paeporn et al. 2004, Yaicharoen et al. 2005, Sathantriphop et al. 2006, Jirakanjanakit et al. 2007, Thanispong et al. 2008), all reports were based on use of "diagnostic" doses established by the World Health Organization (WHO; 1998, 2006).

Pyrethroids are the predominant chemicals used for controlling adult *Ae. aegypti* and *Ae. albopictus* (Skuse), both vectors of dengue viruses in Thailand. Establishing a baseline insecticide discriminating dose is crucial for determining susceptibility status and changing temporal patterns of physiological response over time in mosquito populations. Most insecticides used for the control of anopheline malaria vectors have well-established and recommended discriminating ("diagnostic" doses) for routine monitoring of vector populations. However, currently very few insecticides have analogous discriminating doses by which to test the susceptibility of *Ae. aegypti*. Presently, only 2 pyrethroids (permethrin and λ-cyhalothrin) have published diagnostic dose rates for monitoring *Ae. aegypti*. The objective of this study was to establish the baseline diagnostic concentrations for 6 pyrethroids available in Thailand for dengue vector control. For purposes of accurate comparison, the baseline lethal concentrations derived from a fully insecticide-susceptible laboratory strain of *Ae. aegypti* were subsequently used to assess the susceptibility status of 3 field populations in Thailand.

MATERIALS AND METHODS

Mosquitoes

Four different cohorts of *Ae. aegypti* were used in this study. A susceptible strain of *Ae. aegypti* (US Department of Agriculture laboratory [USDA] strain) was used to establish the baseline 99% lethal concentration (LC_{99}) of 6 pyrethroids. The diagnostic percent concentration for each AI was then used to determine the susceptibility profile of 3 local Thai populations (a "deme," individuals from a given locality that potentially form a single interbreeding community) of *Ae. aegypti*, concurrently with the USDA strain as follows: 1) USDA strain was provided by the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. This inbred strain has been maintained continuously in colony for >40 years and at the Department of Entomology, Kasetsart University (Bangkok) beginning in 2004. 2) Kanchanaburi population was obtained in May 2010 as larvae from outdoor container habitats at Pu Teuy Village, Sai Yok District (14°20′12.1″N, 98°59′19″E), Kanchanaburi Province, an area approximately 150 km northwest of Bangkok. 3) Khon Kaen population was obtained in November 2010 as larvae from outdoor container habitats in Non Ton Village, Muang District (16°24′52″N, 102°51′05.5″E), Khon Kaen Province, northeastern Thailand. 4) Nong Khai population was obtained in November 2010 as larvae from outdoor container habitats in Pa Ngew Village, Tha Bo District (17°15′51.1″N, 102°34′30.5″E), Nong Khai Province, northeastern Thailand.

Mosquito rearing

All mosquito larvae and pupae collected from each site were placed in an environmentally controlled insectary located at Kasetsart University, Department of Entomology, Bangkok, and reared to the adult stage. Adult mosquitoes were identified to species and *Ae. aegypti* males and females were transferred to screened holding cages to allow free mating. Females were provided 10% sugar solution soaked on cotton as sustenance and permitted to feed on live guinea pig blood 3–4 days after emergence. Two days postbloodfeeding, oviposition dishes were placed in the cages with gravid females. Eggs were properly conditioned and larval pans set for the next generation and reared using standard techniques and diet established at Kasetsart University (Kongmee et al. 2004). All 4 cohorts were maintained separately and carefully segregated to avoid cross-genetic contamination and under identical laboratory controlled conditions (25 ± 3°C, 75 ± 5% RH, natural light:dark phase).

Insecticides

Six pyrethroid insecticides were used in this study as follows (including Chemical Abstracts name): 1) α-cypermethrin (Sherwood Chemicals Public Company Limited, Bangkok, Thailand, purity 97.05%); 2) deltamethrin (Sherwood Chemicals Public Company Limited, purity 98.46%); 3) permethrin (Sherwood Chemicals Public Company Limited, purity 97.6%); 4) bifenthrin (Sherwood Chemicals Public Company Limited, purity 95.12%); 5) cypermethrin (T.J.C. Chemical Company Limited, Bangkok, Thailand, purity 92%); and 6) λ-cyhalothrin (Syngenta Company, Bangkok, Thailand, purity 91.8%).

Insecticide-treated paper

Separate rectangular test papers (Whatman® No. 1, GE Healthcare UK Limited, Buckinghamshire, United Kingdom; 12 × 15 cm^2) were impregnated with each chemical AI at a specified

Table 1. Mean percentage mortality of 4 strains of *Aedes aegypti* using an established diagnostic concentration of each insecticide.

Insecticide	Diagnostic dose (%)	USDA[1] No. tested	USDA[1] Dead (%)	Kanchanaburi No. tested	Kanchanaburi Dead (% ± SE)	Khon Kaen No. tested	Khon Kaen Dead (% ± SE)	Nong Khai No. tested	Nong Khai Dead (% ± SE)
α-Cypermethrin	0.086	100	100 (100)	92	90 (97.83 ± 1.11)	104	92 (88.46 ± 1.7)	98	96 (97.96 ± 0.83)
Bifenthrin	0.094	97	97 (100)	92	85 (92.39 ± 1.92)	93	09 (9.68 ± 0.33)	99	14 (14.14 ± 1.81)
Cypermethrin	0.221	99	99 (100)	99	97 (97.98 ± 0.82)	96	96 (100)	98	61 (62.24 ± 2.05)
Deltamethrin	0.005	101	101 (100)	97	92 (94.85 ± 0.84)	99	0 (0.0)	102	04 (3.92 ± 0.62)
λ-Cyhalothrin	0.012	103	103 (100)	94	92 (97.87 ± 0.52)	85	11 (12.94 ± 3.64)	99	11 (11.11 ± 0.79)
Permethrin	0.147	97	97 (100)	100	88 (88.00 ± 1.46)	98	98 (100)	98	06 (6.12 ± 0.51)

[1] USDA, US Department of Agriculture.

serial dilution for use in establishing baseline diagnostic concentration for each insecticide and subsequently a single diagnostic concentration ($LC_{99} \times 2$) as determined from the USDA susceptible strain. All papers were prepared in the laboratory at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand (Table 1). Technical-grade AI was diluted with silicon oil (nonvolatile carrier) for uniform distribution of insecticide on papers. All papers were treated at the rate of 2 ml of the insecticide solution per 180 cm^2.

Establishing baseline diagnostic lethal concentrations

The USDA susceptible strain of *Ae. aegypti* was used in a series of dose–response tests to establish the lethal concentrations required to kill 50% and 99% of the test population using each of 6 AIs (WHO 1981). For all chemicals, excluding α-cypermethrin, 5 different percent concentrations produced in a range of 2-fold serial dilutions were initially tested to determine the range of the 3 final concentrations used for establishing the baseline 50% lethal concentration (LC_{50}) and LC_{99} values. For α-cypermethrin, only 3 initial concentrations in 10-fold serial dilutions were used to arrive at the final three used in establishing the baseline. The subsequent 3 final concentrations (produced as 2-, 3-, or 4-fold dilutions of AI) used in baseline assays and dose–response analysis are presented in Table 2. Twenty-five nonblooded female mosquitoes, approximately 3–5 days old, were tested per exposure tube. Treated papers were used only once and discarded. Four replicate assays were conducted for each dilution to derive a mean response and run concurrently with matching controls (without AI). To avoid spurious reporting of resistance in the field where none may exist, WHO routinely sets the diagnostic concentration at twice the minimum concentration that will kill 100% of susceptible mosquitoes (WHO 2006). The double concentration of the LC_{99} for each AI was designated the ''diagnostic dose'' or discriminating concentration and subsequently used for susceptibility tests using the *Ae. aegypti* field strains.

Susceptibility assays

The susceptibility level of each population to 6 pyrethroids was assessed by exposing 25 nonbloodfed 3- to 5-day-old female mosquitoes to a single established diagnostic dose established from the USDA standard strains. Mosquitoes were not deprived of nutritional sustenance (10% sugar solution) before testing. Standard testing procedures followed WHO recommendations (1998). After 60 min of exposure, test and control mosquitoes were transferred to separate holding

Table 2. Probit dose/mortality analysis of a laboratory susceptible strain of *Aedes aegypti* (US Department of Agriculture) exposed to 6 different pyrethroids using 3 different concentrations[1] establishing lethal concentrations (LC) for each AI.

Insecticide	No. tested	LC_{50} (%)	95% FL[2]	LC_{99} (%)	95% FL	Diagnostic concentration (%)[3]	$P >$ chi square
α-Cypermethrin	302	0.0009	0.0004–0.0013	0.043	0.0220–0.1449	0.0863	0.9419
Bifenthrin	282	0.0185	0.0171–0.0202	0.047	0.0396–0.0599	0.0938	0.3082
Cypermethrin	311	0.0052	0.0031–0.0072	0.111	0.0662–0.2760	0.2212	0.1333
Deltamethrin	297	0.0007	0.0006–0.0007	0.002	0.0020–0.0034	0.0049	0.8955
λ-Cyhalothrin	299	0.0012	0.0010–0.0014	0.006	0.0043–0.0087	0.0116	0.8633
Permethrin	275	0.0379	0.0354–0.0407	0.073	0.0632–0.0922	0.1466	0.9718

[1] Percent concentrations used in dose–response assays as follows: α-cypermethrin (0.00125, 0.005, 0.02); bifenthrin (0.0125, 0.025, 0.05); cypermethrin (0.0077, 0.023, 0.07); deltamethrin (0.00031, 0.00125, 0.005); λ-cyhalothrin (0.00037, 0.0015, 0.006); permethrin (0.03125, 0.0625, 0.125).

[2] FL = fiducial limits at 95% confidence level.

[3] Diagnostic concentration/discriminating dose calculation = $2 \times LC_{99}$.

containers and mortality was recorded after 24 h postexposure. Each trial design (population/chemical) was replicated 4 times using freshly treated papers no more than 3 times and discarded. Replicate trials were combined and a mean susceptibility level derived for each population tested as described by Chuaycharoensuk et al. (2011).

Data analysis

The LC_{50} and LC_{99} values were calculated from a derived dosage–mortality regression line (Finney 1971) using log-probit analysis (Proc Probit, SAS/STAT version 8; SAS Institute Inc., Cary, NC). Pearson chi-square analysis was used for goodness-of-fit tests. The estimate of LC_{50} and LC_{99} was determined from 4 test replicates per chemical concentration using the USDA susceptible strain of *Ae. aegypti*. Interpretation of resistance/susceptibility status followed standard WHO criteria (1998).

RESULTS

The baseline susceptibility levels of pyrethroids currently used in dengue vector control in Thailand were established from the standard susceptible strain (USDA). This establishment was based on the insecticide doses which gave the mortality ranging between 10% and 95% in tzhe USDA susceptible strain. Chi-square values demonstrated that the response of *Ae. aegypti* to each AI fit the linear model ($P = 0.3191$). Individual chemical goodness-of-fit tests ranged from $P = 0.1333$ to 0.9718 (Table 2). The LC_{50} and LC_{99} values of 6 pyrethroids against *Ae. aegypti* (USDA) were determined using the log-probit analysis. Permethrin produced the highest LC_{50} (0.0379%) value, whereas deltamethrin gave the lowest LC_{50} value (0.0007%). At LC_{99} values, cypermethrin had the greatest concentration (0.111%), whereas deltamethrin resulted in the lowest (0.002%). A single diagnostic concentration (double concentration of baseline LC_{99}) of α-cypermethrin (0.086%), bifenthrin (0.094%), cypermethrin (0.221%), deltamethrin (0.005%), λ-cyhalothrin (0.012%), and permethrin (0.147%) was subsequently used to determine the susceptibility of the 3 field populations of *Ae. aegypti* (Kanchanaburi, Khon Kaen, and Nong Khai) (Table 1).

Results of susceptibility tests of 3 field populations and the USDA strain with the established diagnostic dose of 6 pyrethroids showed the ability of mosquitoes to survive the diagnostic dose after 1-h exposure to chemical and 24-h holding period (Table 1). The interpretation and criteria of insecticide susceptibility results were as follows: mosquitoes regarded as fully "susceptible" to an insecticide if the mean percent mortality was between 98% and 100%, as showing "incipient" resistance if between 80% and 97%, and "resistant" in operational terms of effectiveness if <80% kill (WHO 1998, 2006).

In all trials, concurrent control (no insecticide, carrier compound only) mortality did not exceed 5%; therefore, final mean mortality did not require a correction factor. Complete mortality (100%) was observed in the USDA standard strain when exposed concurrently to the established discriminating doses of all 6 chemicals. The 3 field populations showed various levels of tolerance/resistance to the chemicals tested. Low to moderate incipient resistance (tolerance) to all 6 pyrethroids was seen in the Kanchanaburi population, with mortality ranging between 88% (permethrin) and 97.98% (cypermethrin). The Khon Kaen population was found completely susceptible (100%) to cypermethrin and permethrin; however, incipient resistance was detected against α-cypermethrin (88.46% morality) and very strong resistance was seen with deltamethrin (0.0%), bifenthrin (9.7%), and λ-cyhalothrin (12.9%). The Nong Khai strain demonstrated strong resistance to deltamethrin (3.92%), λ-cyhalothrin (11.1%), permethrin (6.12%), bifenthrin (14.14%), and cypermethrin (62.24%). The only chemical showing a

high level of effectiveness with the Nong Khai population was α-cypermethrin (97.9% kill).

DISCUSSION

By applying new, revised diagnostic concentrations of 6 pyrethroids, 3 field-collected *Ae. aegypti* populations demonstrated varying physiological resistance-based origin (geography) and chemical tested. The population from Kanchanaburi proved reasonably susceptible to all 6 insecticides, with the lowest mean mortality against permethrin. These results are compatible with previous work from this same area of Kanchanaburi with only slightly lower levels of resistance to permethrin and deltamethrin reported previously (Thanispong et al. 2008, Chuaycharoensuk et al. 2011). However, the other 2 Thai populations, Khon Kaen and Nong Khai, displayed high levels of physiological resistance to bifenthrin, deltamethrin, and λ-cyhalothrin. Interestingly, the Nong Khai population was also found highly resistant to permethrin (6%) and significantly so (62%) with cypermethrin, while Khon Kaen was completely susceptible to both compounds. In general, these results are consistent with recent resistance patterns seen with *Ae. aegypti* elsewhere in Thailand (Chareonviriyaphap et al. 1999, Prapanthadara et al. 2002, Paeporn et al. 2005, Ponlawat et al. 2005, Jirakanjanakit et al. 2007). Comparing all 3 local populations, α-cypermethrin proved to be the one chemical that provided the best overall mortality (88.46–97.96%).

Aedes aegypti is both a common nuisance mosquito and a constant public health threat in Thailand, serving as the primary vector of dengue/dengue hemorrhagic fever (MOPH 2010). One of the very few methods to effectively curb dengue transmission is to reduce a human–vector contact using insecticides (Reiter and Gubler 1997, WHO 1999, Jacobs 2000). However, a major disadvantage with the routine long-term use of insecticides is the prospect that a vector population may develop resistance to the AI, rendering it operationally useless (WHO 1992, Roberts and Andre 1994, Brogdon and McAllister 1998, Hemingway and Ranson 2000, Thanispong et al. 2008).

In Thailand, information on insecticide resistance in *Ae. aegypti*, the primary vector of dengue/dengue haemorrhagic fever, is relatively limited due to a shortage of studies and comprehensive sustainable monitoring programs within the national public health vector control program. *Aedes aegypti* is one of the most efficient, well-adapted, and widely distributed mosquitoes in the tropical and subtropical zones, and has proven extremely recalcitrant to control (Gratz and Halstead 2008). Among the commonly available control techniques, chemical control remains the most effective method to curb dengue transmission. Of the chemical categories (classes), pyrethroids are the most common and extensively used in both governmental and public sectors and still generally regarded as effective adulticides (Chareonviriyaphap et al. 1999, Kongmee et al. 2004, Jirakanjanakit et al. 2007, MOPH 2010). In Thailand, ultra-low volume application of deltamethrin has been used repeatedly to interrupt dengue transmission soon after the 1st dengue case has been reported. For general household use, a variety of low-concentration, combination pyrethroids are widely available for public to control household arthropod pests. Not unexpectedly, the continuous and repetitive contact with insecticides, especially pyrethroids, has resulted in various degrees of insecticide resistance in *Ae. aegypti* populations throughout Thailand. Admittedly, how resistance has impacted dengue control efforts in Thailand has not been adequately evaluated.

Insecticide resistance in mosquito populations is considered one of the major factors undermining the success and impact of vector control programs (Brogdon and McAllister 1998, Hemingway and Ranson 2000). For several decades, insecticide companies have continued to develop promising synthetic alternative compounds and formulations for public health use in private and governmental sectors to prevent dengue transmission (MOPH 2010). Among the compounds of greatest interest have been pyrethroids such as permethrin, cypermethrin, bifenthrin, deltamethrin, cyfluthrin, resmethrin, α-cypermethrin, and tetramethrin (Chareonviriyaphap et al. 1999, Somboon et al. 2003, Paeporn et al. 2005, Ponlawat et al. 2005, Thanispong et al. 2008). Pyrethroids have earned a more favorable acceptance for the control of mosquitoes primarily because of their inherent properties of relatively low toxicity to humans and being highly effective at low concentrations by quickly immobilizing (knockdown) and killing insects. However, it has been this overreliance on a single class of compounds that has contributed to widespread insecticide resistance in mosquito populations (Roberts and Andre 1994, Hemingway and Ranson 2000). In Thailand, insecticide resistance in *Ae. aegypti* was first reported against DDT (dichlorodiphenyl trichloroethane) in Bangkok and Nakhon Ratchasrima (northeast Thailand) (Neely 1964). Subsequently, resistance to phosphorothioate (organophosphate) compounds was found present throughout the country before being reported in *Ae. aegypti* to pyrethroids (Chareonviriyaphap et al. 1999, Jirakanjanakit et al. 2007, Thanispong et al. 2008, Chuaycharoensuk et al. 2011).

Over the past 60+ years, *Ae. aegypti* and other dengue vectors in different countries have developed resistance to commonly used insecticides (Brown and Pal 1971, WHO 1999). Both baseline data (before the start of control operations), followed by routine or periodic insecticide susceptibility

assays to operational chemicals used in a vector control program are of paramount importance for monitoring vector response over time. Although a number of studies on pyrethroid resistance in *Ae. aegypti* have been published, many have relied on using WHO published diagnostic concentrations and conditions (e.g., exposure times) typically used for monitoring *Anopheles* mosquitoes (WHO 1981, 1998). Surprisingly, there is far less information or data supporting the standard diagnostic criteria for susceptibility testing of *Ae. aegypti* (WHO 1992, 1999, 2006). For pyrethroids, only λ-cyhalothrin (0.03%) and permethrin (0.25%) have recommended diagnostic doses provided by WHO for determining the resistant status of *Ae. aegypti* (WHO 1992, 1998).

Recently, the diagnostic doses of 2 commonly used synthetic pyrethroids, permethrin (0.9%) and deltamethrin (0.06%), were established using a reference susceptible strain (Bora Bora, French Polynesia) of *Ae. aegypti* (Jirakanjanakit et al. 2007). However, both diagnostic doses were derived from a log-probit analysis that had a very high chi-square and low *P*-value (<0.005), indicating a relatively poor goodness-of-fit of the data. In general, susceptibility baselines and diagnostic doses of various compounds used for the control of *Ae. aegypti* are lacking and thus information derived on pyrethroid susceptibility may not be completely accurate or operationally meaningful. Furthermore, the majority of data on insecticide susceptibility is limited to only a few areas in Thailand (Chareonviriyaphap et al. 1999, Prapanthadara et al. 2002, Ponlawat et al. 2005, Jirakanjanakit et al. 2007, Thanispong et al. 2008, Chuaycharoensuk et al. 2011). Therefore, WHO (1998) has repeatedly recommended that baseline data on insecticide susceptibility should be gathered on a reference strain of *Ae. aegypti* before performing tests on field-collected populations.

This study did not investigate the possible metabolic and target site mechanisms involved in the resistance detected in the populations tested. Of those mechanisms most likely to be involved with conferring reduced susceptibility to pyrethroids, elevated or modified activities of esterases and/or monooxygenases involved in metabolic detoxification of insecticides (Paeporn et al. 2004) and the possible presence of the *kdr* (knockdown resistance) mutation (Brogdon and McAllister 1998).

The susceptibility of adult *Ae. aegypti* to the 6 pyrethroids were selected, as these compounds currently represent the predominant chemical class utilized for space spray applications (''fogging'') and treated materials (e.g., window curtains). Space spray (''fogging'') application of pyrethroids remains the method and insecticides of choice for adult *Aedes* control in Thailand (MOPH 2010). However, this was not always the case in Thailand, as decades ago DDT (organochlorine), dieldrin (cyclodiene), and malathion (phosphorothioate) had been extensively used to control vector mosquitoes (Bang et al. 1969, Gould et al. 1970, Lofgren et al. 1970, Chareonviriyaphap et al. 1999). At that time, DDT was also widely used to control *Aedes* mosquitoes in Thailand (Neely 1964, Ponlawat et al. 2005). The first reports of DDT resistance in *Ae. aegypti* in Thailand were published in the 1960s (Neely 1964, Bang et al. 1969). Thereafter, resistance to temephos (larvicide), malathion, and fenitrothion were reported as widespread in Thailand (Chareonviriyaphap et al. 1999), followed more recently by many reports of resistance to pyrethroids (Prapanthadara et al. 2002; Somboon et al. 2003; Paeporn et al. 2004, 2005; Ponlawat et al. 2005; Yaicharoen et al. 2005; Sathantriphop et al. 2006; Jirakanjanakit et al. 2007; Thanispong et al. 2008; Chuaycharoensuk et al. 2011). Although DDT was last used in Thailand in 2000, the current susceptibility status of *Ae. aegypti* to various pyrethroids may have been impacted by persistent cross-resistance mechanisms between the 2 chemicals (Chadwick et al. 1977) that still persist in Thailand (Prapanthadara et al. 2002).

The use of chemicals as contact residual insecticides on indoor walls of homes has not been routinely used to directly control adult *Aedes* mosquitoes, although there is strong enough evidence to show it would likely provide longer-lasting control in some situations (Giglioli 1948, Lien et al. 1992, Sulaiman et al. 1993, Reiter and Gubler 1997, Doke et al. 2000) and even eradication (Halcrow 1954, Brown and Pal 1971) when compared to the far more transient effects of space spray applications. The fact that many pyrethroids also perform as contact excitants and spatial repellents to *Ae. aegypti* (Kongmee et al. 2004, Thanispong et al. 2010), exclusive of direct toxic action, lends further support for use of residual insecticides inside homes to reduce human–vector contact and disease transmission. Whether realistic or cost-effective in control programs has yet to be fully explored.

A dengue control program can be seriously compromised and valuable resource squandered without accurate information on insecticide susceptibility status of local *Aedes* vector populations. As dengue remains a major disease problem throughout much of Thailand, the monitoring of insecticide resistance in *Ae. aegypti* and *Ae. albopictus* should be increased in periodicity, geographical coverage, and range of insecticides to assist vector control programs to anticipate and respond accordingly. Investigations of cross resistance to similar or closely related synthetic compounds and in-depth discovery of the actual mechanisms responsible for resistance are needed. Knowledge of vector/pest susceptibility to pesticides, changing trends of resistance, and their operational implications are basic requirements

to guide optimum chemical use. Insecticide resistance monitoring must be an integral part of a viable vector-borne disease and pest control program.

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Irritant and Repellent Responses of *Anopheles harrisoni* and *Anopheles minimus* upon Exposure to Bifenthrin or Deltamethrin Using an Excito-Repellency System and a Live Host

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IRRITANT AND REPELLENT RESPONSES OF *ANOPHELES HARRISONI* AND *ANOPHELES MINIMUS* UPON EXPOSURE TO BIFENTHRIN OR DELTAMETHRIN USING AN EXCITO-REPELLENCY SYSTEM AND A LIVE HOST

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ABSTRACT. Feeding responses of *Anopheles harrisoni* and *An. minimus* were evaluated following exposure to 2 pyrethroid insecticides, bifenthrin or deltamethrin, using an excito-repellency test system in the presence and absence of live host cues. The results demonstrated that contact irritancy was the primary action of bifenthrin or deltamethrin in both mosquito species. There was no noncontact repellency effect elicited by either insecticide. *Anopheles minimus* showed rapid escape response with high mortality rates following direct contact with deltamethrin in the absence of a host and delayed escape responses when a host was present. Similarly, exposure of *An. minimus* to bifenthrin also elicited a delayed escape response in the presence of a host but with lower mortality rates. In experiments using *An. harrisoni*, the presence or absence of a host had no significant effect on behavioral responses to either insecticide ($P > 0.05$). We conclude that deltamethrin elicited stronger irritant chemical effects than bifenthrin but that behavioral responses in vector populations are dampened in the presence of an available host. This information is useful for estimating probability of pathogen transmission when using irritant chemicals in proximity to a blood-meal source.

KEY WORDS *Anopheles* behavior, bifenthrin, deltamethrin, Thailand

INTRODUCTION

There are currently several vector control options available for disease prevention. However, insecticides continue to be one of the mainstays of operational programs. Among insecticides, pyrethroids are the most commonly used chemicals for indoor residual spraying to reduce the survival of vectors entering houses. Moreover, pyrethroids are the only insecticides currently recommended for insecticide-treated mosquito nets. Two of the pyrethroids are bifenthrin and deltamethrin. Bifenthrin is a non–alpha-cyano pyrethroid, and is used to treat mosquito nets (Hougard et al. 2002, Batra et al. 2005, Chouaibou et al. 2006). Deltamethrin is an alpha-cyano pyrethroid and is a recommended for indoor residual spraying (WHO 2006). Both pyrethroids affect the nervous system, causing paralysis in insects as a knock-down effect (Haug and Hoffman 1990, Nguyen et al. 1996, WHO 2001, Hougard et al. 2002, Doyle et al. 2009). Apart from toxic properties of insecticides, these same compounds can elicit changes in a mosquito's behavioral response. One of those behavioral changes is termed ''irritancy,'' which increases activity of mosquitoes. The disturbance of resting mosquitoes is the most obvious result of irritation, causing a mosquito to leave treated surfaces before acquiring a lethal dose; therefore repeated contact is required before mortality occurs (Kennedy 1947, Muirhead-Thomson 1960, Roberts et al. 2000). Another behavioral change is ''repellency,'' used to refer to the stimulation of a vector by a chemical that results in mosquito movement away from the treatment source; this can occur without direct contact with the treated surface (Dethier et al. 1960, Barton Browne 1977).

Behavioral responses can be objectively and quantitatively assessed by using an excito-repellency test system (Roberts et al. 1997). It was first developed in 1970 in an attempt to access the behavioral responses of mosquitoes to insecticides (WHO 1970). Through several studies, the test system has been further modified and improved to evaluate the behavioral responses of various mosquito species (Quinones and Suarez 1989, Ree and Loong 1989, Evans 1993). In 1997, the improved test system distinguished between 2 distinct types of behavioral responses (Chareonviriyaphap et al. 1997, Roberts et al. 1997): contact irritancy (defined as insects leaving an insecticide-treated surface after tarsal contact with the residual chemical) and noncontact repellency (an insecticide to act from an area effect, diverting insects away from treated surfaces without actual physical contact with the chemical). Later, a portable version was developed that allowed direct assessment of mosquito behavior at field sites (Chareonviriyaphap et al. 2002).

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Mosquito behavioral responses to insecticide have been previously documented by many investigators using experimental huts, semi-field studies, and field studies to describe the movement patterns, host-seeking, and resting behavior of female mosquitoes in the presence of hosts with or without exposure to insecticide (Cullen and De Zulueta 1964, Grieco et al. 2000, Chareonviriyaphap et al. 2005, Suwonkerd et al. 2006, Malaithong et al. 2010). Other studies include determination of the influence of environmental factors (temperature, humidity, precipitation, wind, etc.) on seasonal mosquito biting activity and periodicities (Rowley and Graham 1968, Clements 1999, Suwannachote et al. 2009, Paaijmans and Thomas 2011).

In this study, we introduced a live animal host to the excito-repellency test system to examine the behavioral responses of female *Anopheles harrisoni* Harbach and Manguin and *An. minimus* Theobald following exposure to pyrethroid compounds. Specifically, comparisons of escape rates and time were made under contact (irritancy) and noncontact (repellency) test conditions with and without host cues using bifenthrin or deltamethrin.

MATERIALS AND METHODS

Mosquito populations

Two anopheline species were used in this study: *An. harrisoni* and *An. minimus*. *Anopheles harrisoni* egg batches (F18–F20 generations), originally collected from Pu Teuy, Kanchanaburi Province, Thailand, were received from the insectary at Chiangmai University. *Anopheles minimus* was originally collected from Prae Province, northern Thailand, in 1993 and maintained in insectary-controlled conditions at Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand. Egg batches (F87–F90 generations) were subsequently received from CDC and both species were raised in the insectary at Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Female mosquitoes were permitted to imbibe human blood on the 3rd day after emergence for egg development. Larval stages were reared in enameled pans under identical physical and nutritional conditions throughout the study period. Four- to 7-day-old female mosquitoes were used for all tests. All adult test mosquitoes were denied blood feeding and provided with only 10% sugar solution for nutritional energy. The sugar solution was removed and mosquitoes starved for 24 h before the test.

Insecticide-impregnated papers

Test paper (Whatman® No. 1) 27.5 cm × 35.5 cm was impregnated with standard field dose: 0.025 g/m^2 of bifenthrin and 0.02 g/m^2 of deltamethrin. All treated papers were prepared according to World Health Organization specifications (2006); a filter paper impregnated at 1% contains 6.6 mg of technical insecticide, or 367 mg/m^2. Control paper was impregnated with solvent (acetone and mixed with silicon oil) only.

Excito-repellency test system

The test system has been previously described by Chareonviriyaphap et al. (2002) with recent design changes presented in Boonyuan et al. (2011) (Fig. 1). The system consists of 4 chambers for contact trials and 4 chambers for noncontact trials. For contact trials, the impregnated papers are lined on the inside of the screened inner chamber, allowing mosquitoes to rest directly on the treated inner surface, making tarsal contact. For noncontact trials, the impregnated papers are lined on the outside of screened inner chamber, which prevents mosquitoes resting directly on the surface of the impregnated paper and thereby without making tarsal contact. Each trial comprised 2 paired control (untreated) chambers (with and without host) and 2 paired test (treated) chambers (with and without host) as shown in Fig. 1C and 1D.

Twenty-five female mosquitoes were introduced into each test chamber (control and treated) using a mouth aspirator. Mosquitoes were allowed a 3-min period for adjustment to chamber conditions after which the escape funnels were opened to begin the observation period. The numbers of mosquitoes escaping from the chamber containing the control and treated papers and into the receiving cage were recorded at 1-min intervals for a total 30-min observation period. Immediately after the 30-min exposure time expired, the number of dead or knocked-down mosquitoes that remained inside the test chamber and the number of those that escaped were recorded separately. All mosquitoes that either escaped or remained inside the chamber were collected and held separately by category of response in small holding containers with cotton pads soaked in 10% sugar solution to monitor 24-h mortality rates. All assays were replicated 4 times per treatment condition and conducted between 0800 and 1600 h under laboratory conditions with temperatures ranging from 25°C to 28°C and relative humidity ranging from 45% to 65%.

Host cues were provided by the addition of live adult guinea pigs (*Cavia cobaya* Pallas). One guinea pig was positioned inside the chamber containing the impregnated papers and protected from mosquito biting by placing the host inside a cylindrical cage covered by nontreated netting material (Fig. 1B).

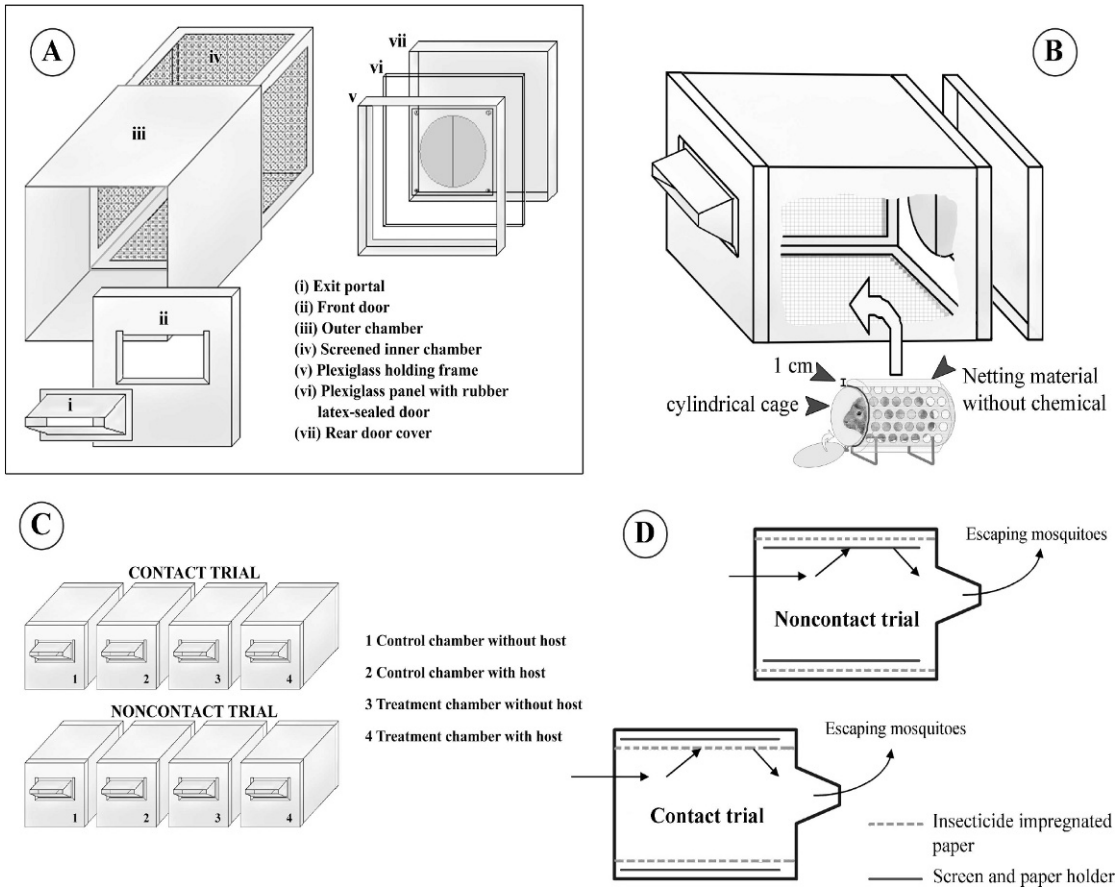


Fig. 1. (A) Excito-repellency test chamber, (B) the chamber with live host (guinea pig), (C) composition of excito-repellency test chamber for behavioral studies, and (D) side view of test chamber for contact and noncontact trials.

Data analysis

Kaplan–Meier survival analysis was used to analyze and interpret the behavioral response data (Kleinbaum 1995). For excito-repellency data, a life table survival analysis approach was used to estimate mosquito escape rates and compare differences between test populations and insecticides (Roberts et al. 1997). For this study, the probability of mosquito response to insecticides was estimated in relation to differences of mosquito escape in the present or absence of live host, in either contact or noncontact trials, including the time in minutes for 25%, 50%, and 75% of the test population to escape into the receiving chamber (ET_{25}, ET_{50}, and ET_{75}). The escape patterns (the time and density of escape from the treated and control chambers) of both mosquito species against bifenthrin or deltamethrin in 2 host conditions (presence or absence) between paired control and treatment in contact trials, paired control and treatment in noncontact trials, and paired contact and noncontact trials were compared.

A log-rank test (Mantel and Haenzel 1959) was used to compare the curves or patterns of escape behavior between different treatments. Statistical significance for all tests was set at $P < 0.05$.

RESULTS

Percentage of escape response

The percentage of escape responses (movement from the chamber with impregnated papers to the receiving chamber) of both *An. harrisoni* and *An. minimus* to bifenthrin or deltamethrin in the presence and absence of a live host were measured using contact and noncontact exposure test conditions.

Our results indicate that escape patterns of both mosquito species under each host condition (with and without host) in contact trials to bifenthrin or deltamethrin were considerably higher than those from matched controls and all test conditions in noncontact trials (Table 1). Overall, there was a lower percentage of escape response from *An. harrisoni* than from *An.*

Table 1. Summary of the percentage of escape response of female *Anopheles harrisoni* and *An. minimus* in contact and noncontact test trials with bifenthrin (0.025 g/m^2) or deltamethrin (0.02 g/m^2).

			An. harrisoni		*An. minimus*	
Trial	Insecticides	Conditions	Tested	% escaped	Tested	% escaped
Contact	Control	With host	99	3.03	99	2.02
	Bifenthrin	With host	99	50.51	98	41.84
	Control	Without host	99	5.05	100	6.00
	Bifenthrin	Without host	98	58.16	99	74.75
	Control	With host	98	4.08	99	2.02
	Deltamethrin	With host	98	54.08	99	94.95
	Control	Without host	100	4.00	100	8.00
	Deltamethrin	Without host	99	49.49	98	98.98
Noncontact	Control	With host	99	0	99	1.01
	Bifenthrin	With host	100	2.00	100	5.00
	Control	Without host	100	2.00	100	5.00
	Bifenthrin	Without host	100	4.00	99	9.09
	Control	With host	98	1.02	98	3.06
	Deltamethrin	With host	99	2.02	99	8.08
	Control	Without host	100	3.00	102	5.88
	Deltamethrin	Without host	99	4.04	101	11.88

minimus after contact with bifenthrin (51–58% and 42–75%, respectively) or deltamethrin (49–54% and 95–99%, respectively) in both the presence and absence of hosts. The highest percentage of escape response of *An. minimus* was observed when exposed to deltamethrin in the absence of host (99%), followed by in the presence of host (95%) with the same compound. In addition, *An. minimus* exhibited greatest escape response to bifenthrin in the absence of host (75%) as compared to when a host was present (42%) (Table 1).

Mortality rates of test mosquitoes

The 24-h mortality rates of *An. harrisoni* and *An. minimus* exposed to either bifenthrin or deltamethrin for each experimental treatment is shown in Table 2. Overall, mortality rates of escaped and nonescaped test populations were higher in *An. harrisoni* (escaped, 16–20%; nonescaped, 10–18%) than in *An. minimus* (escaped, 2–3%; nonescaped, 2–4%) after exposure to bifenthrin. On the contrary, the mortality rates of escaped and nonescaped mosquitoes after exposure to deltamethrin were higher in *An. minimus* (escaped, 34–38%; nonescaped, 80–100%) as compared to *An. harrisoni* (escaped, 8–18%; nonescaped, 40%). For *An. minimus* that remained in the chamber after a 30-min exposure to deltamethrin, 24-h mortality rates were higher than those exhibited in test populations that had successfully escaped and higher in the absence of a host than when a guinea pig was present in the test system (100% vs. 80%, respectively) (Table 2). Mortality rates of *An. harrisoni* following exposure to deltamethrin indicated similar results, with greater mortality occurring in nonescaped test populations than in those that moved to the receiving chamber, but the opposite was true when exposed to bifenthrin. Overall, lowest mortality was seen in noncontact trials and all matched controls in contact trials, with no more than 1 death from each cohort of escaped and nonescaped populations.

Escape time

Escape time (ET), measured at 1-min intervals, was designated based on the percentage of the mosquitoes escaping from chambers treated with bifenthrin or deltamethrin in contact trials with and without hosts, into the receiving chamber at 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of the total test population with the system during the 30-min exposure period (Table 3). A delay in escape response to both insecticides in contact trials was clearly indicated using *An. minimus* in the presence of a host, unlike in *An. harrisoni* where a host presence had no minimal delay effects. Bifenthrin produced a greater duration in time until escape using *An. minimus* under both host treatment conditions, especially for ET_{50} and ET_{75} with a host present, which could not be calculated because of insufficient numbers of mosquitoes exiting the treatment chamber during the testing period. For *An. harrisoni*, there was longer time duration to escape from treated chambers containing either bifenthrin or deltamethrin under both host conditions (presence or absence) when compared with escape times of *An. minimus*.

Escape patterns

Figures 2 and 3 show escape patterns of *An. harrisoni* and *An. minimus* in response to bifenthrin or deltamethrin in contact and noncontact

Table 2. Summary of the 24-h mortality rates of female *Anopheles harrisoni* and *An. minimus* in contact and noncontact trials with bifenthrin or deltamethrin.

			An. harrisoni		*An. minimus*	
Trial	Insecticides	Conditions	Escaped[1]	Nonescaped[2]	Escaped[1]	Nonescaped[2]
Contact	Control	With host	0 (0/3)	0 (0/96)	0 (0/2)	0 (0/99)
	Bifenthrin	With host	20.00 (10/50)	18.37 (9/49)	2.44 (1/41)	1.75 (1/57)
	Control	Without host	0 (0/5)	1.06 (1/94)	0 (0/6)	0 (0/94)
	Bifenthrin	Without host	15.79 (9/57)	9.76 (4/41)	2.70 (2/74)	4.00 (1/25)
	Control	With host	0 (0/4)	0 (0/94)	0 (0/2)	1.03 (1/97)
	Deltamethrin	With host	7.55 (4/53)	40.00 (18/45)	34.04 (32/94)	80.00 (4/5)
	Control	Without host	0 (0/4)	1.04 (1/96)	0 (0/8)	0 (0/92)
	Deltamethrin	Without host	18.37 (9/49)	40.00 (20/50)	38.14 (37/97)	100.00 (1/1)
Noncontact	Control	With host	0 (0/0)	0 (0/99)	0 (0/1)	0 (0/98)
	Bifenthrin	With host	0 (0/2)	0 (0/98)	0 (0/5)	0 (0/95)
	Control	Without host	0 (0/2)	0 (0/98)	0 (0/5)	1.05 (1/95)
	Bifenthrin	Without host	0 (0/4)	0 (0/96)	0 (0/9)	1.11 (1/90)
	Control	With host	0 (0/1)	1.37 (1/97)	0 (0/3)	0 (0/95)
	Deltamethrin	With host	0 (0/2)	0 (0/97)	12.50 (1/8)	0 (0/91)
	Control	Without host	0 (0/3)	0 (0/97)	0 (0/6)	0 (0/96)
	Deltamethrin	Without host	0 (0/4)	0 (0/95)	0 (0/12)	0 (0/89)

[1] Mortality rate (%) of escaped mosquitoes (number of dead mosquitoes/number of escaped mosquitoes).
[2] Mortality rate (%) of nonescaped mosquitoes (number of dead mosquitoes/number of nonescaped mosquitoes).

trials with and without host treatments. There were highly significant differences in observed escape responses of both mosquito species for all treatment pairs comparing contact to noncontact trials ($P < 0.0001$). Highly significant differences in escape responses were also observed when the control was compared to contact trials ($P < 0.0001$), but not significant when the matched control was compared to noncontact treatment trials ($P > 0.05$) (Table 4).

The escape patterns following exposure to bifenthrin or deltamethrin using *An. harrisoni* in contact trials were not significantly different when comparing between trials with hosts vs. contact trials without hosts ($P > 0.05$), whereas *An. minimus* exhibited highly significant differences in escape responses for the same comparison ($P < 0.01$). No significant differences in escape responses to bifenthrin or deltamethrin were observed in noncontact trials with host vs. noncontact trials without host for either mosquito species ($P > 0.05$) (Table 5).

When comparing escape probabilities between *An. harrisoni* and *An. minimus* in contact trials against deltamethrin in the presence of host, *An. minimus* exhibited significantly higher escape probability than *An. harrisoni* ($P < 0.0001$) (Table 6). The same was true in contact trials against both chemicals in the absence of a host, in which response in *An. minimus* was significantly higher than that exhibited by *An. harrisoni* (bifenthrin, $P = 0.0076$; deltamethrin, $P < 0.0001$). In addition, the escape response of *An. minimus* against deltamethrin under both host conditions was significantly higher than when the mosquitoes were exposed to bifenthrin ($P < 0.0001$) (Table 7). In contrast, there was no significant difference in the escape response of

Table 3. Escape time (in minutes) for 25%, 50%, and 75% of *Anopheles harrisoni* and *An. minimus* test populations to escape from excito-repellency chambers containing either bifenthrin (0.025 g/m^2) or deltamethrin (0.02 g/m^2) within a 30-min exposure period in contact trials.

		Deltamethrin			Bifenthrin		
Species	Conditions	ET_{25}[1]	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
An. harrisoni	With host	7	22	—[2]	11	30	—
	Without host	9	—	—	14	25	—
An. minimus	With host	4	7	11	17	—	—
	Without host	3	4	9	11	18	30

[1] ET_{25}, escape time (in minutes) for 25% of mosquitoes; ET_{50}, escape time (in minutes) for 50% of mosquitoes; ET_{75}, escape time (in minutes) for 75% of mosquitoes.
[2] — indicates insufficient number escaped from exposure chambers to estimate ET_{25}, ET_{50} and ET_{75} during the 30-min exposure period.

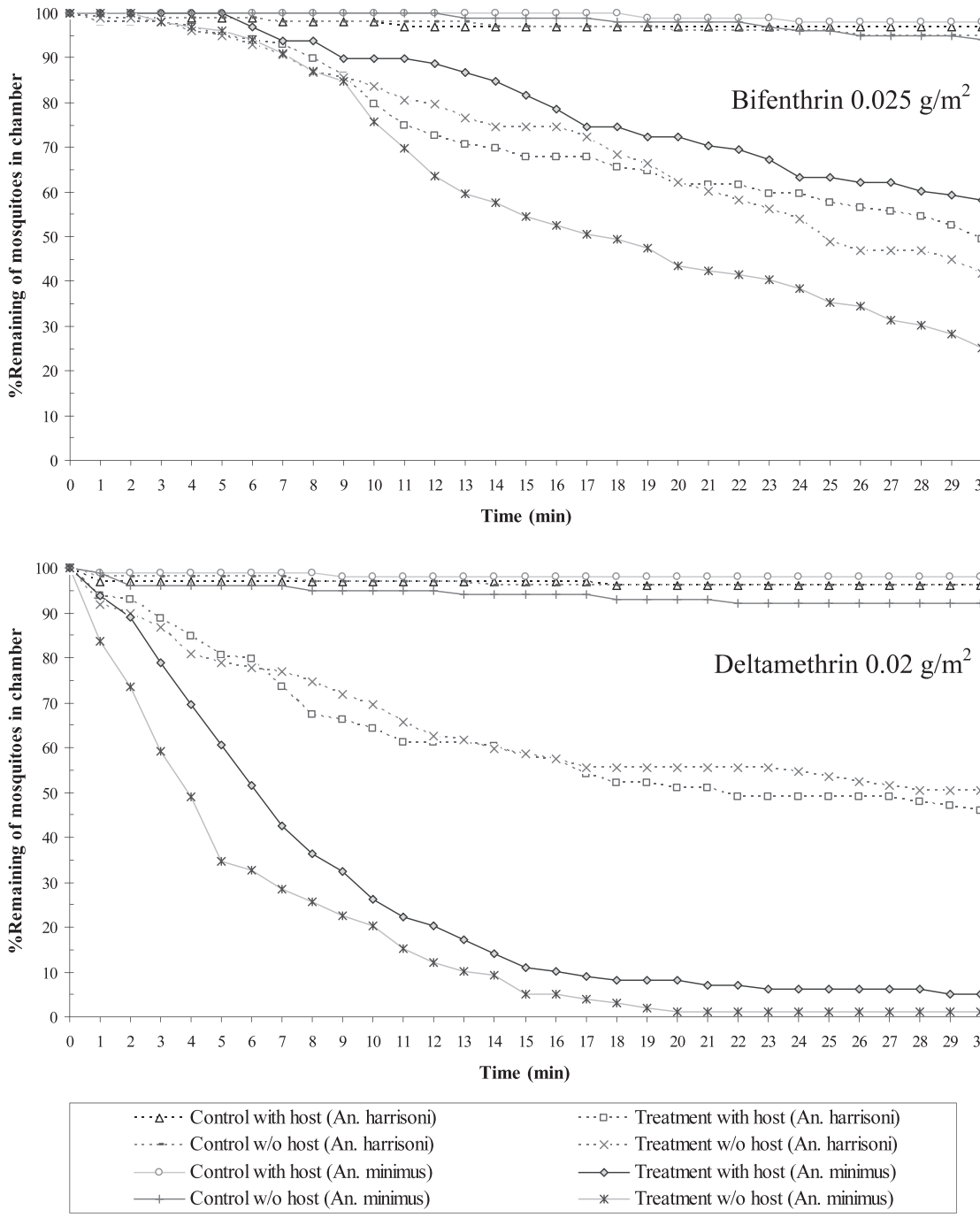


Fig. 2. Escape patterns of *Anopheles harrisoni* and *An. minimus* to bifenthrin 0.025 g/m^2 and deltamethrin 0.02 g/m^2 in contact trial.

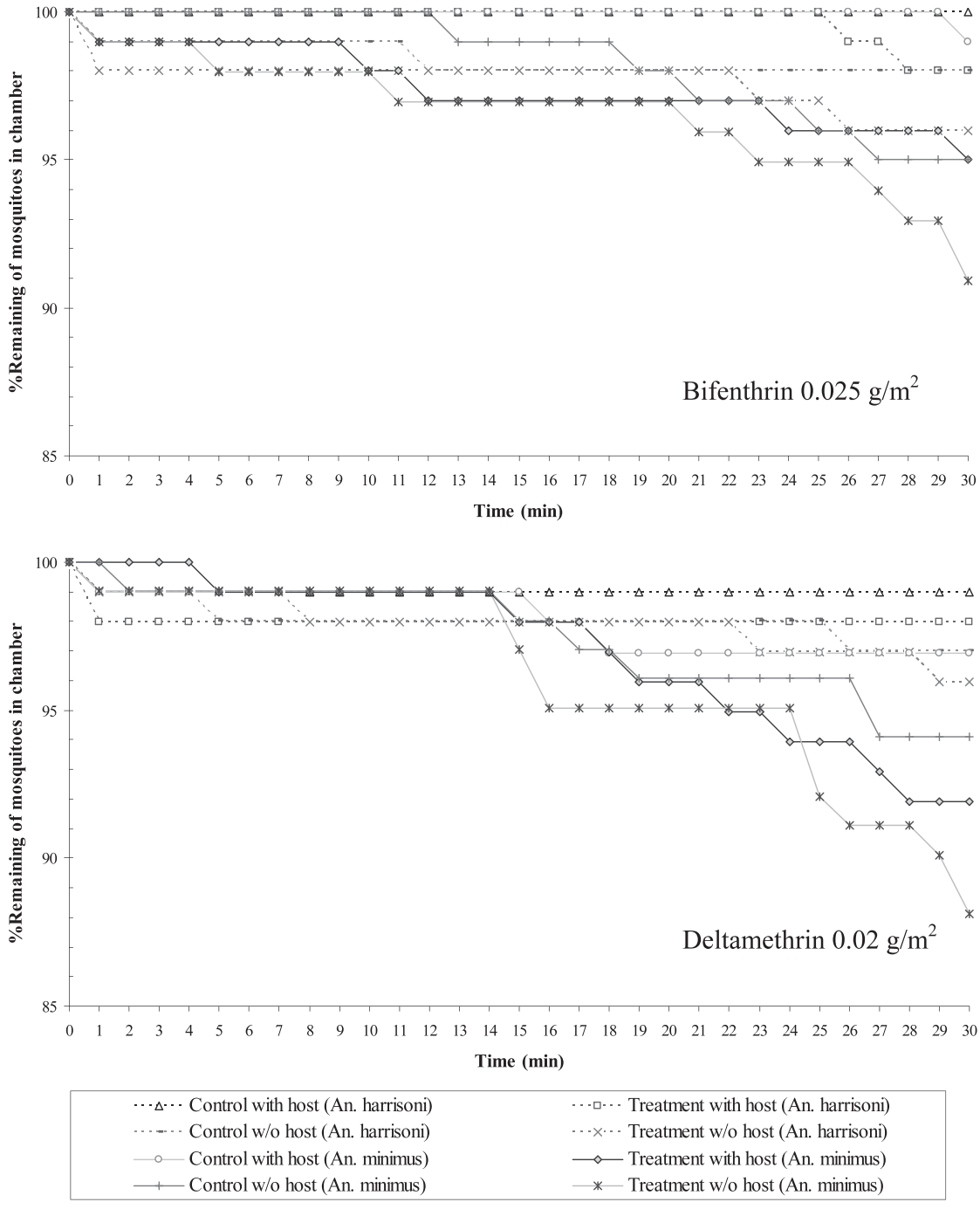


Fig. 3. Escape patterns of *Anopheles harrisoni* and *An. minimus* to bifenthrin 0.025 g/m^2 and deltamethrin 0.02 g/m^2 in noncontact trial.

Table 4. Comparison of escape patterns of *Anopheles harrisoni* and *An. minimus* against bifenthrin or deltamethrin in the presence or absence of a host between paired control and treatment test systems in contact, noncontact, and paired contact and noncontact trials.

		With host			Without host		
Species	Insecticides	Contact control vs. contact treatment	Noncontact control vs. noncontact treatment	Contact treatment vs. noncontact treatment	Contact control vs. contact treatment	Noncontact control vs. noncontact treatment	Contact treatment vs. noncontact treatment
An. harrisoni	Bifenthrin	<0.0001*	0.1583	<0.0001*	<0.0001*	0.4107	<0.0001*
	Deltamethrin	<0.0001*	0.5677	<0.0001*	<0.0001*	0.6952	<0.0001*
An. minimus	Bifenthrin	<0.0001*	0.0987	<0.0001*	<0.0001*	0.2637	<0.0001*
	Deltamethrin	<0.0001*	0.1318	<0.0001*	<0.0001*	0.1377	<0.0001*

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior.

An. harrisoni to bifenthrin vs. deltamethrin under host presence or absence treatment conditions (with host, $P = 0.2230$; without host, $P = 0.8204$).

DISCUSSION

Bifenthrin and deltamethrin are recommended by the World Health Organization for indoor residual spraying against malaria vectors. Deltamethrin has a relatively high irritant and knockdown effect on mosquitoes when compared to bifenthrin, whereas bifenthrin, which is considered to elicit low irritant effects, consistently provides a high kill due to the ability of mosquitoes to rest on treated surfaces for longer periods of time (WHO 2001). However, the kill effect of insecticides also depends on the level of resistance/tolerance of the target vector population to the chemical being applied. Some studies have reported that physiological and biochemical resistance can influence behavioral avoidance as well (Sparks et al. 1989).

The excito-repellency test system reported in the current study with subsequent design modifications has been previously used to evaluate the behavioral responses of mosquitoes to insecticides and provide information on both contact irritancy and noncontact repellency behavioral responses (Chareonviriyaphap et al. 1997, Sungvornyothin et al. 2001, Kongmee et al. 2004, Potikasikorn et al. 2005, Sathantriphop et al. 2006, Pothikasikorn et al. 2007). By adding another variable—a live host—to this test system we were able to measure the effect of host cues on escape response to insecticides under contact or noncontact test conditions with chemicals.

Our results demonstrated that contact irritancy was the primary action of both deltamethrin and bifenthrin to *An. harrisoni* and *An. minimus* test populations with no noncontact repellency effect from either insecticide. Greatest irritancy effects for both chemicals were observed in *An. minimus*, with deltamethrin producing stronger responses than bifenthrin. Overall, 24-h mortality rates of both *An. minimus* and *An. harrisoni* were higher when exposed to deltamethrin than with exposure to bifenthrin. In addition, the mortality rates of *An. minimus* from exposure to bifenthrin were lower than exhibited in *An. harrisoni* populations. As both mosquito populations are susceptible to the chemicals evaluated, these differences may be due to innate behavioral characteristics of each species to avoid or contact insecticide-treated surfaces, resulting in different mortality rates. Previous observations have indicated different responses to DDT (dichlorodiphenyl trichloroethane), deltamethrin, lambda-cyhalothrin, bifenthrin, and deet (diethyl toluamide) between *An. minimus* and *An. harrisoni* (Potikasikorn et al. 2005, Tisgratog et al. 2011). Such behavioral avoidance to bifenthrin may result in mosquitoes moving to a chemical-free surface or area before acquiring a lethal dose (Kennedy 1947, Muirhead-Thomson 1960, Roberts et al. 2000).

Table 5. Comparison of escape patterns of *Anopheles harrisoni* and *An. minimus* from control and treatment chambers against bifenthrin or deltamethrin between paired host conditions in contact and noncontact trials.

		Control		Treatment	
Species	Insecticides	Contact trial with host vs. contact trial without host	Noncontact trial with host vs. noncontact rial without host	Contact trial with host vs. contact trial without host	Noncontact trial with host vs. noncontact trial without host
An. harrisoni	Bifenthrin	0.4789	0.1583	0.4151	0.4005
	Deltamethrin	0.9768	0.3254	0.5840	0.4130
An. minimus	Bifenthrin	0.1462	0.0985	<0.0001*	0.2652
	Deltamethrin	0.0553	0.3448	0.0055*	0.3838

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior.

Table 6. Comparison of escape patterns between paired *Anopheles harrisoni* and *An. minimus* escape patterns in contact and noncontact trials, with host and without host, against bifenthrin or deltamethrin.

Trials	Host conditions	*An. harrisoni* vs. *An. minimus*	
		Bifenthrin	Deltamethrin
Contact	With host	0.1576	<0.0001*
	Without host	0.0076	<0.0001*
Noncontact	With host	0.2449	0.0550
	Without host	0.1526	0.0510

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior.

For *An. harrisoni*, the presence or absence of host had no significant effect on escape responses to both insecticides. The physiological state (mating condition) of test populations could be an influential factor in escape movements because *An. harrisoni* is normally colonized in the laboratory using forced-mating techniques. The tested *An. harrisoni* mosquitoes were mostly unmated mosquitoes. However, in similar evaluations conducted by Pothikasikorn et al. (2007) using the same test system and field-collected *An. harrisoni* exposed to deltamethrin but without any host involved, escape responses were similar to those reported in our study (51% escape in field populations, 49% in colony populations), but with lower mortality (2%) as compared to current results (18–40% mortality).

For *An. minimus*, the presence or absence of a host had significant effects on behavioral responses to both insecticides, with the presence of a host delaying mosquito escape from the treated chamber. This phenomenon may occur when test populations originate from different colonies due to variations in physiological condition of mosquitoes (i.e., mated or unmated) and attractive substances produced by the host (guinea pig) that preferentially attract mated-female mosquitoes. However, all mosquito test cohorts in our study were populations from the same free-mating colony, thereby representing mixed physiological status.

Table 7. Comparison of escape patterns between paired bifenthrin and deltamethrin insecticides in contact and noncontact trials, with host and without host, of *Anopheles harrisoni* and *An. minimus*.

Trials	Host conditions	Bifenthrin vs. Deltamethrin	
		An. harrisoni	*An. minimus*
Contact	With host	0.2230	<0.0001*
	Without host	0.8204	<0.0001*
Noncontact	With host	0.9858	0.3873
	Without host	0.9914	0.5256

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior.

In conclusion, our results clearly demonstrate that deltamethrin elicited stronger irritant effects in both *An. harrisoni* and *An. minimus* than bifenthrin, even in the presence of a host. Such information is beneficial for designing future behavioral studies, either under controlled laboratory or field conditions, to examine responses of other anopheline mosquitoes following exposure to insecticidal compounds used in vector control.

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Pyrethroid induced behavioral responses of *Anopheles dirus*, a vector of malaria in Thailand

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ABSTRACT: Contact and noncontact behavioral actions of wild-caught *Anopheles dirus* in response to the operational field dose of three synthetic pyrethroids (bifenthrin, α-cypermethrin and λ-cyhalothrin) were evaluated using an exito-repellency test chamber. DEET was used as the repellency standard for comparison with the other three synthetic pyrethroids. Results showed that test specimens rapidly escaped from the test chamber when exposed to direct contact with a surface treated with each of the three synthetic pyrethroids and DEET. Alpha-cypermethrin demonstrated the strongest irritant action (84.9% escape), followed by DEET (77.0%), λ-cyhalothrin (68.6%) and bifenthrin (68.3%). In the noncontact configuration, fewer mosquitoes escaped from the test chambers as compared to contact trials, although a significant escape response was still observed as compared to the controls ($P<0.05$). We conclude that *An. dirus* exhibits both irritant and repellent actions in response the three pyrethroids testing in this study. The information obtained will allow us to better understand the behavioral responses of vectors to various chemicals and provide guidance when designing control strategies for targeting specific disease vectors. ***Journal of Vector Ecology* 37 (1): 187-196. 2012.**

Keyword Index: Bifenthrin, DEET, α-cypermethrin, λ-cypermethrin, *Anopheles dirus*, Thailand.

INTRODUCTION

Many people living in tropical and subtropical areas of the world are at risk of infection from a wide variety of vector-borne diseases most notably malaria. Globally, between 100-300 million people live in malaria endemic areas (World Health Organization [WHO] 2009), where the four human malaria parasites (*Plasmodium*) are transmitted by mosquitoes in the genus *Anopheles* (Service and Townson 2002). In Thailand, all major malaria vectors belong to a species complex of which the respective sibling species are not able to be morphologically distinguished from one another (Rattanarithikul and Panthusiri 1994). Among these complexes, members of the *Anopheles dirus* complex serve as the most efficient malaria vectors in Thailand; two of the five species, namely *An. baimaii* and *An. dirus* are the most important (Manguin et al. 2008, Sungvornyothin et al. 2009, Singha and Chandra 2011).Both *An. baimaii* and *An. dirus* have proven extremely difficult to control due to a diverse array of host seeking behaviors and preferences, biting patterns and larval breeding habitats (Pates and Curtis 2005, Sinka et al. 2011). Despite decades of extensive research, efficacious and commercially viable vaccines for malaria are not yet available. Therefore, the prevention and control of malaria in Thailand remains dependent on various vector control measures (i.e. indoor residual spraying (IRS) and use of insecticide-impregnated bed nets) to reduce the risk of transmission by reducing the occurrence of blood feeding. The primary chemicals currently employed for vector control are the synthetic pyrethroids (Roberts and Andre 1994, WHO 1999, Reiter and Gubler 1997, Grieco et al. 2007).

Synthetic pyrethroids elicit a repellent response from many insect species and cause mosquitoes to move away from sprayed areas (Lockwood et al. 1984, Lindsay et al. 1991). The extensive and continued use of pyrethroids should act as a stimulus to increase attention on the behavior of the mosquito vectors. The use of IRS in homes has served as a major means of controlling malaria transmission, yet little is known of the precise role irritant and repellent actions have on specific malaria vectors, especially those that feed and rest indoors prior such as *An. dirus.*

Anopheles dirus was selected as the test species as it represents a major malaria vector in the forest and forest-fringe regions of Thailand and commonly exhibits both endophagic and anthropophilic behaviors in the areas where it occurs (Ismail et al. 1974, Rosenberg and Maheswary 1982, Baimai 1988, Baimai et al. 1988). Little is known or fully appreciated of the behavioral responses of *An. dirus* to the synthetic pyrethroids that are currently being used in public health vector control program in Thailand. Such information is needed to accurately assess the full impact of these chemicals prior to initiating a large scale vector control program using any of these three synthetic pyrethroids. The objective of this study was to use the exito-repellency test system to evaluate behavioral responses in wild-caught populations of *An. dirus* exposed to three commonly used synthetic pyrethroids: bifenthrin, α-cypermethrin and λ-cyhalothrin. Besides, DEET is one of the most commonly used chemicals for disrupting human-

vector contact and was shown to inhibit odor-evoked currents mediated by the insect odorant receptor complex, effectively inhibiting perception of host odors and chemo-attractant cues (Ditzen et al. 2008). In this study, DEET was used as a positive control for repellency and served as a comparison compound for evaluating the other 3 synthetic pyrethroids.

MATERIALS AND METHODS

Mosquito collections

Anopheles dirus was captured from Ban Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14°17'N, 99°11'E). The collection site is located 500 m away from the base of a steep mountainous ridge and is surrounded by natural dense forest (Sungvornyothin et al. 2009). Female mosquitoes were collected from cattle using mouth aspirators during 15 min intervals per hour from 18:00 to 06:00. Collected mosquitoes were then identified, placed in plastic cups covered with netting and provided a cotton pad that was soaked with a 10% sugar solution. All wild-caught adult mosquitoes were transported back to the entomology laboratory in Bangkok for confirmatory identification. Female mosquitoes used in the assay were starved of blood and sugar 24 h prior to testing.

Mosquito identification

Adult mosquitoes were identified using the morphological keys of Sallum et al. (2005) and Rattanarithikul et al. (2006). Female mosquitoes showing a presector dark spot (PSD) on the radius vein that extends basally beyond the PSD spot on the costa vein and a PSD spot that reaches the humeral dark (HD) spot of the costa vein or at least surpasses the middle of the presector pale spot (PSP) of the costa vein of at least one wing were identified as belonging to the *An. dirus* complex.

Insecticide-treated papers

Four chemicals were used in behavioral testing. These were 1) Bifenthrin (BIF) (IUPAC: 2-methylbiphenyl-3-yl-methyl (*Z*)-(1*RS*,3*RS*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate) (Ladda Co., Bangkok), 2) α-cypermethrin (ALP) ((*R*)-cyano(3-phenoxy-phenyl)methyl (1*S*,3*S*)-*rel*-3-(2,2-dichloroethenyl)-2,2-di-methylcyclopropanecarboxylate) (BASF Aktiengesclshaft, Bangkok, Thailand), 3) λ-cyhalothrin (LAM) (Cyano-3-phenoxybenzyl (1s+1r)-cis-3-(z-2-chloro-3, 3, 3-trifluoro-prop-1-enyl)-2, 2-dimethyl cyclopropanecarboxylate) (Syn-genta Crop Protection (Thailand) Ltd, Bangkok, Thailand), and 4) DEET (*N,N*-diethyl-m-toluamide or *N,N*- diethyl-3-methylbenzamide) (United States Department of Agricul-ture, Beltsville, MD).

Standard doses of each compound (0.025 g a.i./m² of bifenthrin, 0.03 g a.i./m² of α-cypermethrin, 0.02 g a.i./m² of λ-cyhalothrin and 2 g a.i./m² of DEET) were impregnated onto 15 x 17.5 cm filter paper. These doses were used based on each chemical's labeled field application rate. All impregnated papers were prepared according to WHO specification (WHO 1998) and were treated at the rate of 2.75 ml of insecticide solution per 180 cm². Control papers (without chemical) were prepared using only a diluent (i.e. acetone or ethanol) following WHO protocol (2006).

Behavioral tests

Tests were conducted to compare two types of behavioral responses (irritancy and repellency) of *An. dirus* in contact and noncontact exposure chambers using four different chemicals (0.025% bifenthrin, 0.03% α-cypermethrin, 0.02% λ-cyhalothrin, and 5% DEET). In each test, 15 female mosquitoes were carefully introduced into each of four chambers by using a mouth aspirator after which the rear door was closed. A receiving cage, measuring 6x6x6 cm³, was connected to the exit portal for collecting exiting mosquitoes (Figure 1). Mosquitoes were allowed a three min adjustment period before the escape funnel was opened to initiate the observation period. Mosquitoes escaping from the chamber into the receiving cage were recorded at 1-min intervals for a period of 30 min. Ambient temperatures (25±5° C) and relative humidity (80±10% RH) were recorded during the experiment. All tests were performed during the day between 08:00 and 16:30 and each test was replicated four times. After each test period, the numbers of dead or knockdown specimens were recorded separately from each exposure chamber. Live escaped specimens and those remaining inside the treatment and control chambers were collected and held separately in clean containers topped with cotton pads soaked in a 10% sugar solution. After 24 h, mosquito mortality was recorded. Details of the chamber design and the methodology are given elsewhere (Tanasinchayakul et al. 2006).

Data analysis

Percentage escape obtained from the treated exposure chambers was adjusted based on paired control escape responses using Abbott's formula (Abbott 1925). Life table survival analysis was used to estimate mosquito escape rates for each test configuration (i.e., by chemical for either contact or noncontact configuration). Kaplan-Meier survival analysis was used to analyze the rates of escaping mosquitoes from each chamber of the excito-repellency system (Kleinbaum 1995, Roberts et al. 1997). Survival analysis was also used to estimate the escape time (ET) at which 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of the test populations escaped from the chamber. Patterns of escape behavior were evaluated within the test cohorts and between difference treatment groups using the log-rank method (Mantel and Haenzel 1959). Mortality was observed after 24 h. Statistical significance for all tests was set at $P < 0.05$.

RESULTS

Two types of behavioral responses, contact irritancy (move away from the treated area after making physical contact with test compound) and noncontact repellency (move away from the treated area before making physical contact with test compound) were observed in *An. dirus*

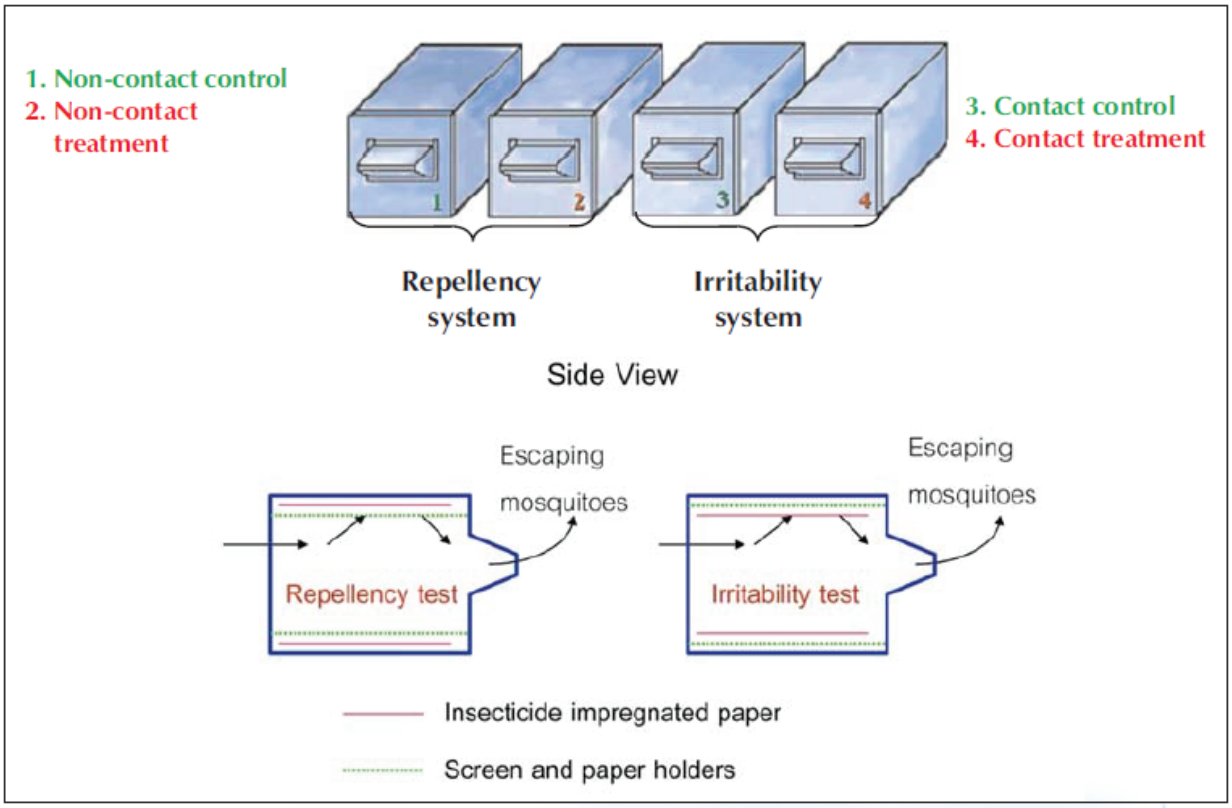


Figure 1. Excito-repellency test chamber used to study insecticide behavioral responses.

that were exposed to three synthetic pyrethroids and DEET using an excito-repellency test system. Percent mortality for escaped and non-escaped mosquitoes from both the control and treatment chambers are presented in Table 1. In general, patterns and rates of escape were much stronger in contact trials (68.32-84.91%) as compared to noncontact trials (0-52.52%) for all four compounds. In contact trials, percent escape of *An. dirus* for α-cypermethrin (84.91%) was higher than for bifenthrin (68.32%), λ-cyhalothrin (68.60%) and DEET (77.01%). Relatively low numbers of mosquitoes escaped from the control chambers (5-20%) in both the contact and noncontact configuration (8.47-17.24%). In noncontact trials, DEET (52.52%) produced the greatest repellent response, followed by bifenthrin (21.88%), α-cypermethrin (11.54%), and λ-cyhalothrin (0%).

Percent mortality of escaped and non-escaped specimens after a 24-h holding period from both the contact and noncontact assays are presented in Table 1. In contact trials, mortality rates of escaped mosquitoes ranged between 6.25 and 25% whereas those remaining in the test chamber showed mortality rates, ranging from 6.12 to 73.33%. The highest mortality rate for escaped mosquitoes from the control chambers was observed in assays matched with bifenthrin (18.18%). In noncontact trials, the percent mortality of non-escaped specimens was generally low, varying between 0 and 7.50. No mortality of escaped mosquitoes was observed from noncontact trials for all four compounds (Table 1).

The escape patterns generated for the insecticide-treated chambers are expressed in 1-min intervals for 25%, 50%, and 75% (ET_{25}, ET_{50}, and ET_{75}) of the test population to exit the test chambers (Table 2). ET values are generally low for contact trials compared to noncontact trails, regardless of test chemical. For contact trails, the ET_{25} values for bifenthrin, α-cypermethrin, λ-cyhalothrin and DEET were 11, 3, 4 and 3 min, respectively. The ET_{50} values for bifenthrin, α-cypermethrin, λ-cyhalothrin and DEET were 26, 5, 10 and 9 min, respectively. The ET_{75} values for bifenthrin and λ-cyhalothrin could not be calculated because of insufficient numbers of mosquitoes exiting during the 30-min test. For the noncontact trails, the ET_{25} and ET_{50} could only be estimated for DEET whereas the exit times for the three synthetic pyrethroids could not be estimated, except bifenthrin (ET_{25}=29) (Table 2). The remaining noncontact trials produced insufficient numbers of escaped mosquitoes to calculate exit times.

Differences in the patterns of escape behavior were analyzed using the log-rank method and statistical significance was set at 0.05 (Tables 3 and 4). Significant differences in escape behavior were observed when comparing paired control versus contact trials and contact versus noncontact trials for all four compounds ($P<0.05$). Significant differences were also observed when noncontact trials was compared to paired control trials for bifenthrin ($P=0.0227$) and DEET ($P<0.0001$). A comparison of the escape response of mosquitoes between pairs of each of the 4 test chemicals in contact and noncontact trials are presented in Table 4. Significant differences in contact trials

Table 1. Percentage escape and 24 h mortality of *Anopheles dirus* exposed to bifenthrin, α-cypermethrin, λ-cyhalothrin and DEET in contact and noncontact trials.

Condition	Test Population	Mosquitoes (No. in test)	% escaped*	Escaped	Remain
Contact	BIF[1]	Treatment (58)	68.32	6.97 (3/43)	73.33 (11/15)
		Control (60)	18.33	18.18 (2/11)	6.12 (3/49)
	ALP[2]	Treatment (58)	84.91	15.68 (8/51)	14.28 (1/7)
		Control (60)	20	0	8.33 (4/48)
	LAM[3]	Treatment (57)	68.60	25 (10/40)	29.41 (5/17)
		Control (60)	5	0	0
	DEET [4]	Treatment (58)	77.01	6.52 (3/46)	66.66 (8/12)
		Control (60)	10	0	12.96 (7/54)
Noncontact	BIF	Treatment (57)	21.88	0	7.50 (3/40)
		Control (59)	10.17	0	3.77 (2/53)
	ALP	Treatment (60)	11.54	0	4.34 (2/46)
		Control (60)	13.33	0	1.92 (1/52)
	LAM	Treatment (57)	0	0	0
		Control (59)	8.47	0	1.85 (1/54)
	DEET	Treatment(56)	52.52	0	4.54 (1/22)
		Control(58)	17.24	0	0

[1]Bifenthrin =0.025 g/m^2, [2]α-cypermethrin =0.03 g/m^2, [3]λ-cyhalothrin =0.02 g/m^2 and [4]DEET =2 g/m^2
*Adjusted rate based on control response

Table 2. Escape time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of *Anopheles dirus* to escape from the excito-repellency test chambers containing one of four chemicals using a 30 minute of exposure period.

Test condition	Bifenthrin			α-cypermethrin			λ-cyhalothrin			DEET		
	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact	11	26	+	3	5	12	4	10	+	3	9	30
Noncontact	29	+	+	+	+	+	+	+	+	8	22	+

+Insufficient number of mosquitoes escaped from test chamber.

Table 3. Comparison of escape responses between contact and control, contact and noncontact and noncontact and control trials for *Anopheles dirus*.

Chemical	Treatment pairs		
	Control vs. Contact	Contact vs Noncontact	Noncontact vs Control
Bifenthrin	<0.0001	<0.0001	0.0227
α-cypermethrin	<0.0001	<0.0001	0.1450
λ-cyhalothrin	<0.0001	<0.0001	0.4618
DEET	<0.0001	0.0227	<0.0001

Significance set at $P < 0.05$.

were observed when α-cypermethrin was compared to bifenthrin (P<0.0001), DEET (P=0.0887) and λ-cyhalothrin (P=0.0076). In noncontact trials, significant differences were observed for all chemical pairs (P<0.05), except when bifenthrin was compared to α-cypermethrin (P=0.4818).

Figures 2 and 3 show the proportions of *An. dirus* mosquitoes remaining in the exposure chamber treated with bifenthrin, α-cypermethrin, λ-cyhalothrin and DEET. These proportions were used to develop escape rate patterns representing the probability of escaping from the exposure chamber in contact and paired control trials (Figure 2) and noncontact and paired control trials (Figure 3). In contact trials, α-cypermethrin produced the greatest irritant response as compared with the other chemicals (P<0.05). In noncontact trials, significantly stronger escape responses were observed in DEET compared to the other compounds (P<0.05) (Figure 3).

DISCUSSION

Although studies have been conducted in Thailand to evaluate the behavioral responses of anopheline mosquitoes to various cyano-pyrethroid insecticides using the excito-repellency test chamber (Chareonviriyaphap et al. 2001, 2004, Muenworn et al. 2006, Thanispong et al. 2009, Mongkalangoon et al. 2009), none have evaluated the active properties of bifenthrin, α-cypermethrin and λ-cyhalothrin, three commonly used synthetic pyrethroids, on field-collected *An. dirus*. In this study, both contact excitatory (irritancy) and noncontact spatial repellency responses of *An. dirus* were quantitatively evaluated using the excito-repellency test system to provide a more complete understanding of vector behavior when exposed to insecticides. All three synthetic pyrethroids produced strong excitation responses among exposed female *An. dirus* without resulting in mortality following contact. Noncontact repellency was also evident but had much less an influence on escape (*avoidance*) behavior than direct contact irritancy. Following the indoor application of excito-repellent compounds, an aversion to normal indoor biting behavior, while potentially reducing disease transmission risk inside the house, may increase the probability for transmission outside the home by promoting greater exophagic behavior. This information should allow for the design of more effective strategies for targeting specific vector species and provides valuable information when selecting the most appropriate interventions for vector control.

For decades, several groups of chemical compounds have been widely used in the national public health vector control program in Thailand (Jirakanjanakit et al. 2007, Thanispong et al. 2008, Chuaycharoensuk et al. 2011). Synthetic pyrethroids represent one of the most commonly used insecticides for controlling all indoor and outdoor blood feeding mosquitoes, in particular *Anopheles* malaria vectors. These compounds have been found to exhibit a certain degree of excito-repellency in many agricultural and medically important insects (Lockwood et al. 1984, Lindsay et al. 1991, Roberts and Andre 1994). The continued use of pyrethroids demonstrate their effectiveness as a vector control tools and supports the need for further evaluations of the avoidance behavior elicited by these compounds across mosquito species and in other arthropod pests. With pyrethroids playing a major role in most indoor residual spray campaigns and as the main active ingredient in most insecticide treated bed nets, the role of the irritant and repellent actions of pyrethriods should be thoroughly defined for specific malaria vectors before beginning any large scale control campaign.

After an excito-repellency test system was introduced

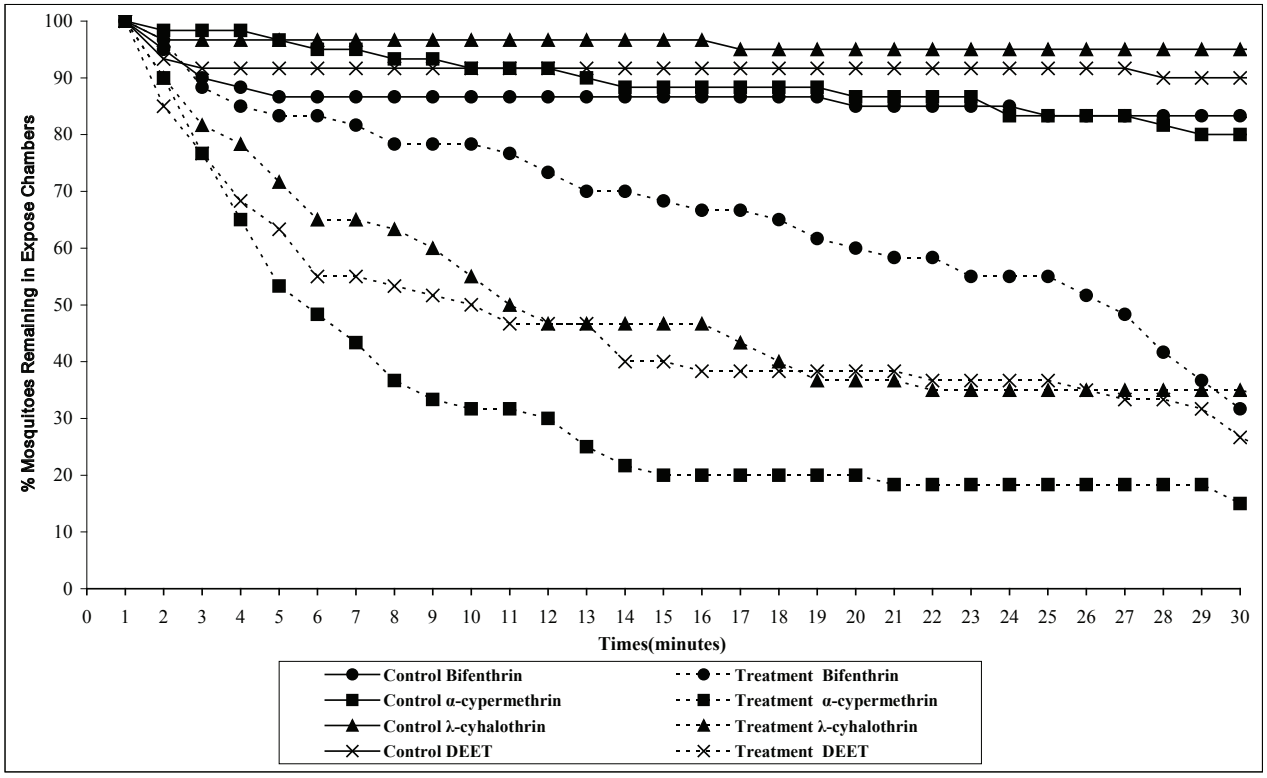


Figure 2. Escape probability of *Anopheles dirus* exposed to bifenthrin =0.025 g/m², α-cypermethrin=0.03 g/m², λ-cyhalothrin =0.02 g/m² and DEET =2 g/m² for treatment and control contact trials.

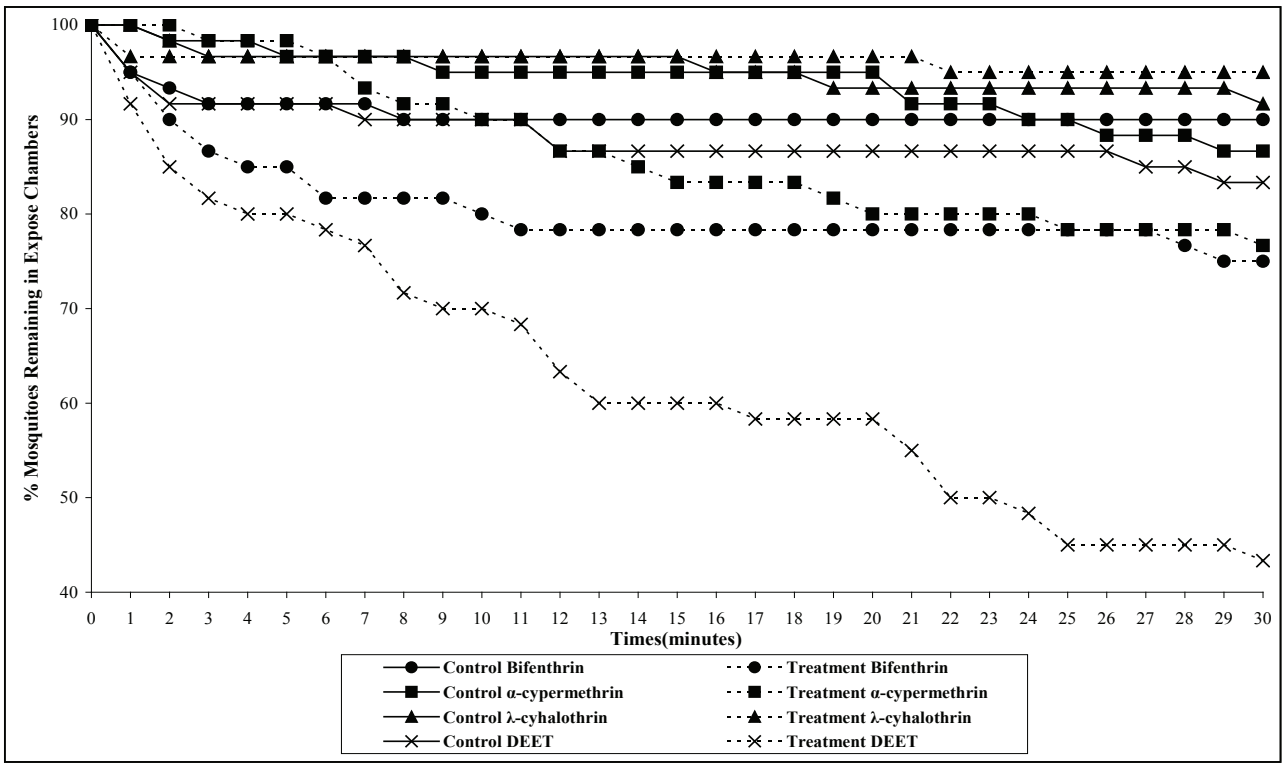


Figure 3. Escape probability of *Anopheles dirus* exposed to bifenthrin =0.025 g/m², α-cypermethrin=0.03 g/m², λ-cyhalothrin =0.02 g/m² and DEET =2 g/m² for treatment and control noncontact trials.

Table 4. Comparison of escape responses of *Anopheles dirus* between pairs of 4 test chemicals in contact and non-contact trials.

Chemical	Treatment pairs	
	Contact	Noncontact
Bifenthrin vs α-cypermethrin	<0.0001	0.4818
Bifenthrin vs λ-cyhalothrin	0.3465	0.0011
Bifenthrin vs DEET	0.0869	0.0010
α-cypermethrin vs λ-cyhalothrin	0.0076	0.0046
α-cypermethrin vs DEET	0.0887	<0.0001
λ-cyhalothrin vs DEET	0.3408	<0.0001

Significance set at $P < 0.05$

and used to separate two types of behavioral actions (Roberts et al. 1997), several studies to evaluate the excito-repellent behavior in *Anopheles* mosquitoes have subsequently been reported (Chareonviriyaphap et al. 1997, 2001, 2004, Pothikasikorn et al. 2005, Muenworn et al. 2006, Pothikasikorn et al. 2007). Overall, synthetic pyrethroids produce much stronger irritant responses in *Anopheles* compared to repellent actions (Chareonviriyaphap et al. 2001, 2004, Muenworn et al. 2006, Pothikasikorn et al. 2007). In this study, a greater avoidance response for *An. dirus* was also observed in contact trials, compared to noncontact and paired control trials. There were also significant differences in the escape responses documented between noncontact trials and their matched controls.

All three pyrethroids produced a rapid and pronounced irritant response in *An. dirus*. The most dramatic avoidance response after physical contact was observed when *An. dirus* were exposed to the operational dose of α-cypermethrin. Similar findings of the irritant actions of pyrethroids were previously observed in both laboratory and field test populations of *An. dirus* (Chareonviriyaphap et al. 2004). Additional laboratory studies also demonstrated a relatively strong irritant effect of α-cypermethrin in three test populations of *Aedes aegypti* from Thailand (Thanispong et al. 2009). These studies also showed that most mosquitoes exited from the treated chamber before receiving a lethal dose of α-cypermethrin. Among the 3 test pyrethriods used in the current study, bifenthrin elicited the weakest escape response. Bifenthrin is a broad spectrum synthetic pyrethroids used for the control of a variety of agricultural pests and exhibits a very low vapor pressure (Hougard et al. 2002). Bifenthrin may exhibit a weaker excito-repellent effect compared to the others due to this low vapor pressure. A low irritant and knockdown response was also found for bifenthrin as compared with permethrin and deltamethrin (WHO 2001). These same studies concluded that although bifenthrin demonstrated an excito-repellent effect, it was a weak enough response to produce consistently high mortality. It was postulated that the weak behavioral response allowed mosquitoes to rest on the treated surface for a sufficient time to acquire a lethal dose of insecticide as compared to other chemicals such as deltamethrin or DEET (Tisgratog et al. 2011). Although noncontact repellency to various synthetic pyrethroids has been recognized in several species of *Anopheles* (Lien 1991, Chareonviriyaphap et al. 1999, 2001, Sungvornyothin et al. 2001), this type of chemical action was not significantly pronounced in our study. Apart from synthetic pyrethroids, DEET is the most commonly used chemical for disrupting human-vector contact and exhibits strong behavioral responses in mosquitoes (Surgeoner 1995, Cox 2005). Although DEET has long been classified as a repellent, more recent studies have concluded that it may act as an inhibitor to mask host cues rather than as a true repellent (Dogan et al. 1999, Dogan and Rossignol 1999). Based on electrophysiological responses, DEET was shown to inhibit odor-evoked currents mediated by the insect odorant receptor complex (Ditzen et al. 2008), effectively inhibiting perception of host odors and chemo-attractant cues. There is no clear conclusion as to whether DEET performs as a true repellent or an inhibitor based on the findings of this study. However, the differences in escape response between contact and noncontact trials did allow us to separate and prioritize the order of action for the irritant or repellent actions of the compounds tested. The intensity of these actions was also determined based on the escape response from matched control and noncontact tests to arrive at an adjusted percent repellency. It must be stated, however, that neither test configuration used a known attractant inside the DEET-treated chamber, a requirement to evaluate if inhibition was a mode of action. Those mosquitoes that escaped from the noncontact test configuration seem to have done so as the result of repellency alone. Regardless, the endpoint obtained from DEET was that it continued to function as a deterrent even in the absence of host cues. Based on our findings from the excito-repellency test system, DEET appears to act as a contact irritant and a moderate spatial repellent.

Significant differences in the escape responses were documented for all paired contact and noncontact trials. In addition, the escape response for all paired noncontact tests were found to be significantly different as compared to their matched controls. Mortality was low for mosquitoes escaping the treated chambers in both the contact and noncontact trials, an indication that behavioral avoidance greatly reduces the opportunity for residual insecticides to impact survival through toxicity. Pothikasikorn et al.

(2005) confirmed that *An. minimus* and *An. harrisoni* are rapidly irritated by λ-cyhalothrin and deltamethrin. Chareonviriyaphap et al. (2004) reported on an extensive study that defined the excito-repellent action of deltamethrin on four anopheline species, all representing important malaria vectors in Thailand. Although repellency was less profound than contact irritancy, the escape responses were statistically significant compared to the matched controls.

Previous studies documented that at least two sympatric species of the *An. dirus* complex, *An. dirus* and *An. baimai*, occur in Pu Teuy Village, Kanchanaburi Province with the most prevalent being *An. dirus* (>95%) (Suwonkerd pers. com). Pu Teuy is considered nearly malaria-free with only a few cases reported. Even then, the area remains vulnerable to increased transmission due to human movement from highly endemic areas (Chareonviriyaphap et al. 2003, Muenworn et al. 2006, Pothikasikorn et al. 2005, Sungvornyothin et al. 2006). In addition, Pu Teuy Village is located in close proximity to intact forest and forest-fringe zones that are ideal habitats for *An. dirus* s.l., one of the efficient vectors of malaria in Thailand (Rattanarithikul et al. 2006).Unfortunately, this study was limited and relied solely on morphological identification which may have resulted in a certain degree of misidentification between these two sibling species (Manguin et al. 2010). We suggest that further evaluations be made to characterize the behavioral responses elicited by vector control insecticides for each species in order to help define the chemicals impact as part of the control programs in Thailand. This will help define the relative risk for malaria transmission associated with particular species that can assist in prioritization and design of appropriate vector prevention and control strategies.

In conclusion, a better understanding of the irritant and repellent actions of chemicals in malaria vectors is essential when evaluating the full impact these compounds may have on both mosquitoes and disease transmission. A standardized approach and quantification of a chemical's primary and secondary mode of action will help optimize our currently available public health tools and hopefully promote the development of newer chemicals and other innovative control methodologies. We believe excito-repellent assays should be an integral component of any insecticide evaluation to determine its full capabilities and potential to mitigate disease transmission.

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Parasites & Vectors

RESEARCH Open Access

Host feeding patterns and preference of *Anopheles minimus* (Diptera: Culicidae) in a malaria endemic area of western Thailand: baseline site description

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Abstract

Background: Host feeding patterns of *Anopheles minimus* in relation to ambient environmental conditions were observed during a 2-year period at Tum Sua Village, located in Mae Sot District, Tak Province, in western Thailand, where *An. minimus* is found in abundance and regarded as the most predominant malaria vector species. Detailed information on mosquito behavior is important for understanding the epidemiology of disease transmission and developing more effective and efficient vector control methods.

Methods: Adult mosquitoes were collected every 2 months for two consecutive nights from 1800 to 0600 hrs. Three collection methods were used; indoor human-landing collections (HLC), outdoor HLC, and outdoor cattle-bait collections (CBC).

Results: A total of 7,663 female *Anopheles* mosquitoes were collected of which 5,392 were identified as members of 3 different species complexes, the most prevalent being *Anopheles minimus* complex (50.36%), followed by *Anopheles maculatus* complex (19.68%) and *Anopheles dirus* complex (0.33%). *An. minimus* s.s. comprised virtually all (> 99.8 percent) of Minimus Complex species captured. Blood feeding behavior of *An. minimus* was more pronounced during the second half of the evening, showing a slight preference to blood feed outdoors (~60%) versus inside structures. Significantly ($P < 0.0001$) more *An. minimus* were collected from human-baited methods compared with a tethered cow, indicating a more anthropophilic feeding behavior. Although a significant difference in total number of mosquitoes from the HLC was recorded between the first and second year, the mean biting frequency over the course of the evening hours remained similar.

Conclusions: The Human landing activity of *An. minimus* in Tum Sua Village showed a stronger preference/attraction for humans compared to a cow-baited collection method. This study supports the incrimination of *An. minimus* as the primary malaria vector in the area. A better understanding of mosquito behavior related to host preference, and the temporal and spatial blood feeding activity will help facilitate the design of vector control strategies and effectiveness of vector control management programs in Thailand.

Keywords: *Anopheles minimus*, Seasonal abundance, Blood feeding behavior, Host preference, Malaria, Thailand

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Background

In recent years, approximately two-thirds of all recorded malaria cases in Thailand have been found along the international border of Thailand-Myanmar [1,2]. Between 2008 and 2010, averages of 7,377 (± 1,662) malaria cases were recorded annually in Mae Sot District [3]. The malaria epidemiology and persistence of disease transmission in the area has been primarily associated with small-scale agricultural activity, both occupationally-related and uncontrolled tribal population movements, and periodic political/civil unrest producing displaced populations in transient conditions more conducive for malaria transmission [1]. The site (Tum Sua Village, Mae Sot District) utilized in this study is considered a perennial malaria transmission area. Several important vectors of malaria are prevalent in the area, including members of the *Anopheles minimus* complex [4], one of the most important malaria vectors in rural forested and scrub areas of western Thailand [1,5,6].

At least two genetically distinct sibling species, *An. minimus* and *Anopheles harrisoni*, belong to the Minimus Complex [7]. These two species are difficult to identify accurately based on morphological characters alone [8,9]. In Thailand, *An. minimus* is regarded as the most predominant malaria vector species and is found throughout the country [10,11], whereas *An. harrisoni* is restricted along the western Thailand-Myanmar border, particularly in Kanchanaburi Province [7,11-13].

Allozyme analysis, once regarded as the gold standard to differentiate members in the Minimus Complex [10] has been replaced by several molecular, nucleic acid based approaches for more accurately separating different species within this complex. Two PCR-based techniques, an allele-specific amplification (ASA) and a single-strain conformation polymorphism (SSCP) assay have been developed for distinguishing members of the Minimus Subgroup and other closely related species [14]. Both multiplex format and a PCR-restriction fragment length polymorphism (RFLP) method were also developed to distinguish species within the Minimus Complex and other related species, namely *An. minimus*, *An. harrisoni*, *Anopheles aconitus*, *Anopheles pampanai* and *Anopheles varuna*, all species are found in Thailand [7,15,16]. A single multiplex PCR assay, using sequence characterized amplified region (SCAR) markers derived from individual random amplified polymorphic DNA is also able to differentiate between *An. minimus* and *An. harrisoni*, and their hybrids, as well as the 3 closely related species within the Minimus Subgroup [17,18].

Details of mosquito biology, especially host feeding activity and preference of individual species within the complex is essential to identify their respective roles in disease transmission and assist vector control operations to apply the most appropriate strategy of vector control management. Other observations on biting cycles and host preferences of *An. minimus* have been made in Thailand [19-24]; however, nearly all ecological and behavioral studies to date have been based on populations identified by morphological traits alone. More recently, the seasonal abundance of *An. minimus* and *An. harrisoni* adults in Kanchanaburi Province were identified using accurate molecular methods [11]. Unfortunately, the low number of *An. minimus* collected in that study area, predominated by *An. harrisoni*, resulted in no conclusive information on trophic behavior and seasonal abundance for this species. Using a PCR-based molecular method to ensure species-specific identification, we described the blood feeding/biting behavior, host preference and seasonal abundance of *An. minimus* over a two-year period in Tum Sua Village.

Methods

Study site

Anopheles minimus populations were collected from the Tum Sua Village (16°4 ° N, 98°41°E), in Mae Sot District of western Thailand. To the west, the border with Myanmar is separated by mountain ranges and the Moei River. Approximately 80 percent of the study site is surrounded by fruit orchards and other agricultural undertakings, while the remaining periphery of the village is composed of intact, sparsely populated, native forest on the west. A 2 meter -wide perennial flowing stream transects the village proper and is bordered by a variety of permanent riparian vegetation along its margins.

Collection methods

Adult mosquito collections were conducted every 2 months for two consecutive nights from 1800 to 0600 hrs, between November 2008 and September 2010. Three collection methods were used; indoor human-landing collections (HLC), outdoor HLC and outdoor cattle-bait collections (CBC). Indoor HLC were conducted in an insecticide-free house. The indoor and outdoor HLC collectors were separated into 2 teams of 4 collectors each, each team working 6 hr evening shifts. While 2 collectors captured mosquitoes inside the house the other 2 remained stationed outside at a distance of approximately 50 m from the same house. The first team worked between 1800 and 2400 hr, followed by the second team beginning at midnight until 0600 hr. Teams were rotated between the first and second halves of the evening on the second collection night to mitigate potential collector bias. Teams also exchanged positions between indoor and outdoor collections each alternate night. Indoor and outdoor HLC took place for 45 min each hour. Outdoor CBC was conducted by a separate team of two collectors, for 15 min each hour following the methods of Sungvornyothin *et al.* [25]. The CBC involved placing a single adult cow under a clean (untreated) cotton bed net measuring 3.6 m x 3.3 m x 2.0 m (L:W:H) with the net suspended 30 cm above the ground level to allow mosquitoes

access inside. The net trap was placed approximately 50 m from the nearest HLC and at equal distances from the perimeter tree line to avoid potential bias in attracting mosquitoes. The cow was exposed to mosquitoes entering the net uninterrupted for 45 min each hour. All mosquitoes, either resting inside the net or on the cow at time of collection, were captured using a mouth aspirator.

All collected mosquitoes were held in plastic holding cups and labeled by hour, location and collector name. Each cup contained cotton soaked with 10% sugar solution for sustenance. Cups were transported back to project site laboratory each hour for initial morphological identification. All *Anopheles* specimens were identified to species following Rattanarithikul *et al.* [8]. Environmental parameters were recorded each hour of collection by teams, using a manual thermo-hygrometer (BARICO GmbH, Villingen-Schwenningen, Germany). Rainfall data were obtained from the local meteorological station, approximately 18 km from the study area. Formal animal/human use approval for this research was granted by the Ethical Research Committee, Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Thailand (KURDI-1/2543- 1421457).

Molecular identification

A multiplex Allele Specific Polymerase Chain Reaction (AS-PCR) procedure was performed for molecular identification of individual adult *Anopheles* initially identified morphologically as *An. minimus* complex species**.** DNA extraction followed the protocol of Linton *et al.* [26] and AS-PCR assay by Garros *et al.* [16] to confirm the species identification.

PCR amplification of ITS-2 region

Following DNA extraction from individual adult mosquitoes, whole or partial specimens (e.g., legs and wings), isolated DNA was subjected to sequential PCR procedures. The PCR mixture contained 17.75 μl ultrapure distilled water, 2.5 μl of 10X reaction buffer, 10 mM of each dNTP, 10 μM of primer, 0.5 units of *Taq* DNA polymerase and 0.5 μl of DNA template. After an initial denaturation step at 94 °C for two min, 40 cycles were programmed as follows: 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 40 sec, and a final extension step at 72 °C for five min. Products were visualized using electrophoresis on a 2% agarose gel. Primer names, sequences and sizes of the PCR products are shown in Table 1. The internal transcribed spacer 2A (ITS-2A) is the universal primer that binds to the same position on the ITS rDNA for 10 closely related species (including 5 anopheline species in the Funestus Group present in tropical Africa only), while the five specific primer (*Pam* to *Mia*) PCR reactions bind at different locations on the ITS-2 sequence of each corresponding species.

Table 1 Primers, sequences, and sizes of PCR products used in the molecular identification of *Anopheles minimus* complex species present in Thailand

Species	Primer name	Sequence (5′ to 3′)	Size of the product (bp)	Tm (°C)
Universal forward primer	ITS2	TGT GAA CTG CAG GAC ACA T		54.5
Anopheles pampanai	PAM	TGT ACA TCG GCC GGG GTA	90	56.0
Anopheles aconitus	ACO	ACA GCG TGT ACG TCC AGT	200	58.2
Anopheles harrisoni	MIC	GTT CAT TCA GCA ACA TCA GT	180	53.2
Anopheles varuna	VAR	TTG ACC ACT TTC GAC GCA	260	53.7
Anopheles minimus	MIA	CCC GTG CGA CTT GAC GA	310	57.6

*The internal transcribed spacer 2 (ITS2A) is the universal primer that binds to the same position on the ITS2 DNA for all 5 species, while the specific primers (PAM to MIA) bind at different places on the ITS2 DNA of the corresponding species. bp = number base pairs; Tm = melting temperature.

Data analysis

Three key factors were chosen for statistical analysis, 1) Climatic seasons - wet period (June to October), dry period (November to February) and a hot period (March to May), 2) Collection periods - early evening (1800–2100 hr), late night (2100–2400 hr), pre-dawn (2400–0300 hr) and dawn (0300–0600 hr) and 3) Collection types - indoor HLC, outdoor HLC and cow-baited captures. The nocturnal biting activity of *An. minimus* was analyzed by mean number of landing mosquitoes captured per human each hour separated by indoor and outdoor collections and by mean number of mosquitoes captured from the CBC per hour. Comparisons of landing capture data were analyzed by a three-way analysis of variance (ANOVA), with year of collection as the blocked factor. Differences among collection groups were compared using Duncan's multiple-range test [27]. The level of significance was set at 5% (P-value < 0.05). All data were analyzed using a SAS statistical package (SAS Release 6.01, SAS Institute, Cary, NC, USA).

Correlation analysis was used to examine the relationship and estimate differences between number of mosquitoes and the independent environmental variables of ambient temperature and relative humidity by hour of collection. Correlation coefficients (r) between number of mosquitoes and hourly means for indoor temperature, outdoor temperature, and indoor humidity were based on the H_0: $r = 0$; H_1: $r \neq 0$, $r > 0$, $r < 0$. The discriminating level for significance for all correlation tests was set at 5% ($P < 0.05$). Correlation analyses were performed using a SPSS statistical program (SPSS version 15.0 Inc. Chicago, IL, U.S.A.).

Table 2 Human-landing collections of adult *Anopheles* species every 2 months from Tum Sua Village, western Thailand between November 2008 and September 2010

Year	Month	*An. minimus*	*An. dirus**	*An. maculatus**	Other *Anopheles* spp.†
One	Nov'08	608	1	84	202
	Jan'09	190	-	54	58
	Mar'09	272	-	50	90
	May'09	366	4	81	205
	Jul'09	30	3	9	269
	Sep'09	6	8	9	123
Two	Nov'09	117	-	62	21
	Jan'10	258	1	38	82
	Mar'10	429	-	23	20
	May'10	995	-	992	66
	Jul'10	303	-	84	781
	Sep'10	285	8	22	336
	Total	3,859	25	1,508	2,271

* Specific species in complex not differentiated by molecular methods.
† Other species: *Anopheles aconitus*, *Anopheles argyropus, Anopheles barbirostris, Anopheles jamesii, Anopheles karwari, Anopheles kochi, and Anopheles peditaeniatus.*

Table 3 Human-landing rates (mosquitoes/person/night) of adult *An. minimus* collected every 2 months from Tum Sua Village, western Thailand between November 2008 and September 2010

Year	Month	Indoor HLC rate per person/-night Mean(SE)	Outdoor HLC rate per person/-night Mean(SE)
One	Nov'08	41(0.60)	42.5(0.33)
	Jan'09	14.5(0.22)	11.5(0.23)
	Mar'09	16(0.31)	32.5(0.40)
	May'09	25.75(0.40)	45.25(0.70)
	Jul'09	3.75(0.09)	0(0)
	Sep'09	0.75(0.03)	0.25(0.02)
Two	Nov'09	15.75(0.30)	8(0.16)
	Jan'10	31.5(0.40)	18.25(0.27)
	Mar'10	19.25(0.30)	57.25(0.82)
	May'10	62.25(1.19)	164.5(2.31)
	Jul'10	37.25(0.69)	35.25(0.55)
	Sep'10	31.5(0.58)	31.25(0.34)
Mean landing rate per person/night:		31.07	

Results

Results of adult anopheline collections performed from November 2008 to September 2010, a total of 24 all-night collections, with matching temporal ambient air temperature, humidity and rainfall data are provided in Tables 2,3,4,5 and 6. From a total of 7,663 anopheline species collected from Tum Sua Village, 5,392 (70.37%) were members within one of 3 species complexes, representing the *An. minimus* (50.36%), *An. maculatus* (19.68%) and *An. dirus* complexes (0.33%), respectively. The remaining anopheline mosquitoes (29.63%, n = 2,271) were regarded as non-malaria vectors (Table 2).

The multiplex AS-PCR assay was used to confirm Minimus Complex species identity. Only 2 species, *An. minimus* and *Anopheles aconitus*, were identified from the collections. A total of 3,859 Minimus Complex species were collected from indoor HLC (1,197:31.02%), outdoor HLC (1,786: 46.28%) and cow baited collections (876: 22.7%). *Anopheles minimus* s.s. represented nearly all (99.8%) of the Minimus Complex members collected (n = 3,854) with only 5 (0.17%) identified as *An. aconitus*. PCR amplified species-specific products of 310 bp and180 bp can clearly separate *An. minimus* from *An. harrisoni*, respectively. *Anopheles harrisoni* was not detected during the entire study period. A total of 2,983 *An. minimus* were collected from indoor and outdoor HLC representing 24 all-night collections using 4 collectors. The total mean number of *An. minimus* per collector over the entire study period was approximately 746 or a mean of 31 landing mosquitoes per person/night. The monthly mean human-landing rates per person/night for indoor and outdoor HLC is shown in Table 3. All data collected regarding *An. minimus*, monthly total indoor and outdoor HLC, mean ambient temperature and relative humidity, and total rainfall used to describe and analyze the trophic behavior and seasonal abundance over a 23-month study is presented in Table 4.

Overall, *An. minimus* demonstrated a slight preference to feed outdoors compared with indoors and fed preferentially on humans compared to cows as offered. Fewer numbers of mosquitoes were captured from the CBC method (n = 876) than combined indoor/outdoor HLC (n = 2,983), although the mean number of mosquitoes captured per person was approximately 746 over the entire study. However, adjusting the HLC numbers to reflect that only 45 min each hour was spent collecting landing mosquitoes, the mean number of *An. minimus* per person was estimated by dividing the mean number by 0.75 to arrive at 994 mosquitoes per person. In the first year, indoor collections peaked in November, whereas two distinct peaks in outdoor activity were observed in November and May that same year (Table 3). With the highest rainfall during the entire study period occurring in July (482.5 mm), *An. minimus* was absent from all human outdoor collections and very low for indoor HLC and outdoor CBC. As the wet season continued, September recorded the lowest total of *An. minimus* for the entire study. During the second year, total outdoor collections of *An. minimus* exceeded indoor collections ($P > 0.05$). Both indoor and outdoor collections peaked in May before the onset of the wet season (Table 3).

Table 4 Total numbers of *Anopheles minimus* captured by location and host matched with contemporaneous environmental parameters from Tum Sua Village, Mae Sot District, western Thailand

Month	Year 1								Year 2								Total No. of mosquitoes
	An. minimus			T (°C)[1]		RH (%)[2]		R^3	*An. minimus*			T (°C)[1]		RH (%)[2]		R^3	
	Indoor	Outdoor	Cow	Indoor	Outdoor	Indoor	Outdoor	Total	Indoor	Outdoor	Cow	Indoor	Outdoor	Indoor	Outdoor	Total	
Nov	164	170	274	19.9	19.6	77.8	76.6	59.9	63	32	22	18.80	17.9	90.15	91.85	0	725
Dec†	-	-	-	-	-	-	-	10.4	-	-	-	-	-	-	-	0	-
Jan	58	46	86	21.55	21.6	79.9	71.55	0	126	73	59	18.30	19	88.8	89.25	11.5	448
Feb†	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	0	-
Mar	64	130	78	23.90	25.6	64.2	74.85	26.8	77	229	123	23.6	23.6	82.8	84.6	5.4	701
Apr†	-	-	-	-	-	-	-	78	-	-	-	-	-	-	-	0	-
May	103	181	82	28.85	26.6	76.45	66.35	155.6	249	658	88	23.8	23.85	93.65	92	104.1	1,361
Jun†	-	-	-	-	-	-	-	418.3	-	-	-	-	-	-	-	87.9	-
July	15	0	15	31.1	23.2	77.5	70.7	482.5	149	141	13	25.35	23.95	92.45	93.3	281.4	333
Aug†	-	-	-	-	-	-	-	272	-	-	-	-	-	-	-	368	-
Sep	3	1	2	24.25	24.5	77.4	79.95	213.9	126	125	34	18.7	23.7	94.4	97.25	182.9	291
Total	407	528	537	-	-	-	-	1,717.4	790	1,258	339	-	-	-	-	1,041.2	3,859

[1] T : Mean ambient temperature.
[2]RH: Mean percent relative humidity (%).
[3]R : Total Rainfall (mm).
† : Non-collection months.

The pattern of mean 'feeding' frequency of *An. minimus* by hour and method of collection are shown in Figures 1, 2, 3. Indoor activity patterns were relatively similar for both years (Figure 1), with greater activity occurring in the second half of the evening with varying points of peak attack. With outdoor HLC, increased activity began earlier in the evening compared to indoor biting with small peaks of activity seen from 2100–2200 and 0100–0200 hrs in the first year, and 2 higher, more notable peaks detected 2300–2400 and 0100–0200 hrs in the second year (Figure 2). For CBC, there were no demonstrable peaks seen throughout the evening although slightly more mosquitoes were caught around midnight during the first year collections, a reverse of what was recorded for outdoor HLC that same time period (Figure 3). *An. minimus* was found to be significantly more abundant in November in the first year and May in the second year (Figure 4).

Total number of landing mosquitoes per hour was analyzed by three-way ANOVA, comparing the 3 climatic seasons (wet, dry, hot), 4 time intervals (early evening, late evening, pre-dawn, and dawn) and 3 collection methods (indoor and outdoor HLC, CBC) (Table 5). There were

Table 5 Three-way ANOVA of total number of landing *An. minimus* by hour, season (dry, hot, and wet), collection method (indoor and outdoor human bait, and outdoor cattle bait) and time interval (early evening, late evening, pre-dawn, and dawn) as discriminating factors

Source	df	Sum of squares	Mean squares	*F*	*P*
Year	1	3876.042	3876.042	18.99	<0.0001
Season	2	14627.620	7313.810	35.83	<0.0001
Time period	3	6030.125	2010.042	9.85	<0.0001
Types of collection	2	5916.954	2958.477	14.49	<0.0001
Season x time period	6	3662.861	610.477	2.99	0.0083
Season x collection methods	4	12326.991	3081.747	15.10	<0.0001
Time period x collection methods	6	1529.639	254.940	1.25	0.2835
Season x Time period x collection methods	12	3463.417	288.619	1.41	0.1629

Year = 1 and 2; season = dry, hot, wet; time period = early evening, late evening, pre-dawn, dawn; types of collection = indoor HLC, outdoor HLC, cow-baited net trap.

Table 6 ANOVA between collection methods, mean ambient temperature, relative humidity and rainfall in Tum Sua Village, Mae Sot District, Thailand. ($P < 0.05$)

correlation	df	*F* value	Sig. (*P*)
Indoor HLC vs. Temp	1	1.154	0.294
Indoor HLC vs. RH	1	0.157	0.696
Indoor HLC vs. Rainfall	1	4.196	0.053
Outdoor HLC vs. Temp	1	0.960	0.338
Outdoor HLC vs. RH	1	0.063	0.804
Outdoor HLC vs. Rainfall	1	1.265	0.273
CBC vs. Temp	1	1.220	0.281
CBC vs. RH	1	0.971	0.335
CBC vs. Rainfall	1	1.908	0.181

Indoor HLC: Indoor human-landing collection.
Outdoor HLC: Outdoor human-landing collection.
CBC: Cow-baited collections.

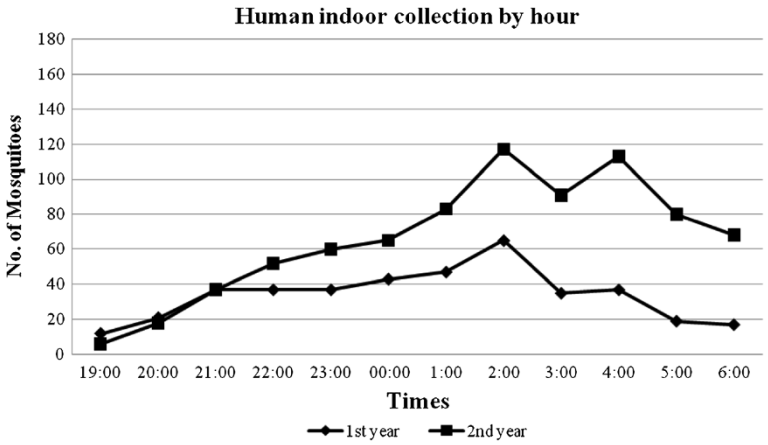


Figure 1 Hourly indoor frequency of human-landing collections of *Anopheles minimus*.

significant differences in overall numbers of *An. minimus* captured between the first year and second year ($F = 18.99$, df = 1, $P < 0.0001$), between seasons ($F = 35.83$, df = 2, $P < 0.0001$), between the 3-hr time periods ($F = 9.85$, df = 3, $P < 0.0001$) and between human and cow-baited collection methods ($F = 14.49$, df = 2, $P < 0.0001$). Analysis revealed a positive association between season and time intervals of collections ($F = 2.99$, df = 6, $P = 0.0083$) and between seasons and collection methods ($F = 15.10$, df = 4, $P < 0.0001$). There was no apparent relationship between time periods and collection method alone ($F = 1.25$, df = 6, $P = 0.2835$) or between season, time period and collection method ($F = 1.41$, df = 12, $P = 0.1629$).

No significant correlation was found between the collection procedures when using either human bait as attractant (indoor or outdoor) or the cow-bait capture method (Table 6). Likewise, there was no correlation (r = 0) found between paired collection method and environmental parameters, i.e., indoor HLC vs. temperature ($P = 0.294$), indoor vs. relative humidity ($P = 0.696$), indoor vs. rainfall ($P = 0.053$), outdoor vs. temperature ($P = 0.338$) outdoor vs. relative humidity ($P = 0.804$), outdoor vs. rainfall ($P = 0.273$), cow-bait vs. temperature ($P = 0.281$), cow bait vs. relative humidity ($P = 0.335$), and cow bait vs. rainfall ($P = 0.181$) (Table 6).

Discussion

The major objectives of the study were vector species identification, description of species diversity and abundance, and host preference/feeding behavior as background and basis for more detailed investigations to follow at the study site. Both morphological and molecular methods of anopheline species identification were used to confirm and attribute observed behavior to particular species, the focus being *An. minimus*. This knowledge is fundamental to understanding the epidemiological importance of vector species and therefore properly targeting protection methods and vector control strategies. At Tum Sua Village, the observations on the adult landing (biting) activity and host preference of *An. minimus* females covered a

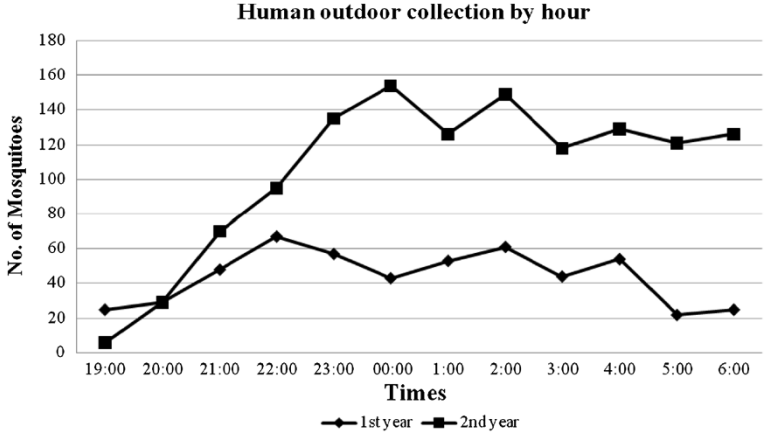


Figure 2 Hourly outdoor frequency of human-landing collections of *Anopheles minimus*.

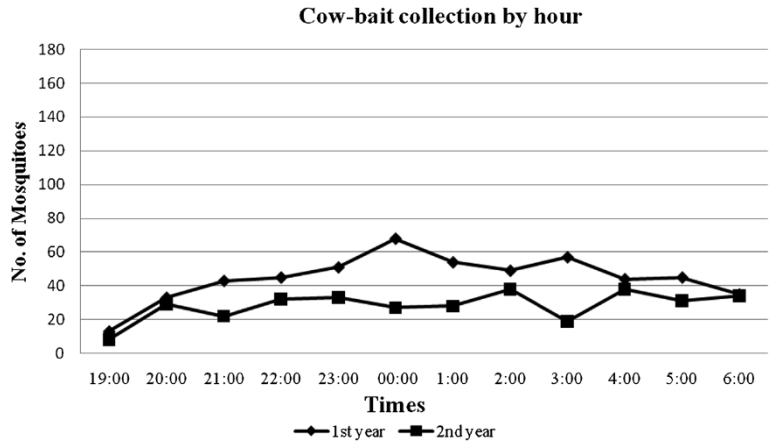


Figure 3 Hourly frequency of landing activity from cow-bait collections of *Anopheles minimus*.

23-month period of periodic sampling. Our findings showed that *An. minimus* has a more anthropophilic tendency as relatively more mosquitoes were caught on human-bait, similar in number both inside and outside of houses, than on a cow located outdoors. This study also demonstrated the impact of increased rainfall, showing a dramatic decrease in adult population numbers during the wet season compared to the dry and hot climatic periods of the year. In general, the greater the propensity to feed on humans compared to other animals is more conducive for efficient and stable malaria transmission. Because *An. minimus* was the predominant and most consistent anopheline collected at this site, malaria transmission in this area is most likely the consequence of this species. This is consistent with previous studies reporting that *An. minimus* complex play a dominant role in malaria transmission in this area of western Thailand as a consequence of relative year-round abundance and characteristics promoting efficient vectorial capacity [5,10,24,28,29].

An. minimus is one of the main malaria vectors on the Southeast Asian mainland [21,30-32]. This species is found from northern India eastwards through Vietnam and across southern China, Laos and Cambodia [7,17,33]. The *An. minimus* complex represents major malaria vectors in tropical and subtropical regions of China [6,24,34]. In northern Vietnam, this species has been shown to differ in some typical behavioral traits associated with the species elsewhere that carry important ramifications regarding malaria transmission and for application of vector control [32]. Whereas *An. minimus* is considered to be an

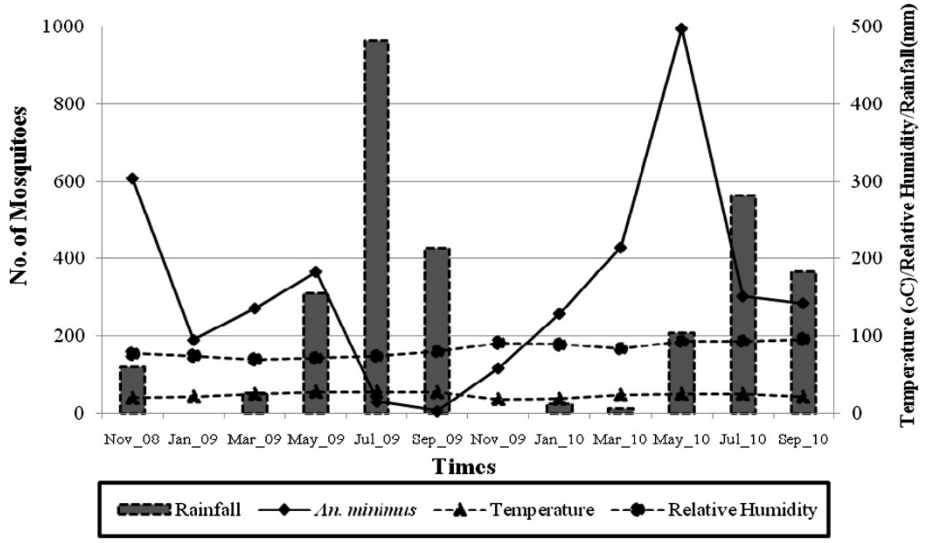


Figure 4 Monthly collection of *Anopheles minimus* in relation to average monthly ambient air temperatures, relative humidity and rainfall in Tum Sua Village, Mae Sot District, Tak Province, western Thailand.

important malaria vector in the Minimus Complex, the role of *An. harrisoni* remains unclear [11], thus the ability to clearly differentiate these 2 sibling species is critical in the interpretation of data and understanding of the role each contributes in the epidemiology of disease. Our ability to clearly distinguish these two species indicated only *An. minimus* was present during the study period. Without accurate species identification, it is extremely difficult to attribute vector capacity and transmission risk to a particular species and further complicates the need and design of target-specific prevention and control measures.

Anopheles minimus complex was the most commonly captured species throughout the study, regardless of collection method and locations, comprising 50.36% of the total anopheline species collected. Among those in the complex, 99.87% were identified as *An. minimus* with no evidence of *An. harrisoni* presence at any time during the study. This observation is consistent with previous findings from the same locality based on adult morphological identification only [20,35,36]. These findings also closely correspond to a larval distribution study by Kongmee *et al.* [37] conducted in the same village. Although a number of studies have reported on female host preference, mosquito density, biting frequency, and behavioral responses to chemicals of *An. minimus* s.l., in only a few exceptions have species-specific identifications been verifiable and confirmed by molecular methods. This is important as many malaria vectors, including *An. minimus* s.l. display a diverse array (plasticity) of host-seeking behaviors, preferences, and larval breeding habitats than commonly assumed [28,29,38]. For example, Harbach *et al.* [20] observed a single feeding peak for *An. minimus* s.l., between 2100 and 2200 hr, whereas, Ratanatham *et al.* [21] reported 2 clear and consistent feeding peaks in biting density, one in the early evening (1900–2200 hr) followed by a second peak before dawn (0500–0600 hr). Rattanarithikul *et al.* [22] reported two prolonged biting periods, the first occurring between 1800 and 2300 hrs and a second, more moderate peak beginning at 0100 hr with a progressive decline in activity throughout the second half of the night.

In recent years, a molecular identification assay has been used to accurately describe the trophic behavior and biting activity of two sibling species within the Minimus Complex from Kanchanaburi Province. Unfortunately, the number of *An. minimus* obtained by Sungvornyothin *et al.* [11] was too low to observe clear biting activity patterns. In our study, indoor human landing by *An. minimus* were pronounced in the early morning hours between 0100 and 0400 hrs with a peak at 0200 hr, whereas an outdoor feeding surge began around 2200 hr, reaching a peak near midnight followed by a gradual decline throughout the second half of the evening. *Anopheles minimus* showed a slight predilection to feed more outdoors than indoors, indicating both exo- and endophagic behaviors. Additionally, a greater number of *An. minimus* were collected from human bait (indoor and outdoor) compared to cow bait, suggesting a more marked anthropophilic behavior by this species.

A significantly greater number of *An. minimus* were collected during the second year of observation compared to the first. The reasons for this difference in adult densities is unclear but is likely related to the differences in local environmental and climatic factors between collection periods and/or by the occurrence of more suitable and productive breeding habitats during the second year. In this study, rainfall appeared to have a profound influence on adult vector densities. This was very evident in the first half of the study where the lowest HLC numbers occurred during the wet months of the year (June-October) but regained adult densities immediately following the rains and into the drier and warmer periods of the second half. During the wet months of the first half of the study, particularly heavy rains occurred resulting in a 51 percent higher precipitation (1,386.7 versus 920.2 mm) than the same period of the second half of the study. As the typical larval habitats of *An. minimus* are associated with small running streams, the heavy and more frequent rainfall would have produced more adverse conditions due to flushing. In addition, higher mean relative humidity was also observed in the second year that may have favored increased vector longevity and thus greater likelihood of human-vector contact over time. A notable reduction in organized vector control measures in the area may also have allowed vector densities to increase more easily. Recently, the Thai Government has encouraged the local community to convert their land to planting rubber trees for greater income generation and may have influenced the population density of *An. minimus*. For example, *An. minimus* populations have decreased significantly in peninsular/southern Thailand following extensive ecological/environmental changes resulting from deforestation and increased urbanization [39]. Recently, *An. minimus* at Pu Teuy Village in Kanchanaburi Province has nearly disappeared, possibly the result of rapid environmental changes and increased agricultural development in the area [11].

This study had several limitations. It was difficult to measure with accuracy the host preference of the population, as the methods used may not have been as comparable as desired. For instance, were four humans the near equivalent of one cow? We presume that the specimens collected from the cow trap represented a full hour of attracted mosquitoes despite the actual collection time by the collectors was only 15 min each hour. Once the mosquitoes entered inside the net, they most likely remained inside regardless of whether or not they blood fed. On the other hand, the HLC took place for only 45 min per hour, thus we adjusted the number of actual landing mosquitoes to reflect the potential mosquitoes missed during those 15 min breaks each hour (assuming

a relative even distribution of landing activity over the entire hour). Secondly, no effort was made to sample surrounding larval habitats concurrently with adult collections in attempt to link larval presence with adult densities. Thirdly, environmental measures (indoor and outdoor air temperature and RH) were routinely recorded each collection period; however, rainfall data was gathered at a station approximately 18 km away from the study site. Although not ideal because of the possibility of varying and patchy rainfall distribution patterns, we believe the relative amount of rainfall per period is still reflective of the relative amounts of rainfall and general seasonality at the study site. Lastly, this study was not an all-inclusive investigation about the vector biology of this species; neither age-grading, (parity) nor determination of malaria infection in *An. minimus* was examined as part of the baseline design. Therefore, we have no information of the change in age structure over the 23-month period or the number of mosquitoes that may have been harboring malaria parasites. Parity (age-grading) has been reported previously for *An. minimus* elsewhere in Thailand [22,40-42]. Despite the above reservations, the study provided clear evidence of the public health importance of this species. Coupled with numerous instances of confirmed vector involvement in western Thailand, our findings reinforce the conclusion regards the primary vector status of *An. minimus* in Tum Sua Village.

The Communicable Disease Control section of the MOPH has described Tum Sua Village as a perennial transmission area, albeit one showing seasonal fluctuations and with generally low transmission most of the year. *Anopheles minimus* has been regarded as the main malaria vector in this area for many decades [5,10]. Our study supports that conclusion based on biting behavior and host preferences compared to other potential vector species present. Vector control using residual application of insecticides remains on the forefront in the fight against malaria at Tum Sua Village. Since 1994, deltamethrin has been the primary insecticide of choice in the National Malaria Control Program [43]. Despite the many years of IRS in the village, the response of *An. minimus* to deltamethrin in Tum Sua is not known and will be the focus of future study.

Conclusions

As *An. minimus* has shown a propensity to feed on humans and indoors, both in substantial numbers and in proportion to outdoor feeding, these findings can help explain the apparent effectiveness and strategy of using IRS for the suppression of malaria transmission indoors by this species. This would also support use of long-lasting insecticide-treated materials to protect people from malaria inside the household. These observations also indicate a high 'residual' transmission potential outdoors where direct control measures are lacking or insufficient. We conclude that the study of vector population behavior is crucial and highly beneficial for a better understanding of the epidemiological factors associated with promoting malaria transmission in a specific setting and in selecting the most appropriate vector control strategy for maximum cost-benefit.

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

All the authors have contributed significantly to this study. TC and MJB contributed to the conceptualization and design of the study. RT, CT and TC did the laboratory and field studies. RT and WJ carry out the molecular genetic studies. RT, MJB, ST, TC and VC prepared and revised the manuscript. All authors read and approved the final manuscript.

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Assessment of geraniol-incorporated polymers to control *Aedes albopictus* (Diptera: Culicidae)

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Summary:

Effective control of mosquito borne diseases has proven extremely difficult with both vector and pathogen remaining entrenched and expanding in many disease endemic areas. When lacking an effective vaccine, vector control methods targeting both larval habitats and adult mosquito populations remain the primary strategy for reducing risk. *Aedes albopictus* from Thailand was used as a reference baseline for evaluation of natural insecticides incorporated in polymer disks and pellets and tested both in laboratory and field conditions. In laboratory and field tests, the highest larval mortality was obtained with disks or pellets containing IKHC (Insect Killer Highly Concentrate) from Fulltec AG Company. This product is reputed to contain geraniol as an active ingredient. With pellets, high mortality of *Ae. albopictus* larvae (92 %) was observed in presence of 1 g of pellets per 500 ml of water at day 1st, and the mortality was 100 % at day 1st for larvae in presence of 5 or 10 g of pellets. Fulltec AG Company has not accepted to give us the exact composition of their IKHC product. Therefore, we cannot recommend it, but the principle of using monoterpenes like geraniol, incorporated into polymer disks or pellets as natural larvicide needs more attention as it could be considered as a powerful alternative in mosquito vector control.

KEY WORDS: *Aedes albopictus*, mosquito vector, larvicide, monoterpene, natural product, vector control.

Résumé : Évaluation du contrôle d'*Aedes albopictus* (Diptera : Culicidae) à l'aide de polymères incorporés de géraniol

Le contrôle effectif des maladies à transmission vectorielle est très difficile, les vecteurs et les pathogènes se maintenant souvent en position retranchée et se développant dans de nombreux foyers endémiques. En l'absence de vaccin efficace, les méthodes de lutte antivectorielle ciblant à la fois les habitats larvaires et les populations d'adultes restent la stratégie de choix pour réduire le risque. Aedes albopictus *en Thaïlande a été choisi pour des tests d'évaluation d'insecticides naturels incorporés dans des disques ou des granulés de polymères, à la fois au laboratoire et sur le terrain. Dans tous les tests, la mortalité larvaire la plus élevée a été obtenue avec les disques et les granulés contenant de l'IKHC (*Insect Killer Highly Concentrate*) de la compagnie Fulltec AG. Ce produit est réputé contenir du géraniol comme matière active. Avec les granulés, une mortalité de 92 % a été observée en présence d'un gramme de granulés pour 500 ml d'eau dès le premier jour, et cette mortalité était de 100 % en présence de 5 et 10 g de granulés dès le premier jour. La compagnie Fulltec AG n'ayant pas accepté de nous donner la composition exacte de leur IKHC, nous ne pouvons recommander ce produit. Mais le principe d'utiliser des monoterpènes incorporés dans des disques ou des granulés de polymères, comme traitement larvicide, nécessite une grande attention, car cela pourrait devenir une méthode alternative efficace de contrôle des moustiques.*

MOTS-CLÉS : Aedes albopictus*, vecteur, larvicide, monoterpène, produit naturel, lutte antivectorielle.*

Since the implementation of the European directive 98/8/EC, known as the "biocide directive", most of the old effective, inexpensive but polluting biocides used for vector control (Rozendaal, 1997) have been withdrawn from the European market. Now, assessment of unwanted side-effects is compulsory for any active biocide substance and, eventually, for any formulated biocide product that contains it, prior to acquiring marketing authorization. Considerations of cost, acceptability, safety and, more recently, respect for the environment, have led companies, research institutes and international agencies to offer new compounds such as insecticides of biological origin, like *Bacillus thuringiensis* ser. *israelensis* (Bti), *Bacillus sphaericus*, Spinosad and growth regulators mimicking insect hormones (Fontenille *et al.*, 2009). Certain active pyrethroid substances show the advantage of being active at low doses and having low persistence (no residual effect), but have low selectivity. Some trials are also under way on natural substances such as essential oils or plant extracts, like geraniol (Khallaayoune *et al.*, 2009; George *et al.*, 2009) or other monoterpenes. Most compounds used for vector control are implemented by trained specialized teams, but after the epidemics of chikungunya in La Reunion Island (2005-2006) and dengue in Martinique and Guadeloupe, personal protection against vectors (PPAV) revealed to be also very important (PPAV Working Group, 2011). The main dengue and chikungunya vectors like *Aedes aegypti*, *Ae. albopictus* (Guzman *et al.*, 2010) and also nuisance caused by *Culex quinquefasciatus*, could be partly controlled by mechanical

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elimination of breeding sites around houses and in gardens, and/or by larvicide treatment and spatial spraying with insecticides. To implement a larvicide treatment by people, some companies are developing monoterpenes-incorporated polymer disks or pellets to be used in gardens or terraces. The efficiency of some of these products has been assessed in Thailand at the Entomology Research Laboratory of Kasetsart University, Bangkok, in laboratory conditions where mosquito colonies are maintained, as well as in field conditions where *Ae. albopictus* is prevalent.

MATERIALS

Aedes albopictus colony

Ae. albopictus MOPH colony (Thailand) was used as a reference baseline for these tests. This colony was originally collected from Nonthaburi Province and has been maintained in a colony at Vector Borne Disease Bureau, Disease Control Deparment, Ministry of Public Health (MOPH, Thailand) for approximately ten years (Chuaycharoensuk *et al.*, 2011). Mosquito colony was carefully segregated to prevent contamination from cross-mating and reared under identical laboratory-controlled condition at 25 ± 5 °C and relative humidity 80 ± 10 % in the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand (Kongmee *et al.*, 2004). Adult mosquitoes were allowed to mate and females permitted to blood feed on restrained live guinea-pigs beginning on the fourth day post-emergence. Moistened oviposition substrates were provided to the females for depositing their eggs. Following conditioning, eggs were re-hydrated and allowed to hatch in pans reserved for each strain. In the present experiment, only stage 3 mosquito larvae were available in sufficient numbers at the time of the test and used.

Geraniol incorporated disks and pellets

Polymer disks and pellets were sent to us under sealed aluminium bags by Valcoplast SA (4, Avenue Bordaberri, 64990 Mouguerre, France) without any indication on their composition for evaluation in blind conditions. The composition was revealed after the tests were done. The disks were made of polymer with incorporation of different products (Table I). IKHC was presented by Fulltec AG (Zug, Switzerland) as a watery concentrate with geraniol (CAS 106-24-1) from natural origin as an active ingredient. Geraniol is extracted by chromatography from palmarosa essential oil (Java Lemongrass, *Cymbopogon martinii*). The final concentration of the products incorporated in the polymer disks was around 10 %. The pellets of 0.5 cm of diameter were made of a mixture of polymer and wood fibers impregnated with IKHC.

METHODS

In the laboratory

Stage 3 mosquito larvae were tested in plastic saucers. In the Experiment A, 50 larvae, 500 ml of filtered water, two tablets of fish food and one polymer disk were added in each saucer. In this experiment, three replicates were done for each of the polymer disk lot. The number of dead larvae was counted every day during one week (day 6 for practical reasons). Afterwards, the polymer disks were kept dry at room temperature and experiment was repeated after one and two months to evaluate the remanence of the effect. A similar experiment (Experiment B) was performed at the same time with polymer disks replaced by IKHC incorporated pellets. One, two or five grams of pellets were added to each saucer containing 500 ml of filtered water. As in Experiment A, 50 stage 3 larvae and two tablets of fish food were added in each saucer. A saucer without pellets was incorporated as a control. Two replicates were done. The number of dead larvae was counted every day until all larvae were dead in all saucers with pellets.

In the field

In this experiment saucers were placed in the garden near the laboratory and protected from rain (Experiment C). Wild populations of *Ae. albopictus* are abundant in this area. The saucers were filled in with 500 ml of filtered water and two tablets of fish food. There were two replicates for each polymer disk lot. The presence of new emerged larvae was checked

		Fulltec AG Company, Switzerland		Penta Manufacturing Company, USA
Disks		**IKHC***	**Synthetic geraniol**	**Synthetic geraniol**
1903/1	Control			
1903/2			100 %	
1903/3				100 %
1903/4		95 %	5 %	
1903/5		95 %		5 %
1903/6		50 %	50 %	
"Juillet2010"		100 %		

* Insect Killer Highly Concentrate G.

Table I. – Details on the products incorporated into the disks.

until Day 18 (D18). This experiment was also repeated after one and two months like Experiment A.

RESULTS

For Experiments A and B, results are expressed as larval reduction rates of stage 3 *Ae. albopictus* for each of the polymer disk or pellet lots, with indication of standard deviation. For Experiment C, results are expressed as presence and abundance of larvae after exposure of the saucers in the garden.

In Experiment A (Table II), a very low larval mortality was noticed in lot 1903/1 (control) until D6. A very low mortality was also observed for lots 1903/2 and /3 with 2.7 % and 4.7 % respectively at D6. On the opposite, a strong mortality was observed with lots 1903/4, /5 and "Juillet2010" with 99 %, 94 % and 100 % respectively at D3 and 100 % afterwards. For lot 1903/6, the mortality was low until D3 and increased dramatically onwards to 50 % at D4 and 91 % at D6.

The results of the same experiment done one month after the previous test, using the same polymer disks that were washed and kept dry at room temperature (Table II bis) showed a very low mortality (below

		Different polymer disks						
	Dates	**1903/1***	**1903/2**	**1903/3**	**1903/4**	**1903/5**	**1903/6**	**"Juillet2010"**
D0	05/04/2011	0 ± 0**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
D1	06/04/2011	0.7 ± 1.2	0 ± 0	0 ± 0	83 ± 29	70 ± 20	0 ± 0	97 ± 5.8
D2	07/04/2011	2 ± 2	0 ± 0	2.7 ± 4.6	96 ± 6.9	89 ± 9.9	4.7 ± 4.6	96 ± 4
D3	08/04/2011	2 ± 2	0.7 ± 1.2	2.7 ± 4.6	99 ± 1.2	94 ± 8.7	4.7 ± 4.6	100 ± 0
D4	09/04/2011	2 ± 2	0.7 ± 1.2	2.7 ± 4.6	100 ± 0	100 ± 0	50 ± 38	100 ± 0
D5	10/04/2011	ND	ND	ND	ND	ND	ND	ND
D6	11/04/2011	2 ± 2	2.7 ± 2.3	4.7 ± 5	100 ± 0	100 ± 0	91 ± 12	100 ± 0

* See text for the identification of polymer disks. ** All figures are means of triplicates ± SD. ND: not done.

Table II. – Experiment A: larval reduction rates (%) of *Ae. albopictus* in presence of geraniol impregnated polymer disks.

		Different polymer disks						
	Dates	**1903/1**	**1903/2**	**1903/3**	**1903/4**	**1903/5**	**1903/6**	**"Juillet2010"**
D0	07/05/2011	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
D1	08/05/2011	0 ± 0	0.7 ± 1.2	0.7 ± 1.2	2 ± 3.5	3.3 ± 5.8	0 ± 0	8.7 ± 1.2
D2	09/05/2011	0 ± 0	0.7 ± 1.2	1.3 ± 1.2	4.7 ± 3.1	6 ± 5.3	2 ± 2	11 ± 3.1
D3	10/05/2011	0 ± 0	0.7 ± 1.2	1.3 ± 1.2	5.3 ± 2.3	8.7 ± 3.1	2.7 ± 1.2	13 ± 3.1
D4	11/05/2011	0.7 ± 1.2	0.7 ± 1.2	1.3 ± 1.2	8.7 ± 3.1	9.3 ± 2.3	4 ± 2	15 ± 1.2
D5	12/05/2011	0.7 ± 1.2	0.7 ± 1.2	1.3 ± 1.2	8.7 ± 3.1	11 ± 3.1	4 ± 2	18 ± 3.5
D6	13/05/2011	0.7 ± 1.2	0.7 ± 1.2	2 ± 0	11 ± 3.1	14 ± 2	4.7 ± 1.2	19 ± 4.2

Table II bis. – Experiment A: larval reduction rates (%) of *Ae. albopictus* with the same geraniol impregnated polymer disks. 1 month after the previous test.

		Different polymer disks						
	Dates	**1903/1**	**1903/2**	**1903/3**	**1903/4**	**1903/5**	**1903/6**	**"Juillet2010"**
D0	05/06/2011	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0 ± 0	0 ± 0
D1	06/06/2011	0 ± 0	0 ± 0	0 ± 0	2 ± 3.5	2 ± 3.5	0 ± 0	8.7 ± 1.2
D2	07/06/2011	0 ± 0	0 ± 0	0 ± 0	4.7 ± 3.1	4.7 ± 3.1	2 ± 2	11 ± 3.1
D3	08/06/2011	0 ± 0	0.7 ± 1.2	0 ± 0	5.3 ± 2.3	8.7 ± 3.1	2.7 ± 1.2	13 ± 3.1
D4	09/06/2011	0.7 ± 1.2	0.7 ± 1.2	1.3 ± 1.2	8.7 ± 3.1	11 ± 3.1	6.7 ± 1.2	15 ± 1.2
D5	10/06/2011	0.7 ± 1.2	0.7 ± 1.2	2.7 ± 1.2	11 ± 3.1	14 ± 2	9.3 ± 1.2	18 ± 3.5
D6	11/06/2011	0.7 ± 1.2	1.3 ± 1.2	2.7 ± 1.2	14 ± 2	22 ± 2	13 ± 3.1	19 ± 4.2

Table II ter. – Experiment A: Larval reduction rates (%) of *Ae. albopictus* with the same geraniol impregnated polymer disks. two months after the first test.

5 %) in lots 1903/1 (control), /2, /3 and /6; and a low mortality of 11 %, 14 % and 19 % for lots 1903/4, /5 and "Juillet2010" respectively at D6. The same experiment done two months later gave similar results (Table II ter) with a very low mortality (below 3 %) in lots 1903/1 (control), /2, /3; and a low mortality of 14 %, 22 %, 13 %, and 19 % for lots 1903/4, /5, /6 and "Juillet2010" respectively at D6.

In Experiment B (Table III), there was a very low mortality (2 % at D3) in the control saucer (without any pellet), but a very high mortality in presence of pellets. With 1 g of pellets in the saucers, the larval mortality was 92 % at D1, 98 % at D2 and 100 % at D3. With 5 and 10 g of pellets, the mortality was 100 % as soon as D1. One and two months later (Tables III bis, III ter), the mortality stays quite high with 84 % and 83 % at D1 for 1g pellets, and 100 % at D1 for both 5 g and 10 g pellets.

Dates	Different mass of geraniol impregnated pellets			
	Control	1 g	5 g	10 g
D0 05/04/2011	0 ± 0*	0 ± 0	0 ± 0	0 ± 0
D1 06/04/2011	0 ± 0	92 ± 1.4	100 ± 0	100 ± 0
D2 07/04/2011	0 ± 0	98 ± 1.4	100 ± 0	100 ± 0
D3 08/04/2011	2 ± 0	100 ± 0	100 ± 0	100 ± 0

* All figures are means of duplicates (± SD).

Table III. – Experiment B: larval reduction rates (%) of *Ae. albopictus* in presence of geraniol impregnated pellets.

Dates	Different mass of geraniol impregnated pellets			
	Control	1 g	5 g	10 g
D0 07/05/2011	0 ± 0	0 ± 0	0 ± 0	0 ± 0
D1 08/05/2011	0 ± 0	84 ± 1.4	100 ± 0	100 ± 0
D2 09/05/2011	0 ± 0	93 ± 0.7	100 ± 0	100 ± 0
D3 10/05/2011	0 ± 0	100 ± 0	100 ± 0	100 ± 0

Table III bis. – Experiment B: larval reduction rates (%) of *Ae. albopictus* in presence of geraniol impregnated pellets, one month after previous test.

Dates	Different mass of geraniol impregnated pellets			
	Control	1 g	5 g	10 g
D0 07/05/2011	0 ± 0	0 ± 0	0 ± 0	0 ± 0
D1 08/05/2011	0 ± 0	83 ± 4.2	100 ± 0	100 ± 0
D2 09/05/2011	0 ± 0	89 ± 1.4	100 ± 0	100 ± 0
D3 10/05/2011	0 ± 0	96 ± 2.8	100 ± 0	100 ± 0
D4 09/04/2011	0 ± 0	100 ± 0	100 ± 0	100 ± 0

Table III ter. – Experiment B: larval reduction rates (%) of *Ae. albopictus* in presence of geraniol impregnated pellets, two months after first test.

In Experiment C (Table IV), young larvae of *Ae. albopictus* were found in the control saucer (with polymer disk 1903/1) at D6 and their number increased onward. The same was found for lots 1903/2 and /3, for which larvae were found as early as D9. For lot 1903/6, larvae were found at D15 onward, but no larva was found in lots 1903/4, /5 and "Juillet2010". One month later (Table IV bis), young larvae of *Ae. albopictus* were found in the control saucer (with polymer disk 1903/1) at D15 and increased, as well as in saucers of lots 1903/3 and /6. Larvae were found earlier for lot1903/2 at D12 and later for lot 1903/4 at D18. And no larva was found in lots 1903/5 and "Juillet2010". Two months later (Table IV ter), young larvae of *Ae. albopictus* were found in the control saucer (with polymer disk 1903/1) and 1903/2 at D9. For lots 1903/3 and /6 larvae were found at D12, for lot 1903/4 at D15, and for lot 1903/5 at D18. No larva was found in lot "Juillet2010".

DISCUSSION AND CONCLUSION

Polymer disks or pellets, impregnated with different geraniol based products, were tested in laboratory and in open field conditions. The main objective of this study was to check if those products could be used on an individual basis to control *Ae. albopictus* or other anthropophilic mosquito populations in gardens or terraces.

The *Ae. albopictus* MOPH colony of the Department of Entomology (Faculty of Agriculture, Kasetsart University, Bangkok, Thailand) was used for laboratory tests where stage 3 larval reduction rates were calculated using different polymer disks or pellets in saucers.

For all tests, the mortality in the controls was very low, thus indicating that colonized mosquitoes used in this study were healthy at time of testing.

With the polymer disks in saucers in presence of 50 stage 3 larvae, we observed a strong mortality with disks impregnated with IKHC. This mortality was maximum with disks lot named "Juillet2010" impregnated with IKHC only, reaching 100 % from D3 onwards. The mortality decreases when IKHC is diluted with different synthetic geraniol. With pure synthetic geraniol (lots 1903/2 and /3), we observed a very low mortality. That means that the geraniol alone is probably not the active larvicide ingredient of IKHC. This test has been repeated after one and two months, using the same disks kept dry at room temperature. The larval reduction rate decreased dramatically from the first month, even with lot "Juillet2010" where the mortality dropped from 100 % to 19 % on day 6. That shows that the disks could not be used several times.

		Different polymer disks											
			1903/2		1903/3		1903/4		1903/5		1903/6	"Juillet2010"	
	Dates	1903/1	a*	b*	a	b	a	b	a	b	a	b	a	b
D0	05/04/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D3	08/04/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D6	11/04/2011	+++	0	0	0	0	0	0	0	0	0	0	0	0
D9	14/04/2011	++++	0	+++	0	+	0	0	0	0	0	0	0	0
D12	17/04/2011	+++++	0	++++	0	++	0	0	0	0	0	0	0	0
D15	20/04/2011	++++++	0	++++	++	+++	0	0	0	0	++	++	0	0
D18	23/04/2011	++++++	++	++++	+++	+++	0	0	0	0	+++	+++	0	0

* a and b are duplicates with similar polymer disks. +: 1-5 larvae; ++: 6-10 larvae; +++: 11-15 larvae; ++++: 16-20 larvae; +++++: 21-25 larvae; ++++++: 26-30 larvae.

Table IV. – Experiment C: presence and abundance of *Ae. albopictus* larvae in saucers with different geraniol impregnated polymer disks in an infested area.

		Different polymer disks											
			1903/2		1903/3		1903/4		1903/5		1903/6	"Juillet2010"	
	Dates	1903/1	a	b	a	b	a	b	a	b	a	b	a	b
D0	07/05/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D3	10/05/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D6	13/05/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D9	16/05/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D12	19/05/2011	0	0	++++	0	0	0	0	0	0	0	0	0	0
D15	22/05/2011	+++	++	+++++	++	+++	0	0	0	0	++++	+++	0	0
D18	25/05/2011	++++++	+++	++++++	++++	++++	++	0	0	0	+++++	++++	0	0

Table IV bis. – Experiment C: presence and abundance of *Ae. albopictus* larvae in saucers with different geraniol impregnated polymer disks in an infested area, one month after the previous test using the same disks.

		Different polymer disks											
			1903/2		1903/3		1903/4		1903/5		1903/6	"Juillet2010"	
	Dates	1903/1	a	b	a	b	a	b	a	b	a	b	a	b
D0	05/06/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D3	08/06/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D6	11/06/2011	0	0	0	0	0	0	0	0	0	0	0	0	0
D9	14/06/2011	+	0	+	0	0	0	0	0	0	0	0	0	0
D12	17/06/2011	++	+	+++	0	++	0	0	0	0	++	+	0	0
D15	20/06/2011	++++	+++	++++	++	+++	+	0	0	0	++++	+++	0	0
D18	23/06/2011	++++++	+++++	+++++	++++	+++++	++	++	+	+	++++++	+++++	0	0

Table IV ter. – Experiment C: presence and abundance of *Ae. albopictus* larvae in saucers with different geraniol impregnated polymer disks in an infested area, two months after the previous test using the same disks.

However, the duration of the effect during the first test (Table II) has not been evaluated beyond six days.

The efficiency of the IKHC is confirmed in experiment B using pellets. The mortality reached 100 % with 1 g of pellets in 500 ml of water at D3 (Table III) and this efficiency was conserved with pellets kept at room temperature in their original bag after one and two months (Tables III bis and III ter). The duration of the effect still needs to be evaluated to determine the frequency of use of pellets in saucers or different water collections in gardens.

The Experiment C was conducted in open field, in an area with wild *Ae. albopictus* populations, and it shows again a high efficiency of IKHC impregnated disks. In the control saucers, we observed new larvae at D6, D15 and D9 during the first, second and third

tests respectively (Tables IV, IV bis and IV ter). In the saucers with IKHC impregnated disks, no larva was observed until the end of the test at D18 in all 3 tests for lot "Juillet2010" (Tables IV, IV bis and IV ter). There are two possibilities for this absence of larvae during the experiment; either the product is a repellent for females *Ae. albopictus* avoiding egg-laying in those saucers, or the product is also ovicide and all eggs have been killed before the emergence of larvae. These two hypotheses need now to be checked.

Finally, these polymer disks or pellets impregnated with natural products with insecticide activity could be an efficient tool to control mosquito populations in gardens, terraces or greenhouses. The pellets made of polymer and wood fibers seem especially practical for an individual usage. Our results showed that IKHC presents a very strong larvicide activity and, on the contrary, synthetic geraniol has no larvicide activity. As we do not know the active ingredient(s) of this product because the manufacturer refused to give us the complete and exact composition of the product, we therefore cannot recommend it for the moment. The way is opened to discover a natural product, probably a mixture of monoterpenes with larvicide activity which could be used with those slow release polymers or wood fiber systems. The advantage of using natural product against mosquito vectors is that they do not develop resistance and they are non-toxic to fishes, mammals and most other non-target organisms in the environment. As larvicide in mosquito control, insect growth regulator, biopesticides (Rozendaal, 1997), essential oil and natural product like mixture of monoterpenes (Ben Marzoug *et al.*, 2011; Guerrini *et al.*, 2011; Mkaddem *et al.*, 2009; Sacchetti *et al.*, 2004) represent alternative products for mosquito vector control.

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DIVERSITY OF *STOMOXYS* SPP. (DIPTERA: MUSCIDAE) AND DIURNAL VARIATIONS OF ACTIVITY OF *STOMOXYS INDICUS* AND *S. CALCITRANS* IN A FARM, IN WANG NAM KHIAO DISTRICT, NAKHON RATCHASIMA PROVINCE, THAILAND

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Summary:

A study of species diversity of *Stomoxys* spp. and diurnal variations of activity of the most abundant was performed during a one year period at a local dairy cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand. Four species of stomoxyine flies were morphologically identified, including *Stomoxys indicus* Picard 1908, *S. calcitrans* (Linnaeus 1758), *S. sitiens* Rondani 1873 and *S. uruma* Shinonaga and Kanao 1966. The most common species were *S. indicus* (50.2 %) and *S. calcitrans* (49.5 %). *S. sitiens* and *S. uruma* were found in small proportions (< 1 %). The number of flies captured was significantly different among the three seasons with the greatest number in the rainy season (mean = 66 %; df = 2, P < 0.05). The variations of diurnal activity were observed during different period of times (06:00 to 18:00) during three seasons. Both sexes of *S. indicus* and males of *S. calcitrans* showed unimodal activity pattern in cool and summer seasons. But a bimodal activity pattern was recorded in rainy season. For females *S. calcitrans*, a unimodal peak of activity was observed in cool season and a constant variation of activity all along the day in summer and rainy seasons, with an increase from the morning to the evening. A better understanding of stomoxyine fly behavior, especially the daily flight activity, can assist in prioritization and design of appropriate vector prevention and control strategies.

KEY WORDS: *Stomoxys* spp., stable fly, diurnal variation of activity, Vavoua trap, Thailand.

Résumé : DIVERSITÉ DES ESPÈCES DE *STOMOXYS* (DIPTERA : MUSCIDAE) ET OBSERVATION DES VARIATIONS DE L'ACTIVITÉ DIURNE DE *STOMOXYS INDICUS* ET *S. CALCITRANS* DANS UNE FERME DU DISTRICT DE WANG NAM KHIAO, PROVINCE DE NAKHON RATCHASIMA, THAÏLANDE

Une étude de la diversité des espèces de Stomoxys *(Diptera : Muscidae) et des variations de l'activité diurne des deux espèces les plus abondantes a été menée pendant un an dans une ferme laitière du district de Wang Nam Khiao, Province de Nakhon Ratchasima, en Thaïlande. Quatre espèces ont été identifiées morphologiquement :* Stomoxys indicus *Picard 1908,* S. calcitrans *(Linnaeus 1758),* S. sitiens *Rondani 1873 et* S. uruma *Shinonaga et Kanao 1966. Les plus abondantes ont été* S. indicus *(50,2 %) et* S. calcitrans *(49,5 %).* S. sitiens *et* S. uruma *ont été trouvés en faible proportion (< 1 %). Le nombre de mouches capturées était significativement différent suivant les trois saisons, avec le nombre le plus important en saison des pluies (moyenne : 66 % ; ddl : 2 ; P < 0,05). Les variations diurnes du cycle d'activité ont été observées de 6 h 00 à 18 h 00 pendant les trois saisons. Les deux sexes de* S. indicus *et les mâles de* S. calcitrans *ont montré une activité unimodale en saison froide et en été, mais une activité bimodale en saison des pluies. Pour les femelles de* S. calcitrans*, une activité unimodale a été observée en saison froide, mais l'activité a été constante tout au long de la journée, avec une augmentation du matin au soir, en été et en saison des pluies. Une meilleure compréhension du comportement de ces mouches, spécialement leur cycle d'activité diurne, pourrait faciliter le développement de méthodes de prévention et de contrôle plus efficaces.*

MOTS-CLÉS : Stomoxys *spp., mouche des étables, cycle d'activité diurne, piège Vavoua, Thaïlande.*

Stomoxyine flies are blood-sucking Diptera belonging to genus *Stomoxys* (Diptera: Muscidae), which contains eighteen different species in the world (Zumpt, 1973). One of these, *Stomoxys calcitrans* (Linnaeus 1758), normally referred as "stable fly", is the most cosmopolitan species and a significant economic pest of livestock and other warm-blooded animals in many parts of the world (Zumpt, 1973; Greenberg, 1971; Harwood & James, 1979; Mullens *et al.*, 1988). Both male and female stable fly feed primarily on a wide vertebrate host range (Wall & Shearer, 1997). In the United Kingdom, *S. calcitrans* preferred to feed primarily on cattle and horses (Warnes & Finlayson, 1987). In Egypt, domestic donkeys and horses remain the most preferred vertebrate hosts (Hafez & Gamal-Eddin, 1959). Although livestock is a major blood source, humans can also be bitten by this species. When under mass attack of stable fly, significant economic losses due to reduction of anticipated gross weight gain and 30-40 % decrease in milk yields have been observed (Hall *et al.*, 1982; Mullens *et al.*, 1988). Campbell *et al.* (2001) reported weigh gains by grazing cattle were reduced an average of 0.20 kg per steer per day by an average of 2.79 flies per leg, representing a 19 % reduction in weight gain or 7 % per stable fly. In addition, stable flies have been known as mechanical vector for several pathogens such as

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Anaplasma marginale (anaplasmosis), *Trypanosoma* spp. (trypanosomosis) as well as different viruses, including bovine leucosis virus, lumpy skin disease virus (Mihok *et al.*, 1995; Torr *et al.*, 2006).

Surveys of adult stomoxyine fly populations can be assessed by different techniques. Various trapping devices have been developed to collect flies. In the United States, sticky traps (Broce trap and William trap) are commonly used for sampling stomoxyine flies (Williams, 1973; Broce, 1988). Recently, a friendlier field trap device, originally designed for tsetse fly collection in Africa, was used for stomoxyine fly collection. This "Vavoua trap", whose name is derived from the name of an African village, has been proved to be a very efficient way to sample *Stomoxys* spp. in many African countries (Holloway & Phelps, 1991; Mihok *et al.*,1995; Mihok & Clausen, 1996), in La Réunion Island (Gilles *et al.*, 2007a) and in Thailand (Tainchum *et al.*, 2010, Muenworn *et al.*, 2010).

In Thailand, five species of stomoxyine flies are known (Zumpt, 1973; Masmeatathip *et al.*, 2006; Muenworn *et al.*, 2010). The presence of *S. pullus* is suspected, but not yet confirmed. *S. calcitrans* has been found in abundance in many parts of Thailand, particularly in the north and northeastern regions where dairy and beef cattle farms are most available (Sucharit & Tumrasvin, 1981). A good knowledge of biological and ecological data is absolutely crucial to understand the epidemiology of pathogen transmission by these flies and to design vector control methods. To assist in improving this base of information, we have studied the seasonal variations of density and daily variations of activity of stomoxyine flies in a local dairy cattle farm during a one year period of time.

MATERIALS AND METHODS

Collection site

Stomoxyine flies collection was made at a local dairy cattle farm in Wang Nam Khiao District, Nakhon Ratchasima Province (14°25'6"N, 101°51'0"E). The majority of Wang Nam Khiao area is covered with organic farms near Tub Lan National Park, one of the biggest national parks in Thailand. Approximately 100 cows are housed in this local farm. Absolutely no insecticide has been used to protect cows from insect bites.

Fly collection

Eight Vavoua traps (Laveissiere & Grebaut, 1990) were placed around the farm and left operational during the night before collection at 06:00 hr. Collections were made at every two hours at 06:00, 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hr (local time) during two consecutive days per month from January to December in the year 2010. Captured flies were preserved in vials, containing 95 % ethanol and recorded by date and hour of capture. Specimens were subsequently brought back to the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand for morphological identification following Zumpt (1973) with some modifications.

Climatic parameters

Ambient air temperature and relative humidity were recorded every two hours at the dairy cattle farm, during the period of collections. And average rainfall was obtained at Nakhon Ratchasima meteorological station. Three seasons were categorized as: cool season (November to February), summer (March to June) and rainy season (July to October). Each season was of four months long and the same effort of fly-collection (64 day-traps) was used per season.

Data analysis

Captured flies were compared by a two-way analysis of variance (ANOVA). Differences among seasons on one side and day time periods on the other were performed, using Fisher's least-significant difference. The accepted level of significance was determined at 5 % (P-value < 0.05). All data were analyzed using SPSS program package (Ver 17, SPSS Inc., Chicago, IL, USA).

RESULTS

A survey of stomoxyine flies was carried out at Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand during a one year period from January to December 2010. Four species of stomoxyine flies were identified in this rural area, including *Stomoxys indicus* Picard 1908, *S. calcitrans* (Linnaeus, 1758), *S. sitiens* Rondani, 1873, and *S. uruma* Shinonaga & Kanao, 1966. A total of 3,449 flies were captured (Table I) with 1,731 specimens of *S. indicus*, representing 50.2 % of the total collection, and 1,707 specimens of *S. calcitrans*, representing 49.5 %. *S. sitiens* and *S. uruma* were found in a relatively low number with eight specimens (0.2 %) and three specimens (0.1 %), respectively.

The variations of seasonal abundance were determined during three different climatic seasons: cool, summer and rainy. In general, flies were found to be more abundant in the rainy period of the year (Table II). The total number of *S. indicus* and *S. calcitrans*

captured in the rainy season was statistically different from other seasons ($P < 0.05$). A total of 75.5 % of *S. indicus* were captured in rainy season (1,307), followed by those captured in summer (221) representing 12.8 %, and in cool season (203) representing 11.7 %. Similarly, the greatest number of *S. calcitrans* were captured in rainy season (958) representing 56.1 % of the captures of this species. Lower proportions were collected in cool season (578) representing 33.9 % and in summer (171) representing 10 % (Table II). During all seasons, more females *S. indicus* were collected than males, while females *S. calcitrans* were collected in lower number compared to males as shown in Table II. The total number of *S. indicus* and *S. calcitrans* for both males and females were statistically different among the three seasons ($P < 0.05$). This analysis could not be made for *S. sitiens* and *S. uruma* because of the relatively low number of flies of these species captured for each season.

Monthly fly collections were made during a one year period, and the total number of flies captured per species, per sex and per month was analyzed (Table III). The peak of abundance was observed in August for *S. calcitrans* (396 specimens: 329 males and 67 females), and in September for *S. indicus* (526 specimens: 127 males and 399 females) (Fig. 1). The lowest number of stomoxyine flies was collected in March in which 41 specimens of three species were captured. Three specimens of *S. uruma* were captured only in November. Significant differences in the total number of flies collected during the 12 months were observed ($P < 0.05$).

Species	Number of flies		
	Male	Female	Total
Stomoxys indicus	556	1,175	1,731
Stomoxys calcitrans	1,193	514	1,707
Stomoxys sitiens	5	3	8
Stomoxys uruma	1	2	3
Total	1,755	1,694	3,449

Table I. – Stomoxyine flies collected by Vavoua traps from a dairy cattle farm, Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from January to December 2010).

Season	*S. indicus*		*S. calcitrans*		*S. sitiens*		*S. uruma*		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
Cool[1]	74	129	386	192	2	0	1	2	786 (22.8 %)
Summer[2]	79	142	90	81	2	0	0	0	394 (11.4 %)
Rainy[3]	403	904	717	241	1	3	0	0	2,269 (65.8 %)

[1] Cool season: November-February, [2] Summer season: March-June, [3] Rainy season: July-October.

Table II. – Total number of Stomoxyine flies (*Stomoxys* spp.) captured per season with Vavoua traps (64 day-traps per season) at Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand.

Month	*S. indicus*		*S. calcitrans*		*S. sitiens*		*S. uruma*		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
January	7	7	32	12	2	0	0	0	60
February	14	25	129	60	0	0	0	0	228
March	8	23	3	6	1	0	0	0	41
April	7	7	20	23	1	0	0	0	58
May	31	66	26	24	0	0	0	0	147
June	33	46	41	28	0	0	0	0	148
July	51	108	75	39	0	0	0	0	273
August	137	293	329	67	0	2	0	0	828
September	127	399	154	74	0	1	0	0	755
October	88	104	159	61	0	1	0	0	413
November	17	40	69	46	0	0	1	2	175
December	36	57	156	74	0	0	0	0	323
Total	556	1,175	1,193	514	4	4	1	2	3,449

Table III. – Monthly captures of Stomoxyine flies (*Stomoxys* spp.) with Vavoua traps (16 day-traps per month) at Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from January to December 2010).

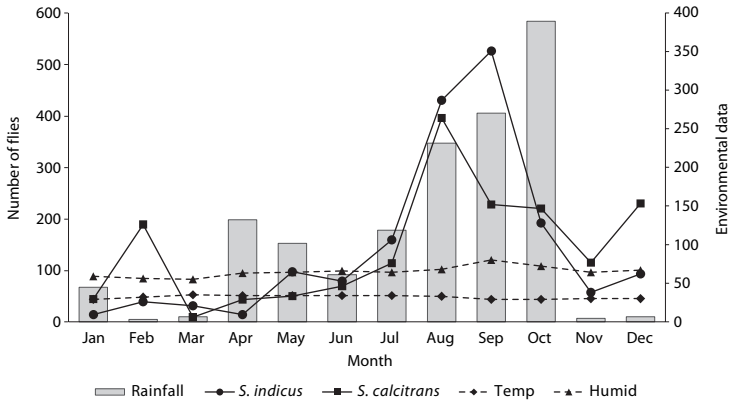


Fig. 1. – Monthly captures of *S. indicus* and *S. calcitrans* with Vavoua traps (16 day-traps per month) at Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand (from January to December 2010), with indications of rainfall, temperature (Temp) and relative humidity (Humid).

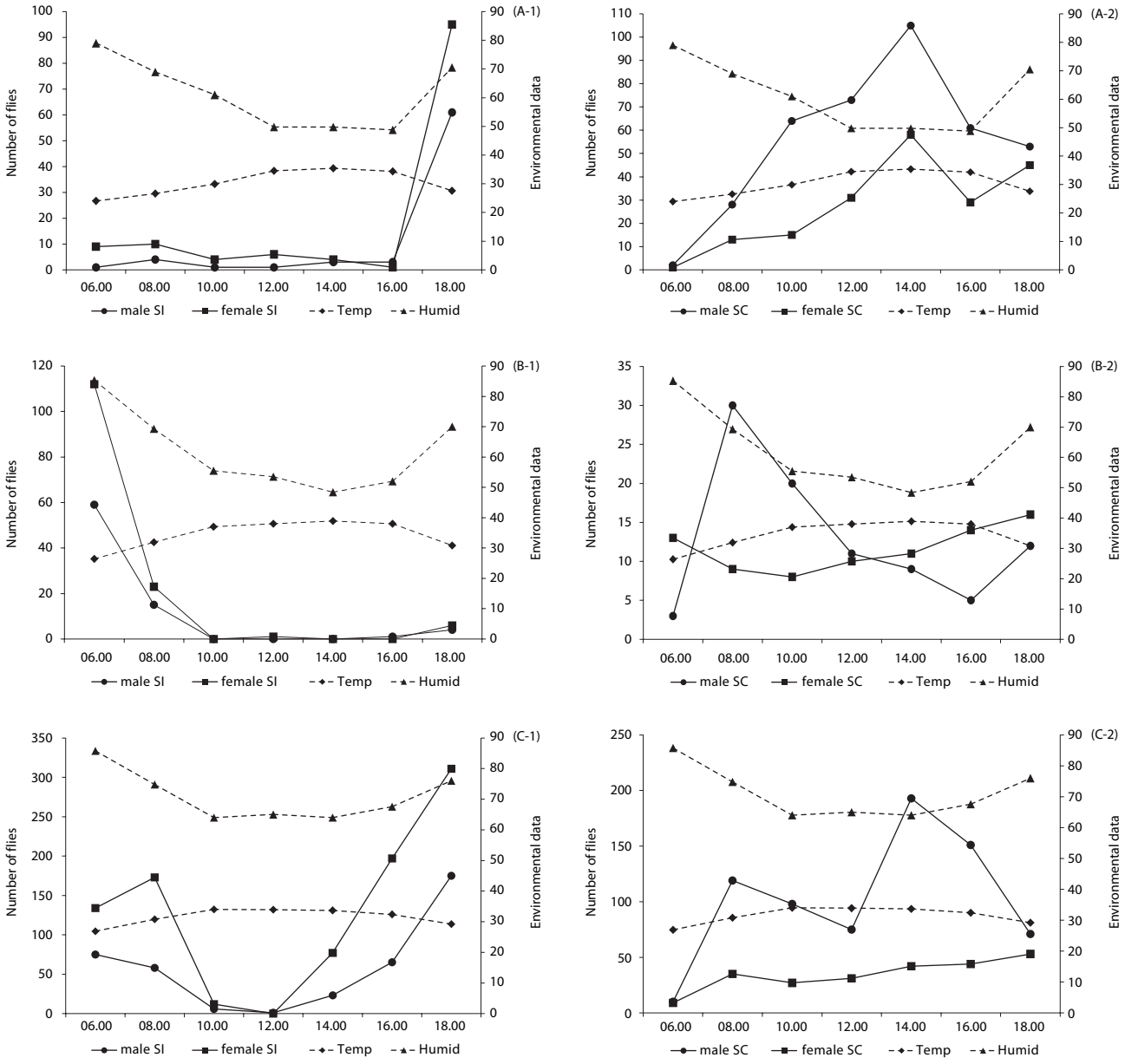


Fig. 2. – Variations of diurnal activity of *Stomoxys indicus* (SI) during cool (A-1), summer (B-1), rainy (C-1) seasons, and of *S. calcitrans* (SC) during cool (A-2), summer (B-2), rainy (C-2) seasons, collected with Vavoua traps (16 day-trap per month) at a Dairy Cattle Farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand.

Diurnal variations of activity of *S. indicus* and *S. calcitrans* during three seasons are given in Figure 2. Flies were collected from different periods of the day with two hours intervals, beginning from 06:00 to 18:00 hr. In cool season, a peak of activity was observed in evening (18:00 hr) for both males and females of *S. indicus* (Fig. 2, A-1). In contrast, a main peak of activity occurred in the afternoon (14:00 hr) for males and females of *S. calcitrans* (Fig. 2, A-2). For summer period, both sexes of *S. indicus* show a peak of activity in the morning at 06:00 hr (Fig. 2, B-1). Males of *S. calcitrans* showed a peak of activity in the morning (08:00 hr) but females of this species showed a constant activity all along the day with a slight increase in the afternoon (Fig. 2, B-2). During the rainy season, two peaks of activity were observed for males and females of *S. indicus*, with the most prominent before sunset (16:00 to 18:00 hr), and a less pronounced peak after sunrise (06:00 to 08:00 hr) (Fig. 2, C-1). Males of *S. calcitrans* showed also two peaks of activity with the most important in the afternoon (14:00 hr) and another one in the morning (08:00 hr), while females showed a constant activity throughout the day (Fig. 2, C-2).

DISCUSSION

Few studies of stomoxyine flies have been conducted in Southeast Asian countries, especially Thailand (Sucharit & Tumrasvin, 1981; Masmeatathip *et al.*, 2006; Muenworn *et al.*, 2010; Tainchum *et al.*, 2010). Most studies of stomoxyine flies have been well documented from Africa, the United States and France (Zumpt, 1973; Jones *et al.*, 1985; Gilles *et al.*, 2007a, 2007b, 2008; Mavoungou *et al.*, 2008; Dsouli *et al.*, 2011a, 2011b). Recently, five species of stomoxyine flies were identified from Thailand in which the prevailing species in the rural setting area is *S. calcitrans* (Masmeatathip *et al.*, 2006; Muenworn *et al.*, 2010). In our study, four species of stomoxyine flies were identified, including *S. indicus*, *S. calcitrans*, *S. sitiens* and *S. uruma*.

S. indicus and *S. calcitrans* were found to be the most abundant in this local farm. *S. indicus* is considered as the most common *Stomoxys* species in the Oriental region (Zumpt, 1973). It has been recorded from many countries, from India and Sri Lanka in the West to Samoa Island in the East, and this species is commonly found in cattle barns (Zumpt, 1973). In Thailand, *S. indicus* was first reported by Masmeatathip *et al.* (2006) from Nakhon Pathom Province. In their work on the phylogeny of *Stomoxys* flies, Dsouli *et al.* (2011a) have shown that *S. indicus* could be the oldest species of *Stomoxys*, indicating that this genus could have originated in the Oriental region. In contrast, *S. calcitrans* is native to the Old World and known as a cosmopolitan species, commonly found in many areas in tropical and temperate zones. This species is regarded as a synanthropic fly, which followed human beings during their peregrinations everywhere in the world (Zumpt, 1973).

S. sitiens has been recorded from many places in the Ethiopian region ranging from the Gambia to Egypt all the way to South Africa, but this species is very rare in collections. It occurs also in the Oriental region from India to the Philippines, but the material is as rare as that from Africa (Zumpt, 1973). And *S. uruma* has been reported from the Iriomote and Ishigaki Islands, Ryukyus, Hong Kong and some specimens from India, Vietnam, Taiwan, and Thailand (Zumpt, 1973).

For the study of seasonal abundance, the results showed statistically different numbers ($P < 0.05$) between seasons. The greatest number of flies was captured during the rainy season while their number during the summer and cool seasons were not different. The high number of stomoxyine flies collected in this local dairy cattle farm is the consequence of appropriate environmental conditions, *i.e.* moisture, light intensity, rainfall, and temperature to maintain suitable breeding habitats. In the USA, a single seasonal peak of density for *S. calcitrans* was observed during the summer season, whereas marked bimodal and trimodal peaks have been documented in other locations (Mullens & Meyer, 1987; Lysyk, 1998). In Thailand, former observations showed that a peak of density of *S. calcitrans* was during the rainy season (Masmeatathip *et al.*, 2006; Muenvorn *et al.*, 2010). In our study, the greatest number of adult stomoxyine flies was captured during the rainy season as well. A major seasonal peak of abundance of *S. indicus* and *S. calcitrans* was found in this season. A minor peak has been observed in February, probably due to unusual important rainfalls in January 2010. The summer and cool seasons showed lower numbers of flies; that could be explained by the very low rainfalls and high temperature, which are unsuitable conditions for larval development (Zumpt, 1973). It should have been useful to extend such a survey on a 14-month period for a better explanation of the variations of fly density throughout the year. The differences observed in sex-ratios of our captures of *S. calcitrans* and *S. indicus* all along the year require further studies.

The variations of diurnal activity have been observed among different period of times (06:00 to 18:00) during three seasons. The patterns of activity between the most abundant species were quite different. Our results confirm the crepuscular activity of *S. indicus* already indicated by Zumpt (1973), who wrote that

those flies are more active in the evening and they are readily collected by using light-traps set in cow-sheds.

For *S. calcitrans*, the patterns during all seasons indicate a variation of diurnal activity. For males, this pattern is unimodal in cool season (peak in the afternoon) and summer season (peak in the morning), but bimodal in rainy season. For females *S. calcitrans*, a more or less constant activity was observed all along the day during all seasons. Many authors who have worked on the activity of stomoxyine flies focused only on *S. calcitrans*. Bimodal feeding activity patterns for *S. calcitrans* were reported by Mitzmain (1913), Simmonds (1944), Labrecque *et al.* (1975), Kunz & Monty (1976), and Charlwood & Lopes (1980). In contrast, Coaker & Passmore (1958) and Harley (1965) observed unimodal feeding activity patterns on daily feeding in Uganda. In Thailand, Masmethathip *et al.* (2006) reported that *S. indicus* showed the highest activity at sunset and dawn; in the same experiment, *S. calcitrans* showed an activity all through the day with a peak between 08:00 am to 10:00 am. Muenworn *et al.* (2010) observed a peak of flight activity of males *S. calcitrans* at 10:00 and 16:00 hr, whereas females showed an increase of activity all along the day until 16:00 hr. Berry & Campbell (1985) found that the pattern of daily activity of *S. calcitrans* was affected by temperature, humidity, and the level of solar radiation. In our study, *S. calcitrans* had the highest activity when temperatures range from 30 to 35 °C. This finding is the same as Hafez & Gamal-Eddin (1959), who worked on diurnal rhythm and seasonal variation, and reported that highest biting activity occurred about 30 °C.

CONCLUSIONS

Our study confirmed that *S. indicus* and *S. calcitrans* are the most abundant species of *Stomoxys* in a local dairy local farm in Wang Nam Khiao District, Nakhon Ratchasima Province, Thailand. *S. indicus* appears in Asia as a vicariant species of *S. niger*, which is abundant in farms in Africa, along with the cosmopolitan *S. calcitrans*. It showed also that both species had their seasonal peak of abundance during the rainy season (August-September). And that their daily variations of activity was different during the seasons. Those results let us propose that, in this environment, control methods should be implemented in summer season to limit the development of their populations at the beginning of next rainy season. A better knowledge of larval breeding sites should help to control at the same time adult and larval stages.

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ORIGINAL PAPER

Behavioral responses of *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) to four essential oils in Thailand

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Abstract The behavioral effects of four essential oils extracted from orange peel (*Citrus aurantium* L.), cinnamon leaf (*Cinnamomum verum* J. Presl), citronella grass (*Cymbopogon winterianus* Jowitt), and clove flower [*Syzygium aromaticum* (L.) Merrill & Perry] were evaluated against two medically important species of mosquitoes, *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say, using an excito-repellency test system. *Ae. aegypti* was collected from a small village in Kanchanaburi Province and *Culex quinquefasciatus* was captured from an urban area of Bangkok. Mosquitoes from the F1–F3 generations were tested in the excito-repellency test chamber for contact excitation and non-contact spatial repellency. Results showed that both species demonstrated varying levels of behavioral escape responses to different essential oils, showing a clear dose response depending on percent w/v concentration used. Orange oil produced the least response in both mosquito species, while citronella and clove the greatest. In general, *Cx. quinquefasciatus* exhibited much stronger behavioral responses to all four essential oils than *Ae. aegypti.* From this study, we conclude that the essential oils from various botanical sources should continue to be screened for protective properties against mosquitoes and other biting arthropods.

Keywords *Aedes aegypti* · *Culex quinquefasciatus* · Essential oils · Behavioral responses · Excito-repellency test system

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Introduction

Approximately 4,000 known species of mosquitoes have been described throughout the world with some species having wide cosmopolitan distributions in the urban and peri-urban settings in close association with humans, most notably *Aedes* (*Stegomyia*) *aegypti* L. and *Culex quinquefasciatus* Say. *Ae. aegypti*, the primary epidemic vector of dengue viruses, is a predominately urban, day-biting mosquito, often found in and around human dwellings and preferentially feeds on humans, whereas *Cx. quinquefasciatus* is a common urban and rural species with strong night biting patterns and is a major vector of Bancroftian filariasis (*Wuchereria bancrofti*) and several arboviruses in various parts of the world (Sasa 1976). Both mosquito species have been extremely refractory to common control

measures and can occur in high-population densities. Despite decades of research, an efficacious and commercially viable multivalent dengue vaccine is not yet available. Therefore, the prevention and control of dengue and many other vector-borne diseases (e.g., malaria and filariasis) remains dependent on various vector control strategies to decrease transmission risk. In some instances this requires the use of chemical insecticides as adulticides for space spray and indoor residual spray applications as well as pyrethroid-impregnated bed nets to control adult mosquito blood-feeding (Roberts and Andre 1994; Reiter and Gubler 1997; Grieco et al. 2007).

A variety of chemical compounds can protect humans from blood-feeding insects by one or more of three identified actions, contact excitation (irritancy), non-contact spatial repellency or physiological knockdown (KD), and toxicity (Grieco et al. 2007). The first two properties are potential outcome behavioral responses of mosquitoes after or before they make physical contact with a chemical (Chareonviriyaphap et al. 1997; Roberts et al. 1997) A lethal or KD response occurs when the mosquitoes receive a toxic dose of an insecticidal chemical (Roberts et al. 2000). Recently, a fourth behavioral action has been described for *N*,*N*-diethyl-*m*-toluamide (DEET), a common synthetic chemical often referred to as having a "repellent" action, in which the chemical actually inhibits an insect's odor activated receptors thereby preventing the location of the host for blood-feeding (Ditzen et al. 2008; Suwannachote et al. 2009). Most studies have focused primarily on the toxic action of chemicals designed to control mosquitoes and disease transmission, whereas relatively few investigations have directed attention to the non-toxic properties, including irritancy and repellency (Grieco et al. 2007; Chareonviriyaphap et al. 1997, 2004). Much of the previous works have focused on synthetic chemicals (Thanispong et al. 2009; Mongkalangoon et al. 2009) that are often non-discriminating in their detrimental effect on non-target organisms and can result in excessive contamination to the surrounding environment. In addition, the development of resistance by mosquitoes to synthetic compounds has resulted in reduced effectiveness of traditional chemical-based prevention and control methods (Thanispong et al. 2008; Chuaycharoensuk et al. 2011). For this reason, the potential for use of native plant derived products as a source of essential oils is an attractive option to replace or supplement synthetic compounds for the control of vectors and prevention of disease transmission.

One of the most common insect "repellent" active ingredients in commercial products is DEET (McCabe et al. 1954; Fradin and Day 2002). This compound has demonstrated to be a broad-spectrum repellent with excellent protection against mosquitoes and other blood-sucking arthropods (Fradin and Day 2002; Yap 1986; Coleman et al. 1993; Walker et al. 1996). However, DEET has been shown to have significant adverse or toxic effects to humans (Brown and Hebert 1997), especially when misapplied and at higher concentrations is a powerful solvent of plastics and other synthetic materials (Odalo et al. 2005). For these reason, there has been an interest on developing insect repellents from natural plant extracts, such as thyme (Park et al. 2005), clove (Bernard 1999; Trongtokit et al. 2004, 2005), celery (Tuetun et al. 2005), citronella (Yang and Ma 2005), and others.

Numerous essential oils extracted from plants have been evaluated for insect repellent activity for protection against mosquitoes and other arthropod pests in Thailand, for example *Ocimum* spp. (Chokechaijaroenporn et al. 1994; Tawatsin et al. 2001) *Nepeta cataria* (Polsomboon et al. 2008b), *Citrus hystrix* (Thavara et al. 2007), *Melaleuca leucadendron*, *Litsea cubeba*, and *Litsea salicifolia* (Noosidum et al. 2008). Recently, over 90 plant species were evaluated as potential repellents or toxicants against blood-feeding invertebrates and pestiferous flies in Lao PDR (De Boer et al. 2010). Although several essential oils have exhibited significant repellent activity against target insects, comparatively little has been done to identify the degree of behavioral responses at varying chemical concentrations or to differentiate the two primary types of behavioral responses (i.e., contact excitation and spatial repellency) to chemical exposure (Polsomboon et al. 2008a; Noosidum et al. 2008). As follow-on to the work conducted by Polsomboon et al. (2008a), we describe the behavioral responses of *Ae. aegypti* and *Cx. quinquefasciatus* based on the two non-toxic properties of four extracted essential oils using an excito-repellency (ER) test system.

Materials and methods

Mosquito populations

Ae. aegypti was collected as larvae and pupae from artificial containers located in Ban Pu Tuey, Ta-Soa Sub-district, Sai-Yok District, Kanchanaburi Province (14°20′N, 98°59′E). Species identification and subsequent colonization was carried out at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. *Ae. aegypti* mosquitoes were reared following the methods of Kongmee et al. (2004).

Culex quinquefasciatus was captured as larvae and pupae from polluted collections of water near houses in Jatuchak District, Bangkok, Thailand (13°50′50.39″N, 100°34′19.44″E). Species identification and subsequent colonization was conducted at the Department of Entomology, Kasetsart University using morphological keys and standard rearing procedures (Kongmee et al. 2004).

For both species, only F1–F3 generation, 3–5-day-old female mosquitoes were used. Mating status was not examined, but *Ae. aegypti* was assumed mated while status of *Cx. quinquefasciatus* was unknown.

Essential oils

The four essential oils used in this study were purchased in maximum extract concentration form from Thai-China Flavours and Fragrances Industry Co., Ltd. Company (TCFF, Phra Nakhon Si Ayutthaya, Thailand) as follows: orange oil 'A' (CA: *Citrus aurantium* L.), by cold expression of outer peel (product code: MK-20024); cinnamon oil (CV: *Cinnamomum verum* J. Presl. [synonym = *C. zeylanicum* Blume]), by water of steam distillation of leaves and twigs (product code: MK-20086); citronella oil (CW: *Cymbopogon winterianus* Jowitt), by steam distillation of citronella grass (product code: MK-40012); and clove oil (SA: *Syzygium aromaticum* (L.) Merrill & Perry [synonym = *Eugenia caryophyllata* Thunb.]), by water distillation of dried flower (product code: MK-20006). All oils were deemed acceptable for use based on shelf life characteristics and storage criteria (tightly sealed bottles and protection from light) from the manufacturer; however, none were reanalyzed for specific gravity and refractive index after initial testing at point of production.

Treated papers

Three different concentrations of each oil product as serial dilutions 2.5, 5.0, and 10.0 % (w/v) were individually impregnated onto filtered test papers (14.7 × 17.3 cm^2) for use in the ER test system, following World Health Organization general procedures for treating papers with insecticidal compounds (World Health Organization 1998). Three serial concentrations were prepared by dilution of individual stock oil in absolute ethanol only. All papers were treated at the rate of 2.8 ml of test solution per 254.3 cm^2 surface area and allowed to air dry. Control paper was treated separately with absolute ethanol only. All papers were allowed to air dry for at least 12-h before use in the ER tests and were used only once per test series.

Excito-repellency test system

An ER test system was used as previously described (Polsomboon et al. 2008a; Noosidum et al. 2008; Chareonviriyaphap et al. 2002). Four test chambers were used per test trial. One pair of chambers served as contact and control, the other pair as non-contact and control configurations. The ER test apparatus consists of a screened inner chamber that is used to prevent mosquitoes coming in direct contact with chemical-treated surfaces and thus serving as a non-contact (repellency) chamber (Fig. 1). Alternatively, contact test designs had treated papers attached inside the chamber so that the exposed surfaces were available for mosquitoes to land on. All females were deprived of sucrose solution and provided only water 12 h before testing.

To begin the test, 15, 3–5-day-old, non-blooded female mosquitoes were introduced per chamber using a mouth aspirator and allowed a 3-min ″resting″ (adjustment) period, after which the escape funnel was opened to begin the observation period. A receiving cage (21.5 × 24.5 × 21 cm^3 paper carton) was connected to the exit portal for collecting all escaped mosquitoes. The number of escaping mosquitoes from each ER chamber was recorded at 1-min intervals for a full test period of 30 min. Immediately following test completion, the number of dead or KD (moribund) specimens either remaining inside the chamber and those that had escaped into the receiving cage were recorded for each of the 4 chambers. All moribund and live specimens that had successfully escaped or remained inside the test chamber were transferred to clean holding cups, labeled and provided with a 10 % sucrose solution for sustenance over a 24-h holding and observation period, at which time mortality was recorded. All test pairs were replicated four times.

Data analysis

A Kaplan–Meier survival analysis method was used to analyze and interpret the behavioral response data (Roberts et al. 1997; Kleinbaum 1995). Mosquitoes that escaped from the chamber were categorized as "dead" and those remaining inside the chamber as "survivors" (Chareonviriyaphap et al. 1997). Survival analysis was used to estimate probability of mosquito escape over time and comparison of differences in escape response between difference test populations, essential oils, and percent concentrations. The use of survival curves minimized loss of valuable information by estimating mosquito escape probability over all time points. The log-rank method (Mantel and Haenzel 1959) was used to compare patterns of escape response

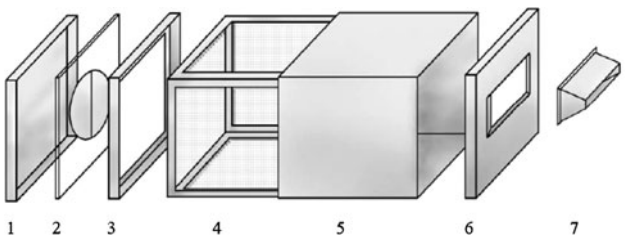


Fig. 1 Breakdown diagram of ER test system used in behavioral response studies (based on Chareonviriyaphap et al. 2002). Rear door cover (1), Plexiglas with rubber-sealed door (2), Plexiglas holding frame (3), screened inner chamber (4), outer chamber (5), front panel (6), and exit portal (7). Receiving cage not shown

within and between different groups by time (Chareonviriyaphap et al. 2004). The discriminating level of statistical significance for all tests was set at 95 % ($p < 0.05$). Mean percent mortality was calculated per test chamber. For those tests in which the control (untreated) mortality exceeded 5 %, a correction factor (Abbott's formula) was applied to the data (Abbott 1925).

Results

ER responses of *Ae. aegypti* and *Cx. quinquefasciatus*, exposed to 2.5, 5.0, and 10.0 % w/v essential oils of orange (CA), cinnamon (CV), citronella (CW), and clove (SA) were evaluated for contact irritancy and non-contact repellency responses using an ER test system. Post-exposure mortality of *Ae. aegypti* and *Cx. quinquefasciatus* after 24-h post-exposure holding period are documented in Tables 1 and 2, respectively. Knockdown data of escape and non-escape specimens were also recorded at the end of the 30 min test. In general, higher mortality was recorded from non-escape mosquitoes compared to mosquitoes successfully exiting the chambers and from exposure to higher concentrations vice lower. In all paired tests, very few mosquitoes (0–5 %) escaped from untreated control chambers and both mortality and KD were very low (data not shown).

The escape response of both species varied significantly, depending upon oil and chemical concentration used. In general, a stronger escape response was observed when both species were tested with clove and citronella compared to orange and cinnamon extracts. A greater escape response was observed in the contact trials compared to the spatial non-contact trial designs. Specifically, a greater overall escape response was recorded for *Cx. quinquefasciatus* compared to *Ae. aegypti*.

For *Ae. aegypti* contact trials, the highest escape responses were seen with 2.5 % clove (36.9 %), the lowest response with exposure to 10 % cinnamon (8.9 %). In non-contact trials, 2.5 % clove and 5 % citronella produced the strongest escape responses (38.8 and 39.0 %, respectively) and 5 % cinnamon gave the lowest escape response (3.3 %) at 30 min. No mortality was observed in escape specimens from contact and non-contact for all three concentrations of orange oil and comparatively low-percent mortality were seen in the non-escape mosquitoes, ranging from 0 to 2 % only. For clove oil, a higher percent mortality was observed in both escape and non-escape *Ae. aegypti* at the concentration of 10 % (Table 1).

Thirty min KD was recorded for all escape and non-escape mosquitoes. For *Ae. aegypti*, KD was very low (0–2 %) for escape and non-escape specimens exposed to orange oil regardless of concentrations. Cinnamon at 10 % concentration produced the greatest percent KD of escaped and non-escaped specimens from both contact and non-contact trials (Table 1). Citronella and clove oil at 10 % gave a significantly higher percent KD as compared to the two lower concentrations.

For *Cx. quinquefasciatus*, the highest escape response in contact and non-contact trials was observed with 10 % citronella (87.7 and 83.0 %, respectively). The lowest percentage escape response in contact trials and non-contact trials were observed with 5 % orange oil (1.7 and 0 %, respectively). No mortality was observed from those escaping from contact and non-contact chambers for the four essential oils, except for a single exception with 10 % orange oil in the contact trials. Comparatively low-percent mortality was seen from all non-escape tests regardless of oil and concentration (ranging 0–7.1 %) (Table 2). Percent KD presented a much different picture. For orange oil, escape and non-escape KD at 1 h it was 0–4.7 %. However, 10 % cinnamon oil resulted in a high-percent KD of non-escape mosquitoes in both contact (82.6 %) and non-contact experiments (96.8 %) (Table 2). In addition, 10 % clove also resulted in relatively high-percent KD in contact (78.6 %) and non-contact (70.6 %) tests. In general, greater than 75 % of KD specimens recovered within 24 h post-exposure holding period, except non-escape mosquitoes from non-contact chamber treated with 5 % citronella oil and contact chamber treated with 10 % orange oil.

The escape patterns based on time (minutes) required for test populations to escape was defined proportionally as 25 % (ET25), 50 % (ET50), and 75 % (ET75) of the exposed females exiting the chamber over the 30 min period (Table 3). Overall, *Ae. aegypti* demonstrated a lower overall escape response than that of *Cx. quinquefasciatus*. In contact trials, *Ae. aegypti* had the greatest ET25 escape values with 2.5 and 5 % citronella (6 and 1 min, respectively), whereas 10 % citronella had a contact ET25 of 30 min. In non-contact trials, 5 % citronella also produced the most rapid escape response at 1 min ET25. In all cases, both ET50 and ET75 values of the four essential oils could not be estimated due to low number of escapees during the 30 min testing period. For *Cx. quinquefasciatus*, ET25 values at 5 and 10 % concentrations of cinnamon, citronella, and clove in contact and non-contact trials were comparatively rapid, ranging between 1 and 5 min for escape. Orange oil produced the slowest escape response even at the highest concentration (contact ET25 at 18 min). *Culex quinquefasciatus* demonstrated a much greater overall escape percentage compared to *Ae. aegypti*. ET50 values were seen for 5 and 10 % cinnamon, and ET50 and ET75 escape times (ET) for citronella and clove were clearly evident (Table 3).

Multiple log-rank comparisons were performed in paired contact, non-contact, and control trials for the four

Table 1 Escape response, KD at 30 min and mortality after 24 h holding period for *Ae. aegypti* following contact and non-contact with three serial concentrations of 4 essential oils in ER tests

Oil	Test	Dose (% w/v)	*N*	Mean # Esc ± SD	Esc (%)*	% KD (30 min)		% Mort (24 h)	
						Esc	Not esc	Esc	Not esc
CA	C	2.5	59	2.25 ± 1.0	12.3	0	0	0	2.0
		5.0	60	4.25 ± 2.1	25.1	0	0	0	0
		10.0	59	2.0 ± 1.6	12.1	0	2.0	0	2.0
	NC	2.5	60	0.75 ± 0.5	3.4	0	0	0	0
		5.0	60	1.75 ± 0.5	7.0	0	0	0	0
		10.0	57	1.0 ± 1.2	7.0	0	0	0	0
CV	C	2.5	59	5.0 ± 2.4	25.9	0	0	0	2.3
		5.0	59	3.0 ± 1.6	9.4	0	83.0	8.3	2.1
		10.0	59	2.0 ± 1.2	8.9	37.5	100.0	0	5.9
	NC	2.5	60	4.75 ± 1.5	8.5	0	0	0	0
		5.0	60	2.5 ± 1.7	3.3	40.0	84.0	30.0	4.0
		10.0	60	3.52 ± 2.2	21.7	30.8	97.9	0	2.1
CW	C	2.5	59	5.25 ± 1.5	30.4	0	0	0	2.6
		5.0	60	5.25 ± 1.5	29.1	0	5.1	0	0
		10.0	57	3.5 ± 1.7	24.6	14.3	58.1	7.1	2.3
	NC	2.5	59	1.5 ± 1.3	10.2	0	0	0	0
		5.0	60	6.0 ± 1.4	39.0	0	0	0	0
		10.0	58	3.0 ± 2.2	20.7	0	73.9	25.0	8.7
SA	C	2.5	60	7.0 ± 0.8	36.9	0	40.6	3.6	0
		5.0	58	4.25 ± 0.5	22.6	0	12.2	0	2.4
		10.0	54	2.75 ± 1.0	17.6	9.1	97.7	0	46.5
	NC	2.5	58	6.25 ± 2.1	38.8	0	32.0	0	6.1
		5.0	59	3.25 ± 1.7	19.3	0	32.6	0	0
		10.0	57	3.25 ± 1.0	22.8	7.7	97.7	23.1	29.6

C contact, *NC* noncontact, *N* combined sample size; *KD* knockdown, *Esc* escaped mosquitoes, *Not esc* non-escaped mosquitoes, *CA Citrus aurantium* (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil)

* Adjusted rate based on paired control response

essential oils by concentration. No statistical differences in pattern of escape were observed in comparisons of contact versus non-contact ($p > 0.05$), except for *Ae. aegypti* at 2.5 % citronella and 5 % orange and for *Cx. quinquefasciatus* at 2.5 % cinnamon oil (Table 4). For all concentrations, *Ae. aegypti* showed no significant differences ($p > 0.05$) in escape response between orange and citronella in contact trials, and orange and cinnamon in non-contact trials, and similarly with *Cx. quinquefasciatus* for cinnamon and clove extracts in both contact and non-contact tests. No significant differences ($p > 0.05$) in escape patterns were observed for *Cx. quinquefasciatus* comparing concentrations at 5 and 10 % for cinnamon, citronella and clove in both contact and non-contact trials. Comparisons between both contact and paired control and non-contact and pair control showed significant differences ($p < 0.05$) for all pairings, except for *Ae. aegypti* in contact and paired control at 5 and 10 % cinnamon and in non-contact and paired control at 2.5 and 5 % orange and 5 % cinnamon oil. No significant differences were seen with *Cx. quinquefasciatus* in both contact and non-contact versus pair controls at 2.5 and 5 % orange oil concentrations.

Using survival analysis, the proportion of mosquitoes remaining in exposure chambers treated with three different concentrations of essential oils in the contact and non-contact test designs during a 30-min exposure period are graphically presented in Figs. 2 and 3. Overall, the escape rate by 1 min intervals was greater in *Cx. quinquefasciatus* than *Ae. aegypti* regardless of test concentration or type of oil used. Orange oil produced the weakest escape response *Ae. aegypti* and *Cx. quinquefasciatus* in both contact (Fig. 2a) and non-contact trials (Fig. 3a), regardless of concentration. Much stronger escape patterns were seen in *Cx. quinquefasciatus* compared to *Ae. aegypti* for all

Table 2 Escape response, KD at 30 min and mortality after 24 h holding period for *Culex quinquefasciatus* following contact and non-contact with three serial concentrations of four essential oils in ER tests

Oil	Test	Dose (% w/v)	*N*	Mean # Esc ± SD	Esc (%)*	% KD (30 min)		% Mort (24 h)	
						Esc	Not esc	Esc	Not esc
CA	C	2.5	58	0.25 ± 0.5	1.7	0	0	0	0
		5.0	60	0.5 ± 1.0	1.7	0	0	0	0
		10.0	58	3.75 ± 3.3	24.6	0	4.7	13.3	4.7
	NC	2.5	60	0.5 ± 0.6	1.7	0	0	0	0
		5.0	60	0 ± 0	0	0	0	0	0
		10.0	60	2.5 ± 2.1	16.7	0	0	0	0
CV	C	2.5	59	9.0 ± 2.9	59.7	0	0	0	0
		5.0	60	10.0 ± 1.8	65.5	0	20.0	0	5.0
		10.0	58	8.75 ± 4.1	60.3	0	82.6	0	0
	NC	2.5	58	6.5 ± 1.7	44.8	0	0	0	0
		5.0	59	7.25 ± 1.7	49.2	0	6.7	0	0
		10.0	60	7.25 ± 3.3	47.5	0	96.8	0	0
CW	C	2.5	59	5.75 ± 3.5	36.9	0	0	0	0
		5.0	60	11.25 ± 2.9	75.0	2.2	6.7	0	0
		10.0	60	13.25 ± 1.3	87.7	0	0	0	0
	NC	2.5	60	5.0 ± 2.9	28.6	0	0	0	0
		5.0	60	11.0 ± 2.8	73.3	0	6.3	0	6.3
		10.0	60	12.5 ± 1.0	83.0	0	30.0	0	0
SA	C	2.5	59	11.25 ± 1.9	76.3	0	0	0	0
		5.0	60	11.75 ± 1.3	76.8	2.1	53.9	0	0
		10.0	60	11.5 ± 2.6	75.4	0	78.6	0	7.1
	NC	2.5	59	8.75 ± 3.9	57.9	0	8.3	0	0
		5.0	58	10.0 ± 1.2	69.0	5.0	44.5	0	0
		10.0	60	10.75 ± 1.7	70.7	0	70.6	0	5.9

C contact, *NC* noncontact, *N* combined sample size; *KD* knockdown, *Esc* escaped mosquitoes, *Not esc* non-escaped mosquitoes, *CA Citrus aurantium* (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil)

* Adjusted rate based on paired control response

concentrations of cinnamon in both contact (Fig. 2b) and non-contact trial configurations (Fig. 3b). For citronella, the escape response was stronger for *Cx. quinquefasciatus* compared to *Ae. aegypti* for all three concentrations in both contact (Fig. 2c) and non-contact (Fig. 3c) tests as was the case with clove oil in contact (Fig. 2d) and non-contact trials (Fig. 3d).

Discussion

The two primary objectives of the study were to (1) evaluate four essential oil extracts for avoidance response in two different species of mosquitoes, and (2) demonstrate that the ER test system can be a useful tool to conduct preliminary screening of different compounds to determine acceptable candidates that could proceed to more advance evaluation. Although this method of evaluation presents limitations for extrapolating the findings to actual applications for protection, ER testing does provide an approach for quickly assessing a compound's potential qualities without the use of live hosts and associated ethical considerations.

All four essential oils showed excito-repellent actions against *Ae. aegypti* and *Cx. quinquefasciatus*. Higher mortality (toxicity) was observed in both escaped and non-escaped *Ae. aegypti* (maximum 46.5 % in contact with 10 % clove oil) as compared to *Cx. quinquefasciatus* (maximum 13.3 %) after a 24-h holding period. Greater mortality was associated with higher concentrations of oils. Citronella and clove were significantly more effective in both excitation and repelling *Ae. aegypti* compared to orange and cinnamon extracts. However, 5 % orange was shown to have more marked contact irritancy properties

Table 3 Estimated ET in minutes for 25 % (ET_{25}), 50 % (ET_{50}), and 75 % (ET_{75}) of *Ae. aegypti* and *Cx. quinquefasciatus* populations to escape from the exposure chambers of ER tests

Oil	% w/v	*Ae. aegypti*			*Cx. quinquefasciatus*		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
CA	2.5						
	CT	–	–	–	–	–	–
	NT	–	–	–	–	–	–
	5.0						
	CT	27	–	–	–	–	–
	NT	–	–	–	–	–	–
	10.0						
	CT	–	–	–	18	–	–
	NT	–	–	–		–	–
CV	2.5						
	CT	13	–	–	18	–	–
	NT	14	–	–	–	–	–
	5.0						
	CT	–	–	–	1	12	–
	NT	–	–	–	3	–	–
	10.0						
	CT	–	–	–	3	7	–
	NT	–	–	–	3	–	–
CW	2.5						
	CT	6	–	–	2		–
	NT	–	–	–	12	–	–
	5.0						
	CT	1	–	–	1	3	28
	NT	1	–	–	1	2	–
	10.0						
	CT	30	–	–	1	4	15
	NT	–	–	–	1	2	19
SA	2.5						
	CT	3	–	–	1	11	26
	NT	8	–	–	1	12	–
	5.0						
	CT	16	–	–	4	8	28
	NT	–	–	–	5	11	–
	10.0						
	CT	–	–	–	1	3	18
	NT	–	–	–	2	9	–

CA Citrus aurantium (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil), *CT* contact, *NT* non-contact

with *Ae. aegypti* in comparison to non-contact and paired controls, suggesting that excitation may be the only major action of orange oil. Similarly, citronella and clove at the lowest concentration were more likely to perform as contact irritants against *Ae. aegypti* than greater concentrations suggesting that non-contact repellency is the predominant escape response as concentration increases. Moreover, the *Aedes aegypti* that successfully escaped the treated chambers of either chemical were far more likely to survive than those remaining inside the contact and non-contact chambers indicating that the lowest dose of citronella and clove were not toxic. Lastly, higher numbers of *Ae. aegypti* were KD as concentrations increased; however, over 52 % recovered during 24-h post-exposure indicating a limited toxic action of these two active oils.

For *Cx. quinquefasciatus*, the lowest concentration of clove produced the strongest escape response in both contact (76.3 %) and non-contact trials (57.9 %), followed by cinnamon and citronella. As no statistical differences were seen among the three concentrations of clove and cinnamon in both contact and non-contact configurations, it appears the lowest concentrations tested are also the most

Table 4 Multiple log-rank comparisons in paired contact, non-contact, and control trials for the four essential oils by concentration

Oils	Dose (% w/v)	Comparisons	*Ae. aegypti* *p* value*	*Cx. quinquefasciatus* *p* value*
CA	2.5	NC vs. NT	0.3080	0.5703
		CC vs. CT	0.0246	0.3132
		NT vs. CT	0.0649	0.5804
	5.0	NC vs. NT	0.1883	NA
		CC vs. CT	0.0007	0.5670
		NT vs. CT	0.0285	0.1556
	10.0	NC vs. NT	0.0392	0.0011
		CC vs. CT	0.0160	0.0002
		NT vs. CT	0.2488	0.2026
CV	2.5	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.8358	0.0093
	5.0	NC vs. NT	0.6594	<0.0001
		CC vs. CT	0.2416	<0.0001
		NT vs. CT	0.6041	0.0912
	10.0	NC vs. NT	0.0002	<0.0001
		CC vs. CT	0.1014	<0.0001
		NT vs. CT	0.2458	0.2423
CW	2.5	NC vs. NT	0.0123	0.0003
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.0013	0.4295
	5.0	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0003	<0.0001
		NT vs. CT	0.5625	0.9741
	10.0	NC vs. NT	0.0002	<0.0001
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.6848	0.4972
SA	2.5	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0003	<0.0001
		NT vs. CT	0.5788	0.2067
	5.0	NC vs. NT	0.0023	<0.0001
		CC vs. CT	0.0042	<0.0001
		NT vs. CT	0.3491	0.2188
	10.0	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0039	<0.0001
		NT vs. CT	0.7914	0.1553

CA Citrus aurantium (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil), *CT* contact treatment, *NT* non-contact treatment, *CC* contact control, *NC* non-contact control, *NA* insufficient numbers of mosquitoes escaping to conduct analysis

* The discriminating level of statistical significance for all tests was set at 95 % ($p < 0.05$)

appropriate for protecting against this species. With the same two oils, the 30-min KD among mosquitoes that did not escape in both contact and non-contact tests showed a clear dose response in relation to increased serial concentrations, yet the majority of escape mosquitoes made a full apparent recovery within the 24-h holding period indicating relatively poor toxicity of clove and citronella to *Cx. quinquefasciatus*.

One of the most significant components of a mosquito abatement program is an understanding of how mosquito vectors behave in response to chemical control measures. These responses can be characterized by three primary actions; excitation ("irritancy"), repellency, and toxicity (Grieco et al. 2007). The combination of the first two behavioral actions is sometimes referred to as "ER," whereas toxicity implies insecticidal action in the form of lethality or reduced survival. Behavioral actions of mosquitoes are part of an innate response repertoire to active ingredients having "irritant" and/or "repellent" properties causing movement away from a point source; whereas, toxicity is a purely physiological response to direct contact with a poison. Numerous attempts have been made to more

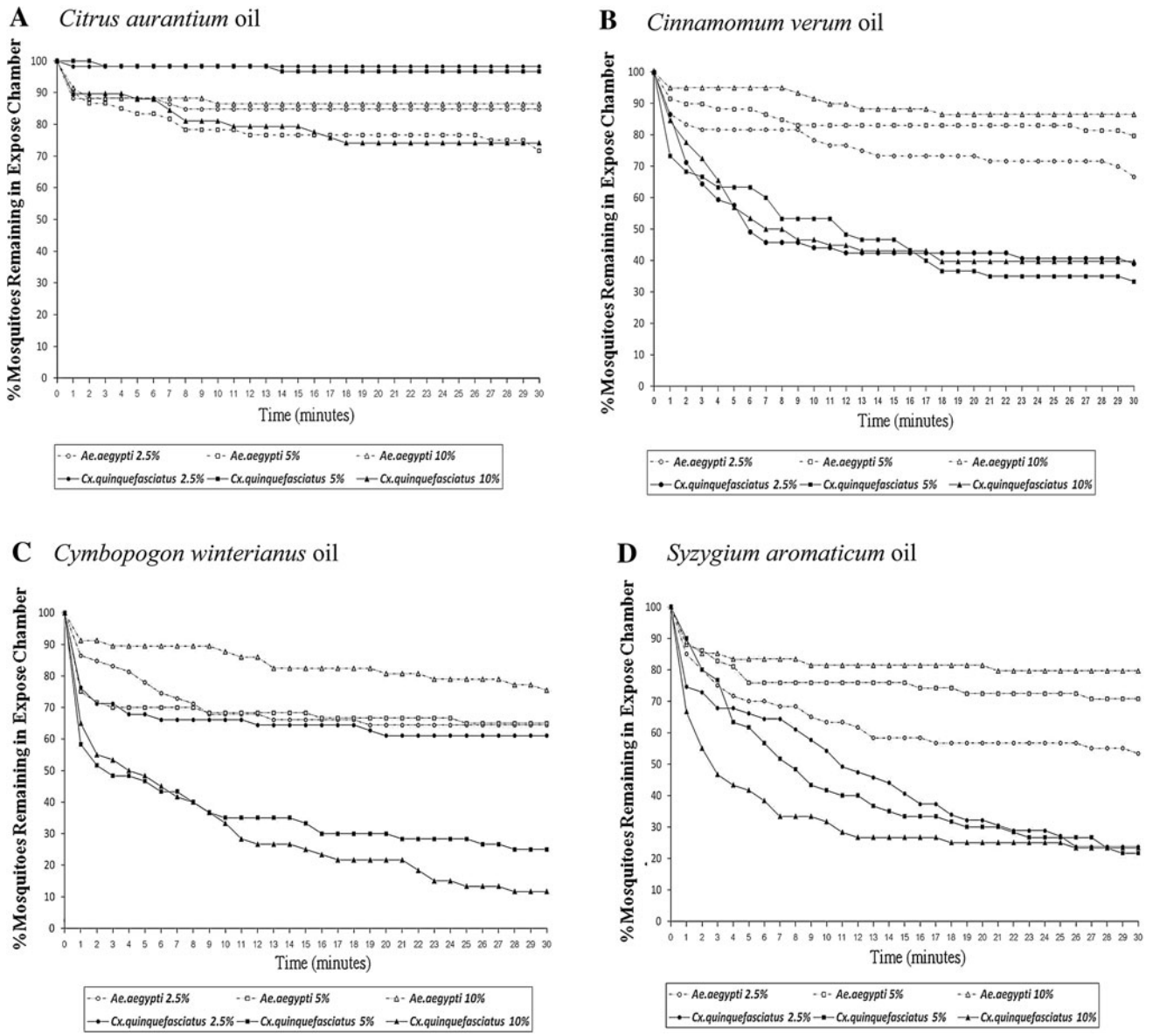


Fig. 2 Escape probability (rate) of female *Ae. aegypti* and *Culex quinquefasciatus* exposed in contact excitation trials using three serial percent concentrations (w/v) of orange (*Citrus aurantium*) oil (**a**), cinnamon (*Cinnamomum verum*) oil (**b**), citronella (*Cymbopogon winterianus*) oil (**c**), and clove (*Syzygium aromaticum*) oil (**d**)

accurately measure the behavioral responses of mosquitoes to test chemicals, as documented in the designs of various ER test systems (Chareonviriyaphap et al. 1997; Roberts et al. 1984; Rutledge et al. 1999; Sungvornyothin et al. 2001; Polsomboon et al. 2008a). In 2005, a modular, high-throughput screening system was developed to observe all three primary actions of test chemicals depending on the assay configuration (Grieco et al. 2005). Although the modular system is smaller in size compared to the previous ER test chambers thereby minimizing the amount of chemical required and the number of test specimens needed per test, this system is more applicable for use in the laboratory setting rather than in the field and more useful for mass screening of different chemical libraries for mode of actions.

In this study, we choose a well-documented ER test system for observing the behavioral responses of two field collected mosquito species, *Ae. aegypti* and *Cx. quinquefasciatus*, to four essential oil extracts produced by a commercial company in Thailand. The value of many essential oils, some traditionally used to ″repel″ or otherwise prevent mosquitoes from biting, has been underestimated as possible alternatives to the most common synthetic and other natural active ingredients currently on the market (Walker et al. 1996; Rutledge et al. 1983; Debboun et al. 2007). Many studies in Thailand and

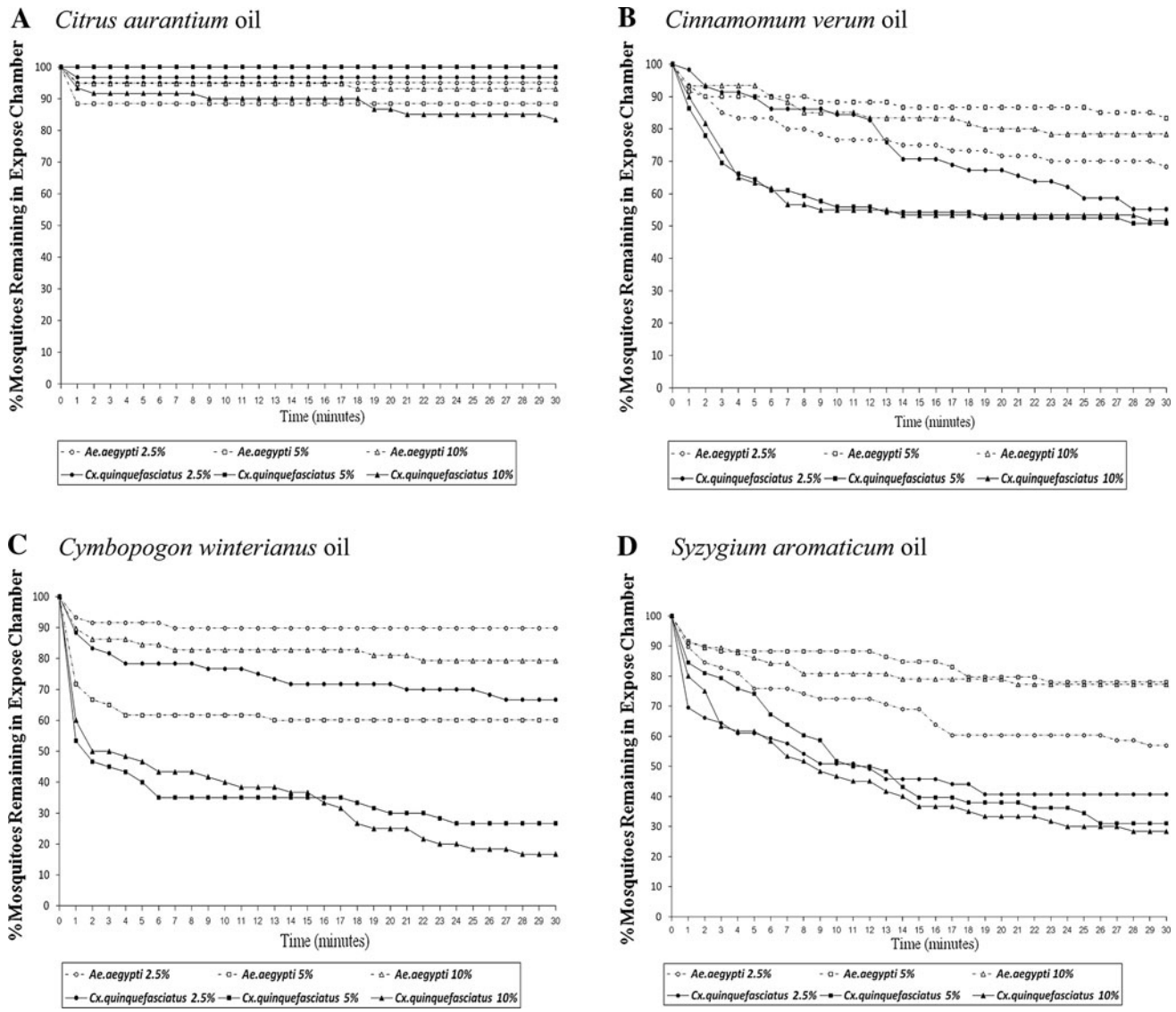


Fig. 3 Escape probability (rate) of female *Ae. aegypti* and *Culex quinquefasciatus* exposed in non-contact repellency trials using three serial percent concentrations (w/v) of orange (*Citrus aurantium*) oil (**a**), cinnamon (*Cinnamomum verum*) oil (**b**), citronella(*Cymbopogon winterianus*) oil (**c**), and clove (*Syzygium aromaticum*) oil (**d**)

elsewhere have shown that various plant species contain select phytochemicals that can perform as insect repellents against mosquitoes and other arthropods (Odalo et al. 2005; Bernard 1999; Trongtokit et al. 2004, 2005; Yang and Ma 2005; Chokechaijaroenporn et al. 1994; Tawatsin et al. 2001; Noosidum et al. 2008; De Boer et al. 2010; Suwonkerd and Tantrarongroj 1994; Tawatsin et al. 2006; Adewoyin et al. 2006; Zhu et al. 2006; Gillij et al. 2008; Kumar et al. 2011). Surprisingly, comparatively few studies have actually demonstrated or identified the precise chemical actions being elicited on mosquitoes and have only focused on the outcome (bite protection) (Polsomboon et al. 2008a; Noosidum et al. 2008).

This study was not designed to measure protection response time of oils on mosquito behavior. However, both citronella and clove oil demonstrated the strongest excitation and repellency actions on both *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes based on comparatively rapid ET. For citronella, a common ingredient in commercial repellent products, this study represents the first observation that shows the marked behavioral effects of this oil on mosquitoes. Clove extract is also capable of eliciting a strong excitation and repellency response in the two species, followed by cinnamon oil. Clove and cinnamon deserve more in depth investigation on their protective properties using other testing methods (e.g., human exposure trials) as possible additives in commercial product formulations.

An understanding of the basic behavioral responses of mosquitoes to natural and synthetic compounds is a more

complete measure of their overall effect and potential for reducing human-vector contact and disease transmission. In this study, two types of behavioral response, contact excitation and spatial repellency, were evaluated with two mosquito species against four essential oils. These responses were quantitatively evaluated and demonstrated that cinnamon, citronella, and clove oils were more effective in eliciting escape responses in mosquitoes than orange oil. This study demonstrated how essential oils can significantly impact mosquito behavior with the possibility they might be incorporated into topical skin repellent formulations or used on other innovative vector control tools. In addition, this study demonstrated varying avoidance responses based on different concentrations that may also prove useful as starting points for determining the lowest effective concentrations and potential synergistic and antagonistic effects against various mosquito species. Lastly, the ER test system appears suitable for use in the preliminary screening of chemicals for behavioral avoidance characteristics by mosquitoes in the absence of host bait. Chemicals that demonstrate significant avoidance response in the test system can be advanced as potential candidates for advanced testing using animal models or human hosts.

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Parasites & Vectors

RESEARCH Open Access

Species diversity and biting activity of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in a malaria prone area of western Thailand

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Abstract

Background: A survey of adult anopheline mosquito diversities, collected from September 2009 to August 2010, was conducted in a malaria endemic area of western Thailand. Two anopheline species complexes, Dirus and Minimus, along with the Maculatus group were observed. Of several species documented from within each complex and group, four important malaria vectors were identified, including *An. dirus*, *An. baimaii*, *An. minimus,* and *An. sawadwongporni*. Information on biting activity and host preference for any single species within the Dirus complex has never been assessed. Using specific molecular identification assays, the trophic behavior and biting activity of each sibling species within the Dirus complex were observed and analyzed for the Kanchanaburi Province, Thailand.

Methods: Adult female mosquitoes were collected for two consecutive nights each month during a one year period. Three collection methods, human landing indoor (HLI), human landing outdoor (HLO), and cattle baited collections (CBC) were applied. Each team of collectors captured mosquitoes between 1800 and 0600 h.

Results: From a total of 9,824 specimens, 656 belong to the Dirus complex (*An. dirus* 6.09% and *An. baimaii* 0.59%), 8,802 to the Minimus complex (*An. minimus* 4.95% and *An. harrisoni* 84.65%) and 366 to the Maculatus group (*An. maculatus* 2.43% and *An. sawadwongporni* 1.29%). Both *An. dirus* and *An. baimaii* demonstrated exophagic and zoophilic behaviors. Significantly greater numbers of *An. dirus* and *An. baimaii* were collected from cattle as compared to humans ($P = 0.003$ for *An. dirus* and $P = 0.048$ for *An. baimaii*).

Conclusions: Significantly greater numbers of *An. dirus* and *An. baimaii* were collected from cattle baited traps as compared to human landing collections ($P < 0.05$), demonstrating that both species show a strong zoophilic behavior. Knowledge of host-seeking behavior helps to define a species' capacity to acquire and transmit malaria and its contribution to the overall risk for disease transmission in the human population, as well as, assisting in the design and implementation of appropriate vector prevention and control strategies.

Keywords: *Anopheles dirus*, *Anopheles baimaii*, Exophagic, Zoophilic, Thailand

Background

In Thailand, malaria remains one of the most important infectious diseases despite years of well-organized disease control in reducing both mortality and morbidity country-wide [1]. Seventy percent of the malaria cases are documented from the relatively undeveloped borders and hill region of eastern Myanmar, whereas the three species complexes, i.e. *Anopheles dirus*, *An. minimus*, and *An. maculatus* are commonly present and some of them are considered as important malaria vectors, including *An. dirus*, *An. baimaii*, *An. maculatus*, *An. sawadwongporni* and *An. minimus* [2,3].

Better understanding of the behavior of each sibling species within the complex is quite important to help identify their respective roles in disease transmission and to assist the vector control personnel in designing the appropriate steps for vector control management.

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Due to some complexity within the *An. dirus* complex, the closely related species cannot be differentiated from each other by morphological characters [4-9]. Some species are regarded as excellent malaria vectors because their anthropophagic nature brings them into frequent contact with humans, maintain high parasite loads, and exhibit a highly endophagic behavior [10,11]. Although various studies on the biting patterns and host preference of these complexes have been described in Thailand [12-17], most observations of mosquito biology and behaviors were based exclusively on the populations in which identification was made by morphological characters, except for one study on the *An. minimus* complex recently carried out by Sungvornyothin *et al*. [9]. Such morphological identification of the sibling species within a complex is not reliable and can lead to a high degree of misidentification [9,18]. The application of molecular techniques has made it possible to reliably identify species from entomological surveys. For the *An. dirus* complex, such molecular methods include an allele-specific polymerase chain reaction (AS-PCR), a sequence characterized amplified region (SCAR) and a rapid polymerase chain reaction, which are all considered useful tools for identifying the species within a complex [8,19,20]. In this study, we identified the species within the three complexes in the sympatric area of Pu Teuy Village, Sai Yok District, Kanchanaburi Province. This area is considered to be a malaria prone region with all three complexes responsible for malaria transmission being documented [21,22]. By using a molecular identification assay we were able to observe the trophic behavior, biting activity, and seasonal abundance in each sibling species within the *An. dirus* complex, one of the most important complexes for malaria transmission in Thailand.

Methods

Study site

Anopheles dirus was collected from the Pu Teuy Village, a village located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 11'E). The collection site is located approximately 1 km away from the village. It is surrounded by deep forest lying at approximately 400 m above sea level. Local residents and their occupations are associated with the forest. A narrow, slow running stream (2 m wide and an average of 0.5 m deep) bordered with native vegetation runs across the village. Surrounding vegetation is primarily agricultural land used for growing papaya, cassava and maize and secondarily forest [17].

Collection methods

Adult female mosquitoes were collected for two consecutive nights each month during a one year period. Three collection methods, including human landing indoor (HLI), human landing outdoor (HLO), and cattle bait collections (CBC) were utilized. Human landing collections were performed in an existing house with all windows closed and one door remaining open throughout the collection period. The entomological team was divided into two groups of four persons each. Two people collected mosquitoes inside the house, whereas the other two people collected outside. The distance between indoor and outdoor collections was set at 100 m. The first team collected from 1800 to 2400 h, followed by the second team that started at midnight and ended at 0600 h. Both teams rotated their starting position between the first and second halves of the evening on each subsequent collection night to avoid collector bias. Collectors also rotated each night between indoor and outdoor locations. Human landing collections occurred uninterrupted for 45 min each hour. Cattle baited collections were conducted by a separate team of two collectors for 15 min each hour [17]. The CBC involved placing a single cow under the untreated cotton bed net, measuring 3.6 m x 3.3 m x 2.0 m (L:W:H) with the net suspended 30 cm above the ground level to allow any hungry mosquitoes to get inside. The same cow was used throughout the study and was placed at least 50 m from the nearest human landing collection site but at equal distances from the forest fringe to avoid a potential distance bias in attracting mosquitoes. All collected mosquitoes were placed in clean, chemical free plastic cups that were labeled by hour and type of collection. Formal human-use approval was granted by the Ethical Research Committee convened by the Research and Development Institute, Kasetsart University, Thailand (KURDI-1/2553-1421457). All collected mosquitoes were held in netting covered plastic cups and were provided with a cotton pad soaked with 10% sugar solution. Each cup was returned to a local processing station in the field for morphological identification. Ambient air temperature and relative humidity were recorded from indoor and outdoor locations and at the site of the cattle baited trap each hour of the collection using a manual thermo-hygrometer (BARICO GmbH, Villingen-Schwenningen, Germany). Daily rainfall data was also recorded at the study site using a manual Rain Gauge (RAIN GAUGE AUGE KIT, England).

Morphological and molecular species identification

An. dirus complex

Members of the *An. dirus* complex were identified to species using illustrated morphological keys for the adult *Anopheles* of Thailand [2,23]. In brief, female mosquitoes with a presector dark spot (PSD) on the radius vein that extended basally beyond the PSD spot on the costa vein and a PSD spot that reached the humeral dark (HD) spot of the costa vein or at least surpassed the middle of

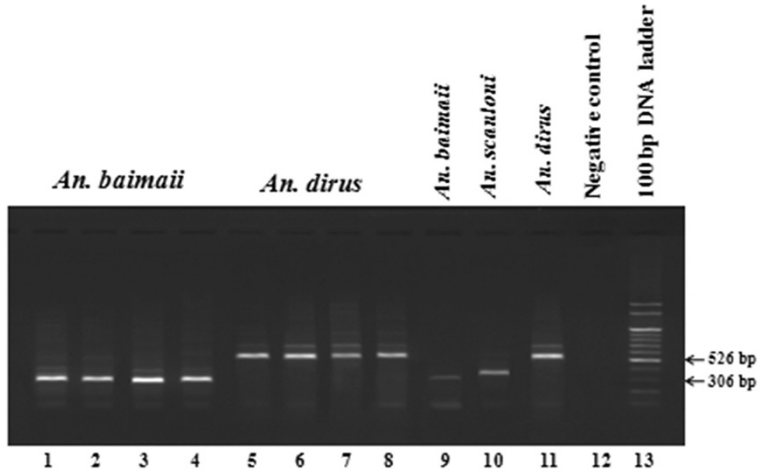


Figure 1 Multiplex Allele-Specific PCR assay. Lanes 1–4: *An. baimaii*; lanes 5–8: *An. dirus*; lanes 9–11: positive of *An. baimaii*, *An. scanloni*, *An. dirus*; lane 12: negative control; lane 13: 100 bp molecular ladder.

the presector pale spot (PSP) of the costa vein of at least one wing were identified as *An. dirus*. Molecular identifications using the AS-PCR assay of Walton *et al*. [8] and Audtho *et al*. [24] were subsequently applied to confirm the species identification, using the specific primers for *Anopheles dirus*, *Anopheles cracens*, *Anopheles scanloni*, *Anopheles baimaii* and *Anopheles nemophilous* (Figure 1).

An. minimus complex

Members of the *An. minimus* subgroup were identified to species using illustrated morphological keys for adult *Anopheles* in Thailand [2]. *An. minimus* was identified when a presector pale spot (PSP) was present on the costal vein of both wings, whereas *An. harrisoni* required, at least, the presence of both presector pale and humeral pale spots (HP) on one wing [25]. Molecular identifications were performed using the AS-PCR assay of Garros *et al*. [26], and was conducted using primers specific for *An. minimus* and *An. harrisoni*.

An. maculatus group

All specimens of the *An. maculatus* group were identified to species using illustrated morphological keys for adult *Anopheles* in Thailand [2]. *An. maculatus* has the R2 + 3 wing veins with one dark spot on both wings and the presector dark (PSD) spot on the R vein is usually shorter than the PSD spots on the subcosta and costa. The R4 + 5 wing veins have 2 dark spots and the abdominal terga segments VII, VIII sometimes on VI are covered with dark scales on the posterolateral corners. Molecular identifications were performed using the AS-PCR assay of Walton *et al*. [27], and were conducted using primers specific for *An. maculatus* and *An. sawadwongporni*.

DNA extraction

DNA for all *Anopheles* specimens was extracted using Genomic DNA from individual adult mosquitoes, according to the procedures of Linton *et al*. [28] and Manguin *et al*. [20].

Data analysis

Three key factors were selected for analysis and included seasonality, biting time and collection method. Seasonal periods were identified as wet (June to October), dry (November to February) and hot (March to May), biting times are separated into early evening (1800–2100 hours), late night (2100–2400 h), predawn (2400–0300 h) and dawn (0300–0600 h), and collection types are listed as indoor HLC, outdoor HLC and cattle baited captures.

The nocturnal biting cycle of *An. dirus* and *An. baimaii* were tabulated by averaging the number of *Anopheles* landing per hour per human by indoor and outdoor location and by averaging the number of mosquitoes captured per cow per hour. Comparisons of landing data were analyzed by non-parametric Kruskal-Wallis tests. The accepted level of significance was determined at 0.05% (P-valve < 0.05), followed by correlation coefficient (r) analysis taking into account the correlation between mosquitoes captured and environmental variables. All data were analyzed using the SPSS statistical package (version 17.0, SPSS, Chicago, IL).

Results

Observations on adult anopheline diversity, collected from September 2009 to August 2010 at Pu Teuy Village, Kanchanaburi Province, western Thailand are given in Table 1. From a total of 9,824 specimens, 656 belong to the *An. dirus* complex (6.68%), 8,802 were from the *An. minimus* complex (89.6%) and 366 were from the *An.*

Table 1 Monthly frequency of *Anopheles* mosquitoes at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, for one year (September 2009-August 2010)

Month	*Anopheles* complexes						
	An. dirus		*An. minimus*		*An. maculatus*		Total
	DIR	BAI	MIN	HAR	MAC	SAW	
September	30	3	9	372	0	0	414
October	54	9	7	234	0	0	304
November	23	4	35	939	0	0	1001
December	0	1	21	676	0	0	698
January	0	0	65	824	0	0	889
February	0	0	64	1115	0	0	1179
March	0	0	34	713	4	0	751
April	4	0	31	554	26	8	623
May	17	4	43	873	43	30	1001
June	51	5	89	783	43	30	1001
July	124	13	74	994	77	42	1324
August	295	19	14	239	50	35	652
Total	598	58	486	8316	239	127	9824

DIR: *An. dirus*, BAI: *An. baimaii*, MIN: *An. minimus*, HAR: *An. harrisoni*, MAC: *An. maculatus*, SAW: *An. sawadwongporni.*

maculatus group (3.72%). Six important species were molecularly identified to include *An. dirus*, *An. baimaii*, *An. minimus*, *An. harrisoni*, *An. maculatus* and *An. sawadwongporni* (Table 1). Among these, four species, *An. dirus*, *An. baimaii*, *An. minimus* and *An. maculatus* are considered to be important malaria vectors in Thailand [1,29]. In the study, the two most recognized malaria vectors, *An. dirus* and *An. baimaii*, were further characterized to obtain some bionomic information on the biting activity, host preference and density.

Table 2 provides the monthly distribution of *An. dirus* and *An. baimaii* collected by the three collection methods during the one year study period. From a total of 656 specimens in the *An. dirus* complex, 598 specimens (91.2%) were *An. dirus* and 58 (8.8%) were *An. baimaii*. Greater numbers of these two species were collected during the rainy season (June to October) with a distinct peak in August. For *An. dirus*, 378 (63.2%) were captured on cattle, 168 (28.1%) were collected from outdoor human landing collection, and 52 (8.7%) were obtained from indoor human landing collection. In contrast, a total of 58 specimens of *An. baimaii* were captured from all three collection methods. Twenty-nine (50%) were captured on cattle, 23 (39.7%) were obtained from outdoor human landing collection, and 6 (10.3%) from indoor human landing collection (Table 2). Our results showed that *An. dirus* was more attracted by cattle than humans and, in the latter case, more outdoors than indoors, regardless of the time periods and climatic seasons.

Total mosquito biting frequencies by hour and collection method for *An. dirus* and *An. baimaii* are given in Figures 2–3. *An. dirus* specimens were found to exceed *An. baimaii* in numbers for all collection periods. The indoor biting activity of *An. dirus* presented one prominent peak between 1900 and 2000 h and a smaller peak between 0200 and 0300 h (Figure 2). The outdoor human landing activity was elevated from 2300 to 2400 h (Figure 2). In contrast, cattle baited collections showed one clear peak for *An. dirus* in the early evening (1900–2000 h) followed by a decline throughout the rest of the night (Figure 2). Although low numbers of *An. baimaii* were obtained, the outdoor activity peaks were clearly defined with a distinct outdoor peak between 2400 and 0100 h whereas the indoor peak was difficult to determine due to the low number of specimens collected (Figure 3). Cattle baited catches showed two clear peaks for *An. baimaii* in the early evening (1900–2000 h) and midnight (2400–0100 h) (Figure 3).

The total number of landing mosquitoes per hour was subjected to a Kruskal-Wallis test, with seasons (dry, hot, and wet), collection methods (indoor and outdoor human bait and cattle bait) and time intervals (early evening, late evening, predawn, and dawn) as discriminating factors. A strong significant difference in the number of *An. dirus* and *An. baimaii* were found between seasons ($\chi^2 = 70.55$; df = 2; $P < 0.0001$ *An. dirus* and $\chi^2 = 27.34$; df = 2; $P < 0.0001$ *An. baimaii*), and between indoor, outdoor human landing and cattle bait ($\chi^2 = 11.59$; df = 2; $P = 0.003$ *An. dirus* and $\chi^2 = 6.07$; df = 2; $P = 0.048$ *An. baimaii*). There was no significant difference in the number of either species collected between the four quarterly evening time intervals ($\chi^2 = 2.21$; df = 3; $P = 0.529$ *An. dirus* and $\chi^2 = 3.68$; df = 3; $P = 0.298$ *An. baimaii*). Data from all collection methods was pooled to determine the correlation between mosquito abundance and environmental variables (Figure 4). *An. dirus* and *An. baimaii* densities strongly correlated with the total rainfall ($r = 0.454$; $P = 0.016$ *An. dirus* and $r = 0.609$; $P = 0.003$ *An. baimaii*) but were not related with relative humidity and minimum or maximum ambient air temperatures ($P > 0.05$).

Discussion

Of 73 known *Anopheles* species found in Thailand, some members of *An. leucosphyrus* and *An. maculatus* groups and *An. minimus* subgroups, are recognized as important malaria vectors [2]. Five species have been incriminated as malaria vectors in Thailand, including *An. dirus* [15], *An. baimaii* [30], *An. minimus* [31], *An. pseudowillmori* [30] and *An. aconitus* [30,32,33]. In this study, the six species found belong to two groups and one subgroup, including two members of the *An. dirus* complex found in the *An. leucosphyrus* group, *An. dirus* and *An.*

Table 2 Total of monthly captures from three collection methods of *Anopheles dirus* and *Anopheles baimaii* from Pu Teuy Village, Sai Yok District, Kanchanaburi Province

Month	*An. dirus*				*An. baimaii*				T	H	R
	In(%)	Out(%)	Cow(%)	Total(%)	In(%)	Out(%)	Cow(%)	Total(%)			
Sep	7(23.3)	9(30)	14(46.7)	30	0(0)	0(0)	3(100)	3	28.97	78.38	168.70
Oct	8(14.8)	4(7.4)	42(77.8)	54	1(11.1)	3(33.3)	5(55.6)	9	28.20	79.44	156.90
Nov	0(0)	5(21.7)	18(78.3)	23	1(25)	3(75)	0(0)	4	20.08	85.44	156.90
Dec	0(0)	0(0)	0(0)	0	1(100)	0(0)	0(0)	1	22.47	78.37	7.00
Jan	0(0)	0(0)	0(0)	0	0(0)	0(0)	0(0)	0	20.37	83.47	12.10
Feb	0(0)	0(0)	0(0)	0	0(0)	0(0)	0(0)	0	26.11	77.63	0.00
Mar	0(0)	0(0)	0(0)	0	0(0)	0(0)	0(0)	0	25.63	83.06	76.50
Apr	0(0)	0(0)	4(100)	4	0(0)	0(0)	0(0)	0	27.33	84.01	26.00
May	2(11.8)	3(17.6)	12(70.6)	17	1(25)	2(50)	1(25)	4	26.36	83.11	63.00
Jun	1(1.9)	14(27.5)	36(70.6)	51	0(0)	0(0)	5(100)	5	26.06	92.08	183.00
Jul	12(9.7)	50(40.3)	62(50)	124	1(7.6)	6(46.2)	6(46.2)	13	24.86	88.83	291.00
Aug	22(7.5)	83(28.1)	190(64.4)	295	1(5.2)	9(47.4)	9(47.4)	19	25.80	87.25	208.00
Total	52	168	378	598	6	23	29	58			
(%)	8.7	28.1	63.2		10.3	39.7	50.0				

R: Rainfall (mm), H: Humidity (%), T: Temperature (°C).

baimaii; two members of the *An. maculatus* group, *An. maculatus* and *An. sawadwongporni*; and two members of the *An. minimus* complex belonging to the *An. minimus* subgroup, *An. minimus* and *An. harrisoni*. Previous studies have successfully described the trophic behavior, biting activity, and seasonal abundance in the *An. maculatus* group and *An. minimus* subgroup [9,34], whereas no such studies have documented these activities for the two most important malaria vectors within the *An. leucosphyrus* group, *An. dirus*, *An. baimaii*.

Anopheles baimaii and *An. dirus,* are considered to be the primary malaria vectors in Thailand [2,35]. These two closely related species cannot be differentiated from each other by morphological characteristics alone [8,25]. Both are forest and forest-fringe mosquitoes that are considered highly anthropophilic [2,11]. The preferred

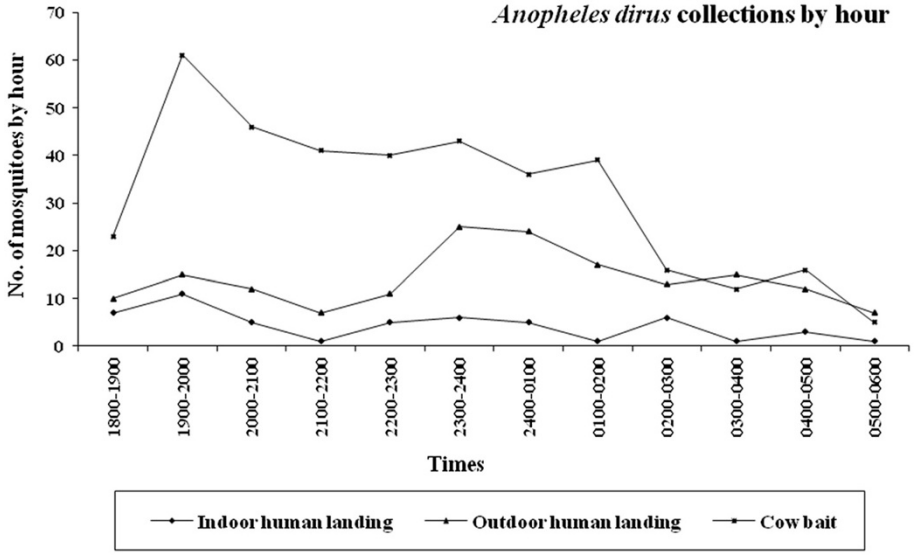


Figure 2 Temporal patterns of *Anopheles dirus* and blood feeding activity for indoor, outdoor human landing and cattle bait collections.

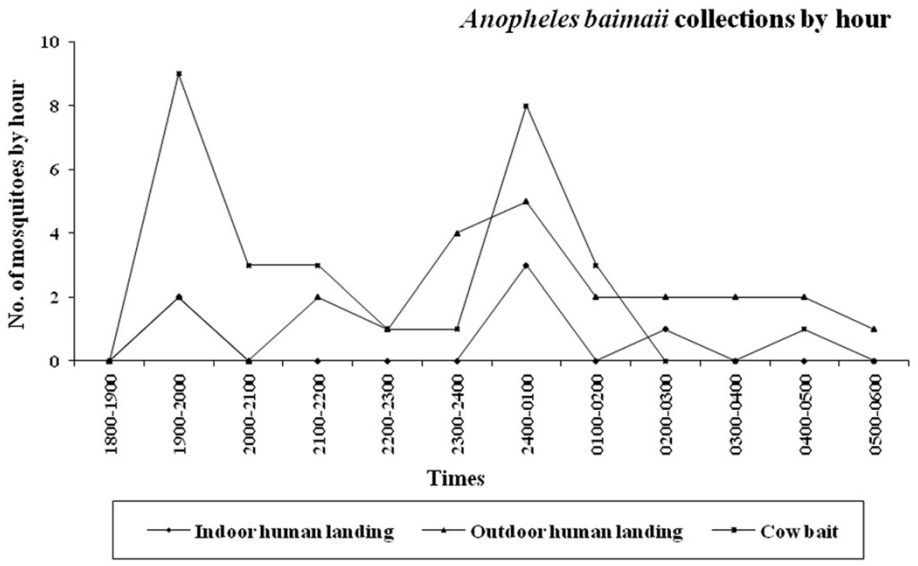


Figure 3 Temporal patterns of *Anopheles baimaii* blood feeding activity for indoor, outdoor human landing and cattle bait collections.

wheel-tracks and temporary ground pools in heavily shaded areas. In addition, larval habitats can be found in water jars, cut tree stumps, and root holes. *An. dirus* is the only species that is found throughout Thailand and often occurs in sympatry with *An. baimaii* in the western part of the country [36]. Both species are considered very anthropophilic in their blood feeding preference and demonstrate both exophagic and endophagic behaviors, and in some cases a generally greater tendency toward exophily [3,5,35]. However, interpretation of these early studies was hampered by species identification that was based on morphological characters, which was unreliable resulting in misidentification of species. This complicated the behavioral analysis of each sibling species within the complex [3,17]. Unlike these earlier studies, species of mosquitoes reported in this study were subjected to a multiplex AS-PCR, providing accurate species identification in order to describe with reliability the trophic behavior, seasonal abundance, and host preference of *An. dirus* and *An. baimaii* in Pu Tuey Village, Kanchanaburi Province. *An. dirus* demonstrated a strong zoophilic behavior. More mosquitoes were caught on cattle bait

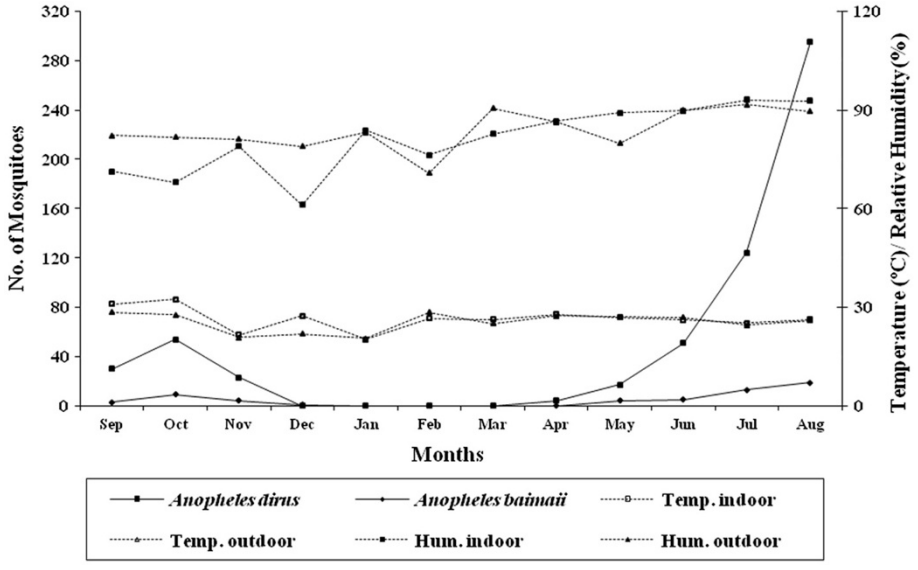


Figure 4 Monthly collections of *Anopheles dirus* and *Anopheles baimaii* in relation to average ambient air temperature and humidity in Pu Teuy Village, Sai Yok District, Kanchanaburi Province.

compared to human landing from both inside and outside houses (63.2% of *An. dirus*). Results of previous studies on biting activity and host preference of *An. dirus* complex in central Thailand demonstrated a delayed and more prolonged feeding peak that occurred between 2000 and 2400 h [13,14]. In southern Thailand, Scanlon and Sandhinand [12] reported the peak biting activity to occur between 2400 and 0300 h. Rosenberg *et al.* [15] observed a biting peak that occurred between 2200 and 0100 h, and Rattanarithikul *et al.* [16] reported a single early-evening biting peak between 2000 and 2200 h. In western Thailand, Sungvornyothin *et al.* [17] observed the biting activity between 2000 and 2100 h for both indoor, outdoor human landing collections and between 2000 and 2300 h in cattle baited collections. All of those biological and behavioral studies for *An. dirus* used only morphological characteristics for identification. This high degree of variability in biting patterns for populations found throughout Thailand might be the result of the mixed populations of species.

Our study described the biting behavior and blood feeding activities based on a clear and reliable identification of the sibling species of the *An. dirus* complex using PCR technology. The indoor biting activity of *An. dirus* recorded in this study demonstrated a peak between 1900 and 2000 h. Outdoor human landing collections identified a prominent peak between 2300 and 0100 h. On the other hand, cattle baited collections showed one clear peak in the early evening between 1900 and 2100 h, followed by a declining trend. *An. baimaii* was collected in a small proportion compared to *An. dirus* and showed an ambiguous pattern of feeding activity. Indoor numbers were too small to evaluate but outdoor activity peaked between 2400 and 0100 h. Cattle baited catches showed two clear peaks at both 1900–2100 h and at 2400–0100 h. Accurate identification of the species using multiplex PCR technology could explain the differences in feeding patterns of these sibling species complexes when compared to earlier published reports. Previous studies have shown that the morphological identification alone of the two sibling species of the *An. minimus* complex is not reliable and can result in nearly 40% misidentification of specimens [9,18].

The seasonal abundance of the *Anopheles* mosquitoes collected in this study appeared to be influenced by several factors. Adult densities were found to be positively associated with increased rainfall (July to August). This is clearly seen from the fact that the greatest numbers of adults were found during the wettest period of the year. Rainfall dependent abundance patterns have previously been reported for Thailand and Bangladesh [5,10,17,37]. This suggests that high rainfall provides adequate larval habitats for *An. dirus* that prefers temporary breeding habitats such as animal footprints, wheel tracks, and temporary ground pools common during the wet season. An inverse relationship with rainfall was documented for *An. minimus* [9] and *An. maculatus* in the same locality [34], wherein these two species have been shown to prefer breeding at the edges of slow-running streams [9,38]. Heavy rainfall would flush these habitats out resulting in reduced adult densities. In contrast, a negative association was found with a higher mean ambient temperature and relative humidity. Although, the number of malaria cases is low in Pu Teuy Village, the area remains at risk for increased transmission due to a high degree of human movement (parasite introduction) from highly malarious areas. Also, the current findings agree with previous work that demonstrated that efficient malaria vectors such as, *An. dirus*, *An. minimus* and *An. maculatus* are commonly found in greater abundance during the rainy periods of the year.

Conclusions

Accurate identification of the species with the use of multiplex PCR technology could explain the differences in feeding patterns of these sibling species, *An. dirus* and *An. baimaii* in Pu Teuy Village as compared to previously published work from other localities in Thailand. The current findings confirm that these species have a higher propensity to feed outdoors compared to indoors as previously published. A better understanding of the members of the Dirus sibling species complex, *An. dirus* and *An. baimaii* behavior related to host preference and both temporal and spatial feeding activity will help facilitate the design and efficiency of malaria vector control operations in Thailand.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors have contributed significantly to this study. TC and JPG assisted with study design. CT, RT and TC did the laboratory and field data collection. SM and WJ carry out the molecular genetic studies. AP performed statistical analysis. All authors contributed to the production of the manuscript and revision, with TC as final guarantor. All authors read and approved the final version of the manuscript.

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SEASONAL ABUNDANCE AND DISTRIBUTION OF *ANOPHELES* LARVAE IN A RIPARIAN MALARIA ENDEMIC AREA OF WESTERN THAILAND

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Abstract. Three taxonomic groups of *Anopheles* larvae were morphologically identified within the Funestus Group (Minimus Subgroup and Aconitus Subgroup) (75.63%), Maculatus Group (20.47%), and Barbirostris Group (0.57%) during a two-year period in conjunction with active malaria transmission in a village near the Thai-Myanmar border in Kanchanaburi Province, western Thailand. The remaining 3.33% of anophelines collected were *Anopheles culicifacies* (3.07%), *Anopheles philippinensis* (0.17%), and *Anopheles vagus* (0.09%). Using an allele-specific multiplex molecular identification assay, the Minimus Subgroup consisted of *Anopheles minimus* (69.83%), and *Anopheles harrisoni* (0.06%) and 2 genetically-related species belonging to the Aconitus Subgroup, *Anopheles aconitus* (0.63%) and *Anopheles varuna* (5.12%). The Minimus and Aconitus Subgroup species were more abundant during the dry season (52.58%) than during the hot (24.95%) and wet (22.46%) seasons. The number of *Anopheles* larvae collected from the stream habitat was significantly higher during the second year than the first year, believed to be due to human environmental changes in the stream habitat from the building of a small check dam, which provided a more suitable and stable habitat for mosquito larval development. This study illustrates the importance of conducting site-specific studies to accurately determine vector bionomics (*eg*, larval habitats) and adult activity patterns and linking observations with malaria transmission dynamics in a given area.

Keywords: *Anopheles*, seasonal abundance, Minimus Complex species, larval abundance, Thailand

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INTRODUCTION

Malaria is one of the most important mosquito-borne infectious diseases in tropical and sub-tropical climes (WHO,

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Diagnostic Doses of Two Pyrethroids Currently Used for Control of *Aedes aegypti* L. (Diptera: Culicidae), a Vector of Dengue

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ABSTRACT

Determining the diagnostic dose is crucial in evaluating the susceptibility of a mosquito population to certain insecticides. Presently, the diagnostic doses of only two pyrethroids (permethrin and λ-cyhalothrin) have been published for monitoring insecticide susceptibility of *Aedes aegypti*. Therefore, the objective of this study was to establish the diagnostic doses for two synthetic pyrethroids—bifenthrin and α-cypermethrin—currently used in dengue vector control, using the United State Department of Agriculture (USDA) standard strain of *Aedes aegypti*. These diagnostic doses were subsequently used to evaluate the susceptibility status in field collected specimens of *Ae. aegypti*. Based on the baseline susceptibility levels, all three field strains demonstrated varying levels of physiological resistance to each compound. Strong resistance to bifenthrin was seen in one strain from Nong Khai resulting in 14.14% mortality whereas complete mortality (100%) was observed in the USDA standard strain when exposed to the established diagnostic doses of these two synthetic pyrethroids. These diagnostic doses could be applied for monitoring the susceptibility status in mosquito populations across Thailand.

Keywords: insecticide, diagnostic dose, *Aedes aegypti*, pyrethroids, Thailand

INTRODUCTION

Over 100 countries around the world are at risk from a wide variety of vector-borne diseases, including dengue fever (DF) and dengue hemorrhagic fever (DHF), with an estimated 2.5 billion people at risk of contracting dengue many of whom live in Southeast Asia (WHO, 2002). Approximately 50–100 million people are infected annually with dengue viruses worldwide, primarily in crowded, impoverished urban regions of the world (WHO, 2009). In Southeast Asia, DHF cases have been increasing from an annual rate of below 10,000 in the 1960s to more than 200,000 in the 1990s (Gibbons and Vaughn, 2002; WHO, 2009). In Thailand, there were 115,434 dengue cases in 2010 with 141 reported deaths (Bureau of the Vector-borne Diseases, 2010)

The four viral serotypes (DEN-1, 2, 3, 4) are transmitted primarily by *Aedes aegypti* (L.), a highly efficient mosquito vector that is often found in and near human dwellings. In Thailand, this mosquito-borne disease causes tremendous morbidity and mortality each year. To date, no effective or commercial multivalent dengue vaccine is available. Prevention of this disease

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remains almost entirely dependent on using various methods of vector control by attacking both the adult and immature stages of the mosquito. Vector control remains the most effective means of reducing the risk of virus transmission (Reiter and Gubler, 1997; WHO, 1999). Unfortunately, *Ae. aegypti* has confounded organized control efforts due to its close association with humans and its exploitation of domestic and peridomestic environments. In Thailand, the standard vector control techniques are based on the use of chemicals and source reduction.

Many chemical compounds, including organophosphates, carbamates, synthetic pyrethroids and so-called bio-rational pesticides (bacterial toxins and insect growth regulators) have long been used in national public health vector control programs (Reiter and Gubler, 1997; WHO, 1998a). In Thailand, several synthetic pyrethroids, for example, deltamethrin, cyfluthrin, bifenthrin, α-cypermethrin, and permethrin, have been introduced into the public market for controlling *Ae. aegypti* (Chareonviriyaphap *et al*., 1999; Somboon *et al*., 2003; Thanispong *et al*., 2010). However, control of this mosquito has been hampered by the development of resistance to these insecticidal compounds in *Ae. aegypti* populations throughout Thailand (Chareonviriyaphap *et al*., 1999; Somboon *et al*., 2003). The selection for resistance to pyrethroids has largely been attributed to the frequent use of the same chemical class of compounds and is believed to have a direct bearing on the effective management and prevention of vector-borne diseases in general (Hemingway and Ranson, 2000). Although, several published reports have described the status of pyrethroid resistance in *Ae. aegypti* populations in Thailand (Chadwick *et al.*, 1977; Chareonviriyaphap *et al.*, 1999; Paeporn *et al.*, 2004; Yaicharoen *et al.*, 2005; Sathantriphop *et al*., 2006; Jirakanjanakit *et al*., 2007; Thanispong *et al*., 2008), these reports were based on the use of 'diagnostic' doses established by the World Health Organization (WHO, 1998b). Baseline insecticide susceptibility has not been established for the detection of insecticide resistance in any populations of *Ae. aegypti* in Thailand. The aim of this study was to establish the diagnostic dosage of two synthetic pyrethroids—befenthrin and α-cypermethin—using the USDA susceptible standard strain of *Ae. aegypti*. These values were then used to determine the susceptibility of field strains of *Ae. aegypti* in Thailand.

MATERIALS AND METHODS

Mosquito strains

A susceptible standard strain of *Ae. aegypti* (USDA) was used to establish the baseline susceptibility levels of two synthetic pyrethroids that were used to determine the susceptibility status of three local strains of *Ae. aegypti* as follows: 1) the USDA laboratory strain was provided by the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida, U.S.A. This colony has been maintained in colony for over 40 y; 2) the Kanchanaburi strain was obtained in May 2010 as larvae from outdoor container habitats at Pu Teuy village, Sai Yok district (14°20′12.1″N, 98°59′19″E), Kanchanaburi province, an area west of Bangkok; 3) the Khon Kaen strain was obtained in November 2010 as larvae from outdoor container habitats in Muang district (16°24′52″N, 102°05′5″E), Khon kaen province, northeast Thailand; 4) the Nong Khai strain was obtained in November 2010 as larvae from outdoor container habitats in Tha Bor district (17°51′51.1″N, 102°34′30.5″E), Nong Khai province, northeast Thailand.

Mosquito rearing

Mosquito larvae and pupae were collected from each site, brought back to the insectary at the Department of Entomology, Kasetsart University, and reared to the adult stage. Adult mosquitoes identified as *Ae. aegypti* were transferred to a mosquito cage and permitted guinea-pig blood on the third/fourth day post emergence. Two

days postbloodfeeding, oviposition dishes were placed in the cages containing gravid females. All strains were colonized separately under laboratory-controlled conditions at 25 ± 3°C and 75 ± 15% RH in the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

Chemicals

Two insecticides—namely, α -cypermethrin (purity 97.05%; Cherwood Chemical Ltd; Bangkok, Thailand) and bifenthrin (purity 95.12%; Cherwood Chemical Ltd; Bangkok,Thailand)—were used in the susceptibility testing.

Insecticide impregnated paper

Test papers 12 × 15 cm^2 impregnated with each chemical at a single diagnostic dose (99% lethal dose (LD_{99}) ×2) established from the USDA standard susceptible strain, were prepared in the laboratory at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand (Table 2). All papers were treated at the rate of 2 mL of the insecticide solution per 180 cm^2.

Susceptibility assay

The level of susceptibility of each population to the two synthetic pyrethroids was assessed by exposing 25 sugar fed 3–5 day-old female mosquitoes to a single diagnostic dose established from the USDA standard strains. Standard testing procedures were followed according to WHO guidelines (1998b). After a 60 min exposure, tested and control mosquitoes were transferred to separate holding containers and mortalities were recorded after 24 h post-exposure. The test was repeated four times and an average susceptibility level was derived for each strain. Based on the percent mortality in the test population, mosquito survival was indicative of the degree of physiological resistance in that population.

Analysis

Control mortality was corrected using Abbott's formula (Finney, 1971). The LD_{99} value was calculated from a dosage-mortality regression line using the log-probit program from the Statistical Analysis System (SAS) software (SAS, 2002). The estimate of the 99% lethal dose (LD_{99}) was determined from four replicates conducted on the USDA susceptible strain of *Ae. aegypti*. Double the concentration for LD_{99} was determined as the "diagnostic dose" and used for susceptibility/resistance tests on *Ae.aegypti* field strains. Determination of resistance/susceptibility status was done according to WHO criteria (WHO, 1998b).

RESULTS

The baseline susceptibility levels to synthetic pyrethroids currently used in dengue vector control in Thailand were established from a standard susceptible strain (USDA). A baseline was established using the insecticide doses which gave mortality ranging between 10 and 95% in the USDA susceptible strain. Small chi-square values (0.9419 for α-cypermethin and 0.3082 for bifenthrin) demonstrated that the responses of *Ae. aegypti* mosquitoes to each compound in the susceptibility trials satisfied a linear model ($P > 0.05$) as shown in Table 2. The 50% lethal dose (LD_{50} value) and the 99% lethal dose (LC_{99} value) to two synthetic pyrethroids against *Ae. aegypti* (USDA) were determined by using log-probit analysis (SAS, 2002). Alphacypermethrin produced an LD_{50} value of 0.0009% whereas bifenthrin gave an LD_{50} value of 0.0185%. With LD_{99} values, α-cypermethrin produced the dose at 0.043% whereas bifenthrin yielded the dose at 0.047%. A single diagnostic concentration (double concentration of LD_{99}) of α-cypermethrin (0.086%) and bifenthrin (0.094%) was obtained (Table 2). The established diagnostic dose was subsequently used to determine the susceptibility level of three field strains of *Ae. aegypti* (Kanchanaburi, Khon

Kaen and Nong Khai) (Table 1).

The results of the susceptibility tests using the established diagnostic dose for the two synthetic pyrethroids are presented in Table 3. The ability of mosquitoes to survive the diagnostic dose after 24 h is indicative of resistance in the population. Based on WHO recommendations, the interpretation of insecticide susceptibility results is divided into three categories: 1) mosquitoes are susceptible to insecticides if the percent mortality is between 98 and 100%; 2) mosquitoes demonstrate incipient insecticide resistance if the percent mortality is between 80 and 97%; or 3) mosquitoes are resistant if the percent mortality is less than 80% (WHO, 1998b).

The results of the current study demonstrated that the USDA standard strain was completely susceptible to all compounds as indicated by 100% mortality to all compounds tested. However, when the *Ae. aegypti* from the three different localities were tested, various levels of tolerance/resistance to the two synthetic pyrethroids were revealed (Table 3). Based on established diagnostic dosages, a trend of tolerance/resistance to the two pyrethroids was identified in the three field strains with mortality rates ranging between 9.68% for bifenthrin and 97.96% for α-cypermethrin. The Khon Kaen and Nong Khai strains were found greatly resistant to bifenthrin as indicated by the low percent mortality results (9.68% for Khon Kaen and 14.14% for Nong Khai). The Kanchanaburi and Nong Khai strains of *Ae. aegypti* were slightly tolerant to α-cypermethrin (97.83% and 97.96% mortality, respectively).

DISCUSSION

Globally, mosquito-borne diseases kill thousands of people each year (WHO, 2009). Mosquitoes are a nuisance and a public health threat and serve as potential vectors of parasitic

Table 1 Description and GPS coordinates of field sites where collections were made.

Collection site/province	Village	District	GPS coordinates
Kanchanaburi	Pu Teuy	Sai Yok	14°20′12.1″N, 98°59′19″E
Khon kaen	Non Ton	Muang	16°24′52″N, 102°05′5″E
Nong Khai	Pa Ngew	Tha Bor	17°51′51.1″N, 102°34′30.5″E

Table 2 Susceptibility data of two pyrethroids based on dose/mortality relationships tested against *Aedes aegypti*, USDA strain.

Insecticide	No. Tested	LD_{50} (%)	95% CI	LD_{99} (%)	95% CI	Diagnostic dose (%) ($2xLD_{99}$)	Chi square
α-Cypermethrin	302	0.0009	0.0004-0.0013	0.043	0.0220-0.1449	0.0863	0.9419
Bifenthrin	282	0.0185	0.0171-0.0202	0.047	0.0396-0.0599	0.0938	0.3082

LD_{50} = 50% lethal concentration; 95% CI = 95% confidence interval; LD_{99} = 99% lethal concentration.

Table 3 Insecticide susceptibility level of four strains of *Aedes aegypti*.

Insecticide	Diagnostic	USDA		Kanchanaburi		Khon Kaen		Nong Khai	
	Dose (%)	No. tested	Dead (%)	No. tested	Dead (%)	No. tested	Dead (%)	No. tested	Dead (%)
α-Cypermethrin	0.086	100	100 (100)	92	90 (97.83)	104	92 (88.46)	98	96 (97.96)
Bifenthrin	0.094	97	97 (100)	92	85 (92.39)	93	9 (9.68)	99	14 (14.14)

diseases such as malaria (Krogstad, 1996; WHO, 2002), dengue/dengue hemorrhagic fever (Gubler *et al*., 1995; Brogdon *et al*., 1998) and filariasis (Thompson *et al*., 1996) among others. One of the keys to preventing disease transmission is the reduction of human-vector contact through the use of insecticides (Roberts *et al*., 1997; Jirakanjanakit *et al*., 2007). However, the long-term use of insecticides may result in insecticide resistance in an insect population of, for example, mosquitoes, body lice, bedbugs, fleas, ticks, flies and cockroaches (WHO, 1992; Roberts *et al*., 1994; Thanispong *et al*., 2010).

The information on insecticide resistance in *Ae. aegypti*, which is a vector of dengue/ dengue haemorrhagic fever, is relatively scarce due to a shortage of comprehensive, sustainable and effective monitoring systems used within the framework of the national public health vector control program in Thailand. *Aedes aegypti* is one of the most efficient, well-adapted and widely distributed mosquito species found throughout the tropical and sub tropical zone and has proven extremely difficult to control. Among several available control techniques, chemical control remains the most effective proven method to reduce dengue transmission. Of the chemicals available to choose from, synthetic pyrethroids are the most common and extensively used in both the government and private sectors. Pyrethroids have the potential to be effective adulticides at present (Chareonviriyaphap *et al*., 1999; Kongmee *et al*., 2004, Jirakanjanakit *et al*., 2007; MOPH, 2010). In vector control programs, ultra-low volume (ULV) application of deltamethrin has been effective at interrupting dengue transmission when used soon after the first dengue case is reported. For household use, several synthetic pyrethroids are currently available for homeowners to control household arthropod pests. However, long-term, continuous and repetitive contact with insecticides has resulted in some degree of insecticide resistance in *Ae. aegypti* mosquito populations.

Although a number of studies on pyrethroid resistance in *Ae. aegypti* have subsequently been published, most have reported findings that follow the WHO standard criteria used for monitoring resistance in *Anopheles* spp. mosquitoes which may not be appropriate or reliable for all vector species. Only diagnostic dosages of lambda-cyhalothrin (0.03%) and permethrin (0.25%) have been reported for detecting the resistance status of *Ae. aegypti* populations (WHO, 2011). Diagnostic tests for insecticide resistance to the currently labeled chemicals used in vector control are of paramount importance in order to monitor and evaluate their effectiveness after long-term use in a vector control program. In recent years, diagnostic doses of two commonly used synthetic pyrethroids—permethrin (0.9%) and deltamethrin (0.06%)—were established for a susceptible reference strain of *Ae. aegypti* in Bora, French Polynesia (Jirakanjanakit *et al*., 2007). However, these two diagnostic doses were obtained from an analytical process of log-probit mortality relationships which gave a very high chi-square and an extremely low *P*-value ($P < 0.005$), indicating a poor goodness of fit (Jirakanjanakit *et al*., 2007). It is still believed that conclusive information on susceptibility baselines and diagnostic doses for various compounds used to control *Ae. aegypti* are lacking and that information on the susceptibility status of *Ae. aegypti* to pyrethroids may not be completely accurate. Furthermore, data on insecticide susceptibility levels are limited to only small focal areas of Thailand (Chareonviriyaphap *et al*., 1999; Prapanthadara *et al*., 2002; Ponlawat *et al*., 2005; Jirakanjanakit *et al*., 2007; Thanispong *et al*., 2008; Chuaycharoensuk *et al*., 2011). In addition, WHO (2005) recommended that baseline insecticide susceptibility levels should be determined on a reference strain prior to evaluating the susceptibility level in any field mosquito populations. A global dengue control program may not succeed without adequate information on insecticide susceptibility levels in *Ae. aegypti* populations.

The current study used three field-collected *Ae. aegypti* strains to determine the possibility of applying the established diagnostic doses for two synthetic pyrethroids. The results indicated that the physiological resistance in Thai *Ae. aegypti* populations varies depending upon the mosquito strain and the test chemical. A strain from Kanchanaburi province demonstrated similar mortality levels to the susceptible USDA reference strain with only low levels of resistance to α-cypermethrin and bifenthrin as reported by Thanispong *et al.* (2008) and Chuaycharoensuk *et al.* (2011). Furthermore, the current findings show similar results to previous reports on the resistance levels of various strains of *Ae. aegypti* to synthetic pyrethroids (Chareonviriyaphap *et al*., 1999; Prapanthadara *et al*., 2002; Ponlawat *et al*., 2005; Jirakanjanakit *et al*., 2007; Thanispong *et al*., 2008; Chuaycharoensuk *et al*., 2011).

Synthetic pyrethroids are currently the insecticides of choice for dengue control in Thailand. The monitoring of insecticide resistance should be increased in consistency, periodicity, and geographic coverage and should be an integral part of any vector/public health program. The investigation of cross resistance to the same, similar or related synthetic compounds should also be included in any insecticide evaluation program. Knowledge of vector/pest susceptibility to pesticides, changing trends of resistance and their operational implications are basic requirements to guide pesticide use in vector-borne disease and pest control programs.

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Effects of Previous Exposure of *Aedes aegypti* (Diptera: Culicidae) Mosquitoes to Spatial Repellent Chemicals on BG-Sentinel™ Trap Catches

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ABSTRACT

The use of the BG-Sentinel™ (BGS) trap as the pull component in a push-pull strategy to control *Aedes aegypti* mosquitoes needs to include studies on the effects of previous exposure of the mosquito to spatial repellents (the push component). The study was conducted from January 2010 to May 2011 in Pu Teuy Village, Kanchanaburi, Thailand. *Aedes aegypti* females were exposed to spatial repellents ([1, 1, 1-trichloro-2,2-bis (4-chlorophenyl) ethane] (DDT), metofluthrin and transfluthrin) and then released within a screen house containing four BGS traps to determine the effect of the spatial repellent exposure on BGS trap capture rates from non-exposed control females. This included: (i) an immediate release experiment where females exposed during 0600–1200 hours were released at 1200 hours and (ii) a delayed release experiment where females exposed during 1200–1800 hours were released at 0530 hours the following day, thus having a recovery period of nearly 12 hours. Exposure of *Ae. aegypti* females to DDT or metofluthrin did not significantly impact BGS trap capture rates compared to non-exposed control females. However, exposure to transfluthrin resulted in a significantly lowered number of recaptured treated versus control females in the immediate release trial but not in the delayed release one. The findings indicated that previous exposure of *Ae. aegypti* to spatial repellents has minor, short-lived impacts on the capture success with BGS traps.

Keywords: *Aedes aegypti*, spatial repellents, screen house, experimental huts, BG-Sentinel™ trap, push-pull stategy, Thailand

INTRODUCTION

Push-pull strategies are effective in the control of some agricultural pests (Miller and Cowles, 1990). A push-pull system combines the use of: (i) behavioral manipulation of the target pest species to repel or deter (push) them away from a resource (in the case of agricultural pest species, this would be a crop) using stimuli that render the resource unsuitable or unattractive and (ii) a device, for example a trap, which is used to remove (pull) them from the environment

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(Miller and Cowles, 1990). This approach may also prove effective in the control of pathogen-transmitting mosquitoes, especially in and around the home where many vector-borne pathogens are predominantly transmitted in the developing world.

In the specific case of *Aedes aegypti*, the trap or pull component is the most problematic part of a push-pull system. Although traps for mosquito adults are commonly used for surveillance purposes, most are ineffective against a day-biting mosquito such as *Ae. aegypti* (Facchinelli *et al.*, 2008). Currently, a new trap, BG-Sentinel™ (BGS) trap, targets the most important elements of *Ae. aegypti* host-seeking behavior by combining an olfactory cue (BGS Lure) with a visual cue (black and white contrast) to attract the mosquito. This trap has proven to be an effective tool for surveillance of *Ae. aegypti* adults, out-performing other collection devices and traps such as the Center for Disease Control (CDC) backpack aspirator, the Fay-Prince trap, the Encephalitis Virus Surveillance trap and the Mosquito Magnet Liberty™ trap (Krockel *et al.*, 2006; Maciel-de-Freitas *et al.*, 2006; Williams *et al.*, 2006). Subsequent screen house studies in Thailand confirmed that this mosquito species can be effectively removed from a controlled environment using the BGS trap (Salazar *et al.*, 2012).

The push component of the system uses spatial repellent chemicals to deter *Ae. aegypti* from host-seeking within and around homes. The BGS trap provides the pull component to remove *Ae. aegypti* from the peridomestic environment thereby further reducing human-vector contact. However, for the pull component to be most effective, previous exposure to the spatial repellent should not substantially decrease the likelihood of the mosquito being trapped. To complement a reported earlier finding that BGS traps effectively capture *Ae. aegypti* that were not previously exposed to spatial repellents (Salazar *et al.*, 2012), the current study involved a follow-up experiment aiming to quantify the effect of previous exposure of *Ae. aegypti* to spatial repellents on BGS trap catch rates.

MATERIALS AND METHODS

Study area and experimental huts

Studies were conducted near Pu Teuy (14º17' N, 99º11' E), which is a small agricultural village (less than 1,500 inhabitants) located 150 km northwest of Bangkok in Sai Yok district, Kanchanaburi province, Thailand. *Aedes aegypti* is prevalent in Pu Teuy village. The experimental site was located more than 800 m from the closest indigenous home, creating a distance buffer for mark-release-recapture mosquito behavioral studies which exceeded the normal flight range of *Ae. aegypti* (Harrington *et al.*, 2005). The experimental huts used in the study mimic indigenous Thai homes in materials and dimensions (Chareonviriyaphap *et al.*, 2010).

Source and use of mosquitoes

Immatures of *Ae. aegypti* were collected weekly from Pu Teuy village and reared to adults in an on-site field insectary. Nulliparous, 3-5 d old sugar-starved females were used for all experimental trials. Mosquito test cohorts (control/treatment) were marked with a unique fluorescent powder color prior to use in studies following previous dusting protocols (Achee *et al.*, 2005). One day pre-trial cohorts of female mosquitoes (n = 50) were placed in individual mesh screen cages (26 × 26 × 30 cm) and provided access to water-soaked cotton pads. For each repellent treatment, two separate mosquito cohorts were used: (i) an immediate release (IR) cohort exposed during 0600–1200 hours and then released into the screen house containing BGS traps at 1200 hours and (ii) a delayed release (DR) cohort exposed during 1200–1800 hours and then released at 0530 hours the following day, thus having a recovery period of nearly 12 hr (with access to water-soaked cotton pads).

Description of the BG Sentinel™ trap

All BGS traps were baited with the BGS Lure and operated according to the manufacturer's instructions. The trap consists of a collapsible container made of a white, plastic-sack material. The BGS trap was aptly described by Salazar *et al*. (2012).

Chemical exposure of mosquitoes

Repellent compounds used in the study were selected based on their spatial repellent characteristics, recognized standards in household protection (as opposed to topical repellents that are applied directly on human skin) and high potential for successful use in a push-pull system. [1, 1, 1-trichloro-2,2-bis (4-chlorophenyl) ethane] (DDT) belongs to the organochlorine group of synthetic insecticides and is known for its environmental persistence (Agency for Toxic Substances and Diseases Registry [ATSDR], 2002). Metofluthrin [2,3,5,6-tetrafluoro-4-methoxymethylbenzyl (*E, Z*)(1*R*, 3*R*)-2,2-dimethyl-3-(prop-1-enyl) cyclopropanecarboxylate (S-1264)] is a more recently synthesized compound produced by Sumitomo Chemical Co. Ltd., Osaka, Japan (Shono *et al.*, 2004; Ujihara *et al.*, 2004). This compound has a high vapor pressure (1.87×10^{-3} Pa at 25 ^{o}C), twice that of *d*-allethrin and 100 times greater than permethrin (Ujihara *et al.*, 2004), and vaporizes at room temperature. Transfluthrin [cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-(2,3,5,6-tetrafluorophenyl)methyl ester, (1R,3S)] is a fast-acting insecticide that exhibits high volatility (9.4×10^{-4} Pa at 20 ^{o}C) and knockdown activity (WHO, 2002).

For the experimental evaluation, the repellent treatment consisted of either chemical-treated polyester fabric (DDT or transfluthrin) or a standard mosquito coil (metofluthrin) that produces smoke to distribute the active ingredient in a given space. Matched huts containing either untreated fabric (solvent only) or blank coil (coil without metofluthrin, but being burned and thus providing smoke) were used simultaneously as negative controls. For trials with mosquito coils, an additional hut containing no coil was used as a second control. A total of four replicates were performed for each chemical treatment.

DDT exposure was conducted using a field application rate (FAR; 2g active ingredient (*ai*).m^{-2}) at 75%, 50% and 25% treatment surface area coverage [SAC]. Assessments were also performed using 25% SAC against 0.5 FAR (1g *ai*.m^{-2}). Transfluthrin evaluations included 1.0 (40 µg *ai*.m^{-2}), 0.50 (20 µg *ai*.m^{-2}), 0.125 (5 µg *ai*.m^{-2}), and 0.062 FAR (2.5 µg *ai*.m^{-2}) using 25% surface area coverage. The surface area coverage for transfluthrin was based on data from studies evaluating the push-pull system that showed 25% coverage to be as effective as coverages of 50% and 100% (Achee *et al.*, 2009). Metofluthrin coils were burned on top of a metal dish and positioned in the center of the hut. Coils were lit 15 min before the mosquitoes were taken into the hut. High (0.006% *ai*) and low (0.003% *ai*) dose coils were evaluated.

Trapping of mosquitoes previously exposed to spatial repellents

BGS trap catch rates for mosquitoes previously exposed to the spatial repellents were evaluated in a screen house, measuring 4 m (width) × 3.5 m (height) × 40 m (length), which was located on-site for use in semi-field experiments and to facilitate the recapture of released *Ae. aegypti* (Salazar *et al.*, 2012). The screen house was subdivided into four 10 m long cubicles, each with a space volume of 140 m^3, and separated by folding metal screen partitions.

Screen house cubicles were designated as control (for release of unexposed cohorts (previously held within chemical-free huts) or treatment (for release of repellent-exposed cohorts). Within each cubicle, four BGS traps were operated simultaneously. The traps were checked for mosquitoes following a timeline established in a previous study (Salazar *et al.*, 2012).

Environmental parameters (temperature, relative humidity and light intensity) were recorded inside each cubicle using HOBO data loggers (HOBO U12-012 Model, Onset Computer Corporation, Bourne, MA, USA).

Data analysis

The percentages of released mosquitoes that were recaptured were corrected by adjusting for the number of knocked down mosquitoes after exposure for IR cohorts, and before releasing them into the screen house for DR cohorts. Percentages of recaptured mosquitoes were quantified cumulatively for the time points when the traps were examined. The Kruskal-Wallis test was used to compare the effects of repellent exposure on the recapture of released mosquitoes between the treatment and control cohorts. The comparison of cumulative BGS trap recaptures for IR versus DR cohorts was performed using the Mann-Whitney test. For all analyses, a *p*-value of 0.05 or less was considered statistically significant. Statistical analyses were performed using the statistical software STATA (version11.2; Stata Corp., College Station, Texas, USA.) using the ranksum and kwallis syntax for the Mann-Whitney test and the Kruskal Wallis test, respectively.

RESULTS

[1, 1, 1-trichloro-2,2-bis (4-chlorophenyl) ethane] (DDT) exposure

Evaluations for DDT at 1.0 FAR (2 g *ai*.m^{-2}) and 75, 50 or 25% SAC produced no significant differences in BGS recapture rates for DDT-exposed mosquitoes versus control ones for either the immediate release or delayed release trials (Table 1). The cumulative BGS recaptures, across FAR and SAC values, ranged from 86 to 94% for DDT-exposed mosquitoes and from 85 to 95% for control mosquitoes (Table 1).

Metofluthrin exposure

There were no significant differences in recapture rates of metofluthrin-exposed mosquitoes and control mosquitoes (kept in huts without coils or exposed to metofluthrin-free burning coils) for either immediate release or delayed release trials with high dose (0.006%) or low dose (0.003%) metofluthrin coils (Table 2). However, delayed release resulted in significantly higher recapture rates compared to immediate release for both low and high dose metofluthrin coils (Table 2). Cumulative recaptures ranged from 77 to 93% for mosquitoes kept in huts without coils to 73–90% for those exposed to metofluthrin-free burning coils and 80–93% for mosquitoes exposed to low or high dose metofluthrin coils (Table 2).

Environmental/ climatic data

No significant difference ($P > 0.05$) was found among mean daily temperature, relative humidity or light intensity measured between the control and treatment screen house cubicles for DDT, metofluthrin and transfluthrin trials (Tables 1–2).

DISCUSSION

Many chemicals can elicit repellent behavioral responses in *Ae. aegypti* at doses well below those required for toxic outcomes (Grieco *et al.*, 2007; Achee *et al.*, 2009; WHO, 2009), but the effects of such exposures on the mosquito's host-seeking behavior are poorly understood. For another important dengue virus vector, *Ae albopictus*, changes in both host-seeking and blood-feeding behaviors upon exposure to plant volatiles under laboratory conditions have been described (Hao *et al*., 2008). *Ae. albopictus* females surviving exposure to geraniol, citral, eugenol or anisaldehyde for 24 and 48 hr all showed different degrees of reduction in host-seeking ability (for example, increased times to reach a target location and to search for a suitable feeding site and insert the stylet). After 48 hr of exposure to 0.250 μg.cm^{-3} of anisaldehyde, 100% of the mosquitoes showed loss of host-seeking

Table 1 Cumulative BG-Sentinel™ trap catches for immediate release (IR)[1] and delayed release (DR)[2] trials with *Ae. aegypti*[3] exposed to ([1, 1, 1-trichloro-2,2-bis (4-chlorophenyl) ethane] (DDT)-treated fabrics.

Surface area coverage	Release[5] and treatment	Cumulative mean percentage (± SD) of released *Ae. aegypti* recaptured by time point[4]										N[6]	Mean daytime (12 hr) conditions		
		Day 1		Day 2				Day 3							
		1330 hours	1730 hours	0530 hours	0930 hours	1330 hours	1730 hours	0530 hours	0930 hours	1330 hours	1730 hours		Temp (°C)	RH (%)	Light intensity (candela.m^{-2})
75%	(IR)[a] Control	91.9[a] (±4.5)	92.9[a] (±3.5)	92.9[a] (±3.5)	93.4[a] (±3.5)	93.4[a] (±3.5)	93.4[a] (±3.5)	-	-	-	-	185/198	33.4	25.9	35.5
	DDT (2g *ai.*m^{-2})	89.2[a] (±5.3)	89.2[a] (±5.3)	90.7[a] (±4.1)	90.7[a] (±4.1)	91.8[a] (±3.8)	91.8[a] (±3.8)	-	-	-	-	178/194	31.9	28.6	30.84
	(DR)[a] Control	-	-	-	89.3[a] (±4.8)	90.3[a] (±3.7)	90.3[a] (±3.7)	90.3[a] (±3.7)	90.3[a] (±3.7)	90.3[a] (±3.7)	90.3[a] (±3.7)	177/196	33.5	25.9	34.5
	DDT (2g *ai.*m^{-2})	-	-	-	90.7[a] (±5.2)	91.8[a] (±3.8)	91.8[a] (±3.8)	91.8[a] (±3.8)	91.8[a] (±3.8)	91.8[a] (±3.8)	91.8[a] (±3.8)	178/194	31.9	28.4	29.6
50%	(IR)[a] Control	79.9[a] (±10.6)	83.9[a] (±4.9)	84.4[a] (±5.9)	85.4[a] (±5.5)	85.4[a] (±5.5)	85.4[a] (±5.5)					170/199	28.7	31.9	44.5
	DDT (2g *ai.*m^{-2})	87.4[a] (±9.6)	89.9[a] (±7.7)	90.4[a] (±7.7)	90.4[a] (±7.7)	90.4[a] (±7.7)	90.4[a] (±7.7)	-	-	-	-	178/198	29.7	69.1	42.2
	(DR)[a] Control				79.2[a] (±10.8)	85.3[a] (±5.9)	85.3[a] (±5.9)	85.3[a] (±5.9)	85.3[a] (±5.9)	85.8[a] (±6.3)	85.8[a] (±6.3)	169/197	30.8	66.7	45.9
	DDT (2g *ai.*m^{-2})				76.5[a] (±11.1)	84.5[a] (±3.4)	84.5[a] (±3.4)	85.0[a] (±3.5)	85.0[a] (±3.5)	85.5[a] (±3.5)	85.5[a] (±3.5)	171/200	29.2	71.8	46.5

Table 1 (Cont.)

Surface area coverage	Release[5] and treatment	Cumulative mean percentage (± SD) of released *Ae. aegypti* recaptured by time point[4]										N[6]	Mean daytime (12 hr) conditions		
		Day 1		Day 2				Day 3							
		1330 hours	1730 hours	0530 hours	0930 hours	1330 hours	1730 hours	0530 hours	0930 hours	1330 hours	1730 hours		Temp (°C)	RH (%)	Light intensity (candela.m^{-2})
	(IR)[a]														
	Control	94.4[a] (±4.7)	95.9[a] (±4.5)	96.4[a] (±4.6)	96.4[a] (±4.6)	96.4[a] (±4.6)	96.4[a] (±4.6)	-	-	-	-	189/196	28.1	24.4	17.9
	1.00 FAR (2g *ai.*m^{-2})	93.9[a] (±3.0)	93.9[a] (±3.0)	93.9[a] (±3.0)	93.91[a] (±2.95)	93.9[a] (±3.0)	93.9[a] (±3.0)	-	-	-	-	185/197	27.4	26.0	11.7
	0.50FAR (1g *ai.*m^{-2})	87.2[a] (±14.6)	93.3[a] (±3.9)	93.9[a] (±3.6)	93.9[a] (±3.6)	93.9[a] (±3.6)	93.9[a] (±3.6)					183/195	27.1	31.7	7.1
25%	(DR)[a]														
	Control	-	-	-	77.2[a] (±8.9)	91.2[a] (±3.7)	91.2[a] (±3.7)	91.2[a] (±3.7)	91.2[a] (±3.7)	91.2[a] (±3.7)	91.2[a] (±3.7)	176/193	28.4	25.3	19.1
	1.00 FAR (2g *ai.*m^{-2})	-	-	-	90.0[a] (±5.4)	95.5[a] (±2.6)	95.5[a] (±2.6)	95.5[a] (±2.6)	95.5[a] (±2.6)	95.5[a] (±2.6)	95.5[a] (±2.6)	190/199	27.8	26.6	12.5
	0.50FAR (1g *ai.*m^{-2})	-	-	-	89.6[a] (±4.7)	92.8[a] (±4.11)	92.8[a] (±4.1)	92.8[a] (±4.1)	92.8[a] (±4.1)	92.8[a] (±4.1)	92.8[a] (±4.1)	181/195	27.5	32.8	7.9

[1] Cohort exposed from 0600–1200 hours and released immediately afterwards.

[2] Cohort exposed from 1200–1800 hours but released only after a holding period of 12 hr.

[3] 3–5 d old starved females.

[4] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females within release trial types (IR or DR; Kruskal-Wallis 95% confidence limit) or between IR and DR trials (Mann-U Whitney Test, 95% confidence limit)

[5] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).

[6] Total recaptured ÷ (Total released - Total knock down).

Table 2 Cumulative BG-Sentinel™ trap catches for immediate release (IR)[1] and delayed release (DR)[2] trials with *Ae. aegypti*[3] exposed to burnable metofluthrin coils.

Dose	Release[5] and treatment	Cumulative mean percentage (± SD) of released *Ae. aegypti* recaptured by time point[4]							N[6]	Mean daytime (12hr) conditions		
		Day 1	Day 2			Day 3						
		1730 hours	0530 hours	1230 hours	1730 hours	0530 hours	1230 hours	1730 hours		Temp (°C)	RH (%)	Light intensity (candela.m^{-2})
Low dose (0.00312%)	(IR)[a] Metofluthrin Control	57.9[a] (±5.6)	69.0[a] (±6.5)	71.0[a] (±6.3)	75.2[a] (±7.9)	75.2[a] (±7.9)	76.6[a] (±7.9)	76.6[a] (±7.9)	111/145	24.9	60.2	15.6
	(IR)[a] Metofluthrin Blank Coil	52.7[a] (±7.9)	59.6[a] (±13.6)	69.2[a] (±18.7)	71.2[a] (±6.3)	72.6[a] (±8.7)	72.6[a] (±8.7)	72.6[a] (±8.7)	106/146	25.2	59.9	16.5
	(DR)[b] Metofluthrin Control	53.1[a] (±11.0)	64.8[a] (±8.2)	74.2[a] (±8.9)	83.5[a] (±1.3)	84.2[a] (±0.3)	84.8[a] (±1.1)	84.8[a] (±1.1)	123/145	24.1	61.8	6.2
	(DR)[b] Metofluthrin Blank Coil	-	-	66.4[a] (±18.8)	87.7[a] (±9.5)	92.5[a] (±2.6)	93.2[a] (±3.4)	93.2[a] (±3.4)	136/146	26.2	63.0	16.6
	(IR)[a] Metofluthrin Control	-	-	77.2[a] (±14.6)	86.9[a] (±3.7)	86.9[a] (±3.7)	89.9[a] (±1.7)	89.9[a] (±1.7)	130/145	26.3	62.7	16.2
	(IR)[a] Metofluthrin Blank Coil	-	-	56.6[a] (±7.4)	74.8[a] (±10.3)	76.9[a] (±11.5)	79.7[a] (±13.8)	79.7[a] (±13.9)	113/143	25.2	65.7	5.7
	Control	85.8[a] (±5.3)	87.2[a] (±5.8)	87.2[a] (±5.8)	87.2[a] (±5.8)	87.2[a] (±5.8)	87.2[a] (±5.8)	87.2[a] (±5.8)	123/148	28.1	65.6	15.6
	Blank Coil	81.2[a] (±8.9)	81.9[a] (±7.9)	82.6[a] (±6.9)	82.55[a] (±7.0)	82.6[a] (±6.9)	82.6.[a] (±6.9)	82.6[a] (±6.9)	123/149	28.0	65.8	17.1
	Metofluthrin	85.9[a] (±6.0)	91.3[a] (±1.1)	92.0[a] (±1.9)	92.0[a] (±1.9)	92.0[a] (±1.9)	92.0[a] (±1.9)	92.0[a] (±1.9)	137/149	26.7	69.2	5.9

Table 2 (Cont.)

Dose	Release[5] and treatment	Cumulative mean percentage (± SD) of released *Ae. aegypti* recaptured by time point[4]							N[6]	Mean daytime (12hr) conditions		
		Day 1	Day 2			Day 3				Temp (°C)	RH (%)	Light intensity (candela.m^{-2})
		1730 hours	0530 hours	1230 hours	1730 hours	0530 hours	1230 hours	1730 hours				
High dose (0.00625%)	(DR)[b]											
	Control	-	-	92.7[a] (±4.2)	92.7[a] (±4.2)	92.7[a] (±4.2)	92.7[a] (±4.2)	92.7[a] (±4.2)	144/150	28.0	65.8	18.1
	Blank Coil	-	-	77.7[a] (±1.6)	85.8[a] (±5.4)	85.8[a] (±5.4)	85.8[a] (±5.4)	85.8[a] (±5.4)	126/148	29.7	65.9	17.3
	Metofluthrin	-	-	89.2[a] (±5.4)	89.2[a] (±5.4)	93.2[a] (±3.0)	93.2[a] (±3.0)	93.2[a] (±3.0)	138/148	26.7	69.4	5.7

[1] Cohort exposed from 0600–1200 hours and released immediately afterwards.

[2] Cohort exposed from 1200–1800 hours but released only after a holding period of 12 hr.

[3] 3-5 d old starved females.

[4] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females within release trial types (IR or DR; Kruskal-Wallis 95% confidence limit) or between IR and DR trials (Mann-U Whitney Test, 95% confidence limit)

[5] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).

[6] Total recaptured ÷ (Total released - Total knock down).

ability, through impacts on the time to host-seeking activation, orientation, probing and engorgement compared to unexposed controls.

Holding female *Ae. aegypti* in experimental huts together with fabrics treated with different concentrations of DDT did not significantly impact subsequent capture success with the BGS trap, compared to non-exposed females, regardless of whether the exposed females were released in the environment containing the traps immediately following DDT exposure or following a 12 hr recovery period in a repellent–free setting. Similarly, mosquitoes exposed to coils with high (0.006%) or low (0.003%) concentration of metofluthrin were as likely to be captured with the BGS traps as non-exposed control mosquitoes. Previous exposure to transfluthrin at 0.125 and 0.062 FAR resulted in significantly lower trap catches, compared to control mosquitoes, for mosquitoes released immediately following exposure but not for those allowed to recover for 12 hr before being released into the environment with the BGS traps. Transfluthrin seemed to have the strongest impact on the mosquitoes in the trials, with higher concentrations (1.0 FAR) inducing near universal mortality and lower ones (0.125 and 0.062 FAR) leading to short-term reduction in BGS trap catches. Transfluthrin and metofluthrin are known as highly volatilized actives compared to DDT which has a higher vapor pressure than these pyrethroids and so does not volatize as easily. However, DDT still has spatial repellant qualities as indicated in previous experimental hut studies (Grieco *et al*., 2007).

The comparison of results for mosquitoes released immediately following exposure versus those allowed to recover for 12 hr before being released into the environment with the BGS traps indicates that even when an effect on host-seeking behavior resulted from the exposure, this was a temporary phenomenon. This is suggestive of effects on sensory pathways used to detect host cues that resolve following the 12 hr holding period. Similarly, Hao *et al*. (2008) noted that a reduction in host-seeking ability in *Ae. albopictus* in the laboratory was reversible following recovery times that were dependent on chemical- and concentration-specific exposure conditions. However, it is not possible to rule out the possibility that the observed increase in host-seeking activity/trap catch rates for the delayed release females resulted, in part, from the fact that they were only supplied with water during the recovery phase and thus were more motivated to locate a food source at the end of the recovery period.

CONCLUSION

The study showed that previous exposure to DDT, metofluthrin or transfluthrin resulted in no more than minor and short-lived reductions in the efficacy of BioGents-Sentinel™ traps to recover host-seeking *Ae. aegypti* females. Exposure to highly volatile pyrethroids (metofluthrin and transfluthrin) appeared to have some impact on the host-seeking ability of *Ae. aegypti* immediately following exposure, but this effect was absent following a 12 hr recovery period. The study produced new knowledge: (i) to inform the selection of the spatial repellent chemicals best suited for inclusion in a push-pull strategy where traps are used as the pull component and (ii) to estimate the length of time a vector will require to return to active host-seeking following repellent exposure.

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Parasites & Vectors

REVIEW Open Access

Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand

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Abstract

Physiological resistance and behavioral responses of mosquito vectors to insecticides are critical aspects of the chemical-based disease control equation. The complex interaction between lethal, sub-lethal and excitation/repellent ('excito-repellent') properties of chemicals is typically overlooked in vector management and control programs. The development of "physiological" resistance, metabolic and/or target site modifications, to insecticides has been well documented in many insect groups and disease vectors around the world. In Thailand, resistance in many mosquito populations has developed to all three classes of insecticidal active ingredients currently used for vector control with a majority being synthetic-derived pyrethroids. Evidence of low-grade insecticide resistance requires immediate countermeasures to mitigate further intensification and spread of the genetic mechanisms responsible for resistance. This can take the form of rotation of a different class of chemical, addition of a synergist, mixtures of chemicals or concurrent mosaic application of different classes of chemicals. From the gathered evidence, the distribution and degree of physiological resistance has been restricted in specific areas of Thailand in spite of long-term use of chemicals to control insect pests and disease vectors throughout the country. Most surprisingly, there have been no reported cases of pyrethroid resistance in anopheline populations in the country from 2000 to 2011. The precise reasons for this are unclear but we assume that behavioral avoidance to insecticides may play a significant role in reducing the selection pressure and thus occurrence and spread of insecticide resistance. The review herein provides information regarding the status of physiological resistance and behavioral avoidance of the primary mosquito vectors of human diseases to insecticides in Thailand from 2000 to 2011.

Keywords: *Anopheles*, *Culex*, *Aedes*, Control, Insecticide, Susceptibility, Behavior, Thailand

Introduction

A number of insect species can transmit pathogens to humans resulting in significant morbidity and mortality as well as placing a profound burden on human productivity and development. Transmission of these vector-borne diseases is related to the complex interplay of three primary components; pathogenicity/virulence of the infectious agent, vector competence (infectivity) and host (human) susceptibility. This transmission cycle is directly and indirectly driven by a diverse number of inter-related environmental factors. Successful control of human diseases requires an understanding of the interaction among these three components and the various other biological, environmental, and socio-economic factors that influence transmission. Such a task often requires or benefits from the full participation of both governmental and private sectors, sufficient numbers of trained personnel, adequate and sustained financial support, and a well-designed, evidence-based vector control program. Despite decades of organized vector control efforts, malaria, dengue, lymphatic filariasis and Japanese encephalitis, remain real threats in various areas of Thailand. One of the most effective means of prevention of these diseases involves vector control to reduce the risk of transmission. In some instances, this requires the use of various chemical compounds as larvicides applied to aquatic habitats and adulticides as outdoor space

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applications and indoor residual sprays (IRS), and the use of insecticide-treated bed nets [1-4] in order to reduce vector survival and density and thus human-vector contact.

At least four groups of synthetic compounds, organochlorine (DDT), organophosphates, carbamates and pyrethroids, have been extensively used in Thailand for the control of both agricultural pests and human/animal disease vectors. DDT was introduced for agricultural pest control in 1934 but was later banned from all agricultural use in 1983. For public health, the use of DDT was launched in 1949 in an indoor residual spray (IRS) pilot campaign to control malaria transmission after Thailand agreed to participate in the malaria eradication program outlined by the World Health Organization (WHO) [5-7]. However, the use of DDT began a gradual decline in use in the later decades and was completely removed for malaria control in the year 2000 due to its perceived adverse impact on the environment and declining public acceptance for indoor residual spraying [7-9]. Before 2000, the extensive use of DDT resulted in the development of physiological resistance in populations of *Anopheles aconitus*, *Anopheles culicifacies*, *Anopheles nivipes* and *Anopheles philippinensis*, all non-malaria vector species in Thailand [8].

For many decades, various synthetic insecticidal compounds have been used extensively in the private sector, agri-business and in the national public health vector control programs in Thailand. The vast majority of compounds, by number and volume, are pyrethroid-based formulated combinations (Table 1). Synthetic pyrethroids have become the most popular and prevalent active ingredients for public health use due to their relatively low mammalian toxicity but high invertebrate potency at low levels, resulting in rapid immobilization ('knockdown') and killing [10]. Most of them have been used to control insect pests such as cockroaches, ants, bedbugs, and mosquitoes [11]. Compared to pyrethroids, relatively few organophosphate and carbamate-based insecticides remain available in the Thai public market. For example, the amount of pyrethroids used for dengue and malaria control in 2007 (excluding treated bed nets) was approximately 1,127 tons whereas that of OP and carbamates together was virtually nil [11].

The extensive use of pyrethroids for vector control has raised major concerns over the selection pressure induced by the insecticides on resistance gene mechanisms [4,12-15]. In addition to insecticides, topically applied repellents such as DEET (*N*, *N*-diethyl-*meta*-toluamide), one of the most effective insect repellent active ingredients [16,17], is available in most local markets and is used extensively to protect against biting mosquitoes and other insects (Table 1), despite potential negative health effects in humans associated with continuous or over application on the skin.

Insect resistance to insecticides has been observed for all classes of compounds, including microbial-based agents and insect growth regulators (hormone mimics) [18]. In general, response to insecticides can be categorized into two major types: physiological resistance and behavioral avoidance [19]. Physiological resistance is the ability of an insect population to survive exposure to a concentration of insecticide that would normally result in complete kill [1]. One or more mechanisms may be involved in physiological resistance, including alteration of target site nerve receptors (e.g., *kdr*, *Rdl* and *Ace.1R*) and detoxification via increased enzyme activity of non-specific esterases, glutathione *S*-transferases and P-450 mediated monooxygenases (mixed function oxidases) [20].

In contrast, behavioral avoidance (deterrence) is defined as the ability of an insect to move away (escape) from an insecticide-treated area, often without lethal consequence [4]. This type of response can be further divided into direct contact excitation (sometimes referred to as 'irritancy') and non-contact spatial repellency [12]. The term 'contact irritancy' involves an insect leaving an insecticide treated area only after making physical (tarsal) contact with the chemical, whereas 'spatial repellency' is when insects move away from the insecticide-treated area without making direct contact [12,19]. Lastly, some chemicals, such as DEET, can elicit a fourth action by effectively masking/jamming the presence of a host through the inhibition of odor-activated receptors [21].

The review herein has compiled information on the use of public health insecticides in Thailand since 2000 and summarizes the primary insecticidal and behavioral responses of disease vector mosquitoes of importance elicited by these chemicals [8]. This should assist in guiding national authorities in the rational and target-specific use of insecticides for effective control of mosquito vectors.

Review

Insecticides used for the control of insect vectors and pests

Insecticides have been widely used to control both urban and peri-urban insect pests [1,2,22]. Although the use of DDT has been completely halted (banned) in many countries, recent allowances have been made for its renewed use in malaria control programs in a number of African nations because of its superior attributes compared to most alternative active ingredients [23]. The marked impact of DDT on mosquito populations in terms of both toxicity and modified behavioral responses that suppress disease transmission is well known despite lacking a clear understanding of the actual mechanisms and interactions at work in some instances. Most studies

Table 1 Twenty-three commercial products and active ingredients for household pest control in Thailand (2012)

Product name (Trade name)	Compounds	Concentration
Shieldtox (odorless I)	Bioallethrin	0.241%w/w
	Bioresmethrin	0.046%w/w
Shieldtox (odorless II)	Prallethrin	0.0729%w/w
	Phenothrin	0.1003%w/w
Shieldtox (Ultra I)	Bioallethrin	0.209%w/w
	Bioresmethrin	0.039%w/w
Shieldtox (Ultra II)	Tetramethrin	0.230%w/w
	Deltamethrin	0.015%w/w
Shieldtox (Ultra Green I)	Tetramethrin	0.230%w/w
	Deltamethrin	0.015%w/w
Shieldtox (Ultra Yellow 1)	Prallethrin	0.055% w/w
	Permethrin	0.100% w/w
	Tetramethrin	0.184%w/w
Raid Insect Killer 4	Propoxur	0.75% w/w
	Tetramethrin	0.30% w/w
	Cypermethrin	0.10%w/w
Raid X-tra	Tetramethrin	0.30%w/w
	Permethrin	0.10%w/w
	Transfluthrin	0.05%w/w
Raid X-tra Plus	Prallethrin	0.06% w/w
	Permethrin	0.20% w/w
Raid Insect Killer (water based)	Prallethrin	0.06% w/w
	Permethrin	0.24% w/w
Raid (water based)	Tetramethrin	0.35% w/w
	Allethrin	0.10% w/w
	Permethrin	0.10% w/w
ARS	Tetramethrin	0.07% w/w
	Dichlovos	0.50% w/w
ARS 3	Tetramethrin	0.02% w/w
	Permethrin	0.10%w/w
	Dichlovos	0.05%w/w
ARS (water based)	Allethrin	0.06%w/w
	Tetramethrin	0.06%w/w
	Permethrin	0.18%w/w
ARS Jet Pro	Imiprothrin	0.20%w/w
	Cypermethrin	0.10% w/w
BYGON (Blue)	Cyfluthrin	0.025% w/w
	Transfluthrin	0.040%w/w
BYGON (Yellow)	Transfluthrin	0.04%w/w
	Cyfluthrin	0.025%w/w
BYGON (Green)	Cyfluthrin	0.025%w/w
	Propoxur	0.500%w/w
	Dichlorvos	0.500%w/w
Kincho	Tetramethrin	0.20%w/w
	Permethrin	0.14%w/w
JUMBO	Bioallethrin	0.200%w/w
	Deltamethrin	0.012%w/w
	Permethrin	0.100%w/w
Sheldrite	Permethrin	0.25%w/w
	Bioallethrin	0.10%w/w
	Dichlovos	0.5%w/w
Atsawin	Tetramethrin	0.20%w/w
	Permethrin	0.10%w/w
GY-15	DEET	4%

on insecticides have focused exclusively on the direct toxicological effects of the molecule on mosquitoes whereas comparatively fewer investigations have accurately measured the behavioral responses resulting from sub-lethal exposure to the active ingredient [12,19,24,25]. Observations on behavioral responses of vectors began with the early use of DDT to control *Anopheles* mosquitoes [1], avoidance outcomes which resulted in the recognition of two different types of non-toxic actions: excitation and repellency, often termed together as 'excito-repellency' [1,4,26-33]. The importance (either benefit or drawbacks) of avoidance behavior without killing or reducing survival of the vector outright has produced plenty of debate and controversy for impact in controlling disease transmission [4,32].

In Thailand, many compounds have been used for the control of medically important insects in both the private and public sectors. In the private sector, several dispensing designs for household pesticides are available [13,14], including space sprays (aerosols), released as a vapor phase (mosquito coils, electric mats), direct applications (creams), and residual liquids. Often, these various formats contain more than one active ingredient and include synergists to enhance knockdown response and effectiveness. Over 80% of the active ingredients currently used in homes are pyrethroids that are used in low concentrations in the form of aerosols. By market volume, both organophosphates and carbamates (Table 1) are used to a far lesser extent. The most common use for home-based insecticides is for control of mosquitoes and other flying insects (house and filth flies) followed by termites, ants, cockroaches, and bedbugs. Among the pyrethroids, permethrin, deltamethrin and cypermethrin are the predominant active ingredients used (Table 1). In the business sector, pest control operators (PCOs) with proper training and special licensure, allows professionals to apply a wider (and more toxic) variety of chemicals to control a broader range of pests (e.g.,

structural pests such as termites and ants). Such PCO training programs, under the support and guidance of the Food and Drug Administrative Office, Ministry of Public Health, has been carried out by the Department of Entomology, Faculty of Agriculture, Kasetsart University at least twice each year since 2003. In the public (government) sector, a wider array of chemicals are used for vector control purposes including organophosphates, carbamates, pyrethroids and so-called 'bio-rational' pesticides and biological agents, such as natural predators, bacterial toxins, insect growth regulators (hormone mimics) and botanical repellents, depending on the target species and circumstances [2,8,34]. For routine dengue vector control in Thailand, an organophosphate-based larvicide (temephos) has been commonly used for the control of *Aedes aegypti* larvae in container habitats since 1950 [8]. Although it remains mostly effective for *Aedes* larval control, evidence of temephos resistance in *Ae. aegypti* has recently been observed in some localities of Thailand [13]. The other organophosphates such as malathion, fenitrothion and pirimiphos-methyl were commonly used as either IRS or fogging agents before being replaced by pyrethroids [8]. In 1994, deltamethrin, a newer, more potent pyrethroid was introduced to Thailand for controlling indoor biting mosquitoes, including *Ae. aegypti* [8]. This compound remains the standard for the control of dengue vectors during dengue outbreaks although recent reports have identified deltamethrin resistance in several populations of *Ae. aegypti* [35]. Deltamethrin and permethrin have also been widely used in the malaria control program [36]. Deltamethrin is used for IRS once or twice a year, depending upon the intensity of malaria transmission in the area (based on endemic malaria zoning categories) determined by the Bureau of Vector Borne Disease [36]. Permethrin is still commonly used to impregnate fabric materials such as clothing, screens, blankets, and bed nets. These insecticide-treated materials can be more easily shipped to malaria endemic areas that may be difficult to access for spray teams because of relative isolation, poor roads and/or on-going civil insurgency and security disruptions. For example, in the four southern-most provinces of Thailand, cases of malaria have risen to nearly 4,000 a year in the area adjacent to the Thai-Malaysian border where rebels have been engaging Thai authorities since 2004. Similarly, the number of malaria cases reported along the Thai-Myanmar border (e.g., Tak and Mae Hongsorn provinces) has been aggravated due to the presence of refugee camps and intense migration from the adjacent country where malaria is still highly prevalent [37].

History and organization of the vector-borne disease control program

Over the 60 year history of the malaria control in Thailand, a number of policy changes have occurred to adjust to new developments in technology and the shifting landscape of malaria epidemiology in the country. Since the implementation of the national malaria control program in the 1950s, malaria associated morbidity and mortality has been reduced dramatically in Thailand except along the border areas. However, in the last decade, a shortage of trained public health personnel and vector control specialists, together with flagging financial support has resulted in dramatic changes in the vector control policies in Thailand. In October 2002, the Department of Disease Control (DDC) reorganized the Vector-Borne Disease Control (VBDC) Program by merging the Malaria Control Unit (MCU) with other vector-borne disease control programs. The DDC was further consolidated to include other non-communicable diseases under its direction. The restructuring helped ease constraints on staffing, budget and equipment for all VBDC elements, and eliminated many of the redundancies and relatively high costs previously incurred by each of the former independent control programs. At the national level, the Bureau of Vector Borne Disease (BVBD) is under the direction of the DDC, in the Ministry of Public Health. The program comprises 12 regions under the direction of a Medical Officer, Director of the Office of Disease Prevention and Control (DPC). Under the DPC, the Vector-Borne Disease Section (VBDS) was set up to respond to the major vector-borne disease issues in each Regional DPC. In 2003, 39 Vector-Borne Disease Control (VBDC) Centers and 302 Vector-Borne Disease Control Units (VBDU) are set up at provincial and district levels, respectively, but recently these local VBD sectors were reduced to 38 VBDCs and 165 VBDUs, respectively. In addition, there are a number of district and sub-district municipalities that operate under the direction of the Ministry of Interior. In October 2011, the 12 DPC regional offices underwent further restructuring, and the VBDSs were merged with and renamed either Technical Support Sections or Emergency Response for Public Health Disasters, depending on the individual DPCs. This resulted in some confusion with the other Public Health Sectors with regard to knowing the correct individuals to contact for advice on insecticide usage and proper vector control application and monitoring techniques. This also included local district and sub-district administrative offices under the Ministry of Interior (Figure 1).

The Ministry of Interior is now responsible for developing their own policies for both vector and pest control activities, including planning and budget allocation. Little is done, however, in regard to pest control evaluation and monitoring. Moreover, each municipality has decision-making power for the local vector control operations, including budget allocations for purchasing insecticides. As a result, each local office can directly

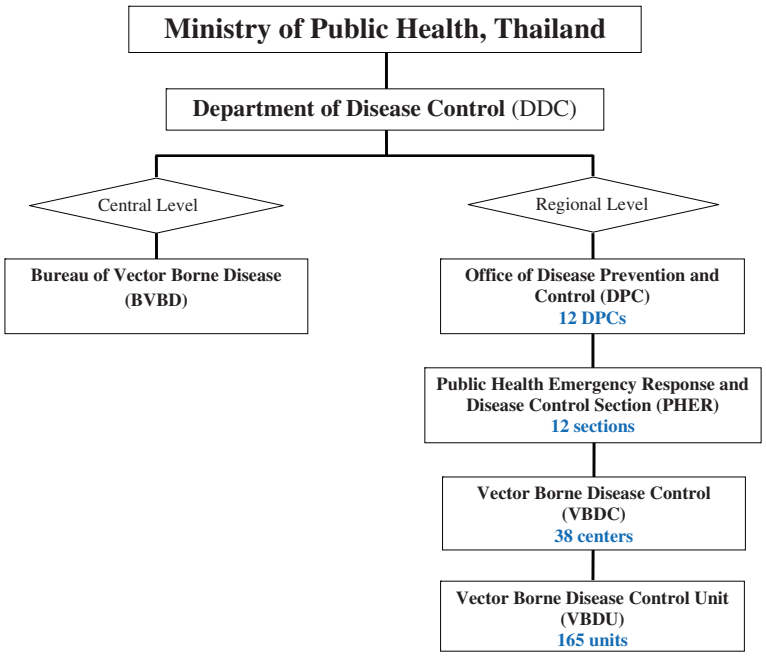


Figure 1 Diagram of the organization of the Vector-Borne Disease Control Program, Thailand.

approach an insecticide company without a clear policy and evidence-based rationale for insecticide choice from the Department of Disease Control, MOPH. The majority of products purchased are typically pyrethroids that include deltamethrin, permethrin, cypermethrin, and alpha-cypermethrin (Wannapa, personal communications).

Mosquito-transmitted diseases in Thailand

Thailand continues to face endemic transmission and the re-emergence of mosquito-borne diseases, principally malaria, dengue fever and dengue hemorrhagic fever (DF/DHF), lymphatic filariasis, Japanese encephalitis and more recently Chikungunya virus [36]. All these parasites and viruses are transmitted to humans by suitable vector mosquito species, some of which are capable of transmitting more than one disease pathogen [38]. The current distribution of these diseases in Thailand is presented in Figure 2. Like other countries in this region, malaria displays significant geographical heterogeneity and is exemplified by more intensified "border malaria", with most of the malaria cases concentrated along the Thai borders with Myanmar and Cambodia [7,39-41], and more recently with the upsurge of malaria near the border with northern Malaysia [36]. According to [42], 8% of the total Thai population (~ 5 million inhabitants) resides in high risk areas for malaria (i.e., 1 case per 1000 population), 42% occupy lower risk areas (29 million population), while 50% are free from exposure to active malaria transmission (34 millions). The predominant malaria parasite species are *Plasmodium falciparum* and *Plasmodium vivax* but *P. vivax* has become slightly more prevalent than *P. falciparum* since 2000 [43].

Dengue viruses (*Flavivirus*) are responsible for one of the most prevalent vector-borne disease entities (DF/DHF) of public health importance in much of the tropical and subtropical world [44]. Each year, between 50–100 million dengue infections occur and nearly half the world population lives in the countries where dengue transmission is now endemic [45]. Much of urban Thailand is regarded as hyper-endemic for dengue infection with all four serotypes (DEN-1, DEN-2, DEN-3, DEN-4) regularly circulating and now occurring more commonly in rural areas. Unlike the majority of countries in the Southeast Asian Region, the total dengue cases reported in 2011 (78,337), 2010 (68,386) and 2009 (115,768) greatly exceeded that recorded in 2008 (56,651) with an increase in the proportion of severe dengue cases being reported [46]. Chikungunya (an *Alphavirus*) is another arboviral disease, very similar in epidemiology and clinical presentation to dengue, but occupying different antigenic families, Togaviridae vs. Flaviviridae, respectively. Compared to dengue, chikungunya is a relatively uncommon reported disease and likely misdiagnosed as classic dengue infection where they co-exist [47,48]. Although periodic outbreaks occur throughout Africa and Southeast Asia, they are typically self-limiting infections and rarely result in severe disease. After years of apparent quiescence, chikungunya emerged in 2004 in Kenya and subsequently invaded the Indian Ocean islands of Comoros and Réunion in 2005

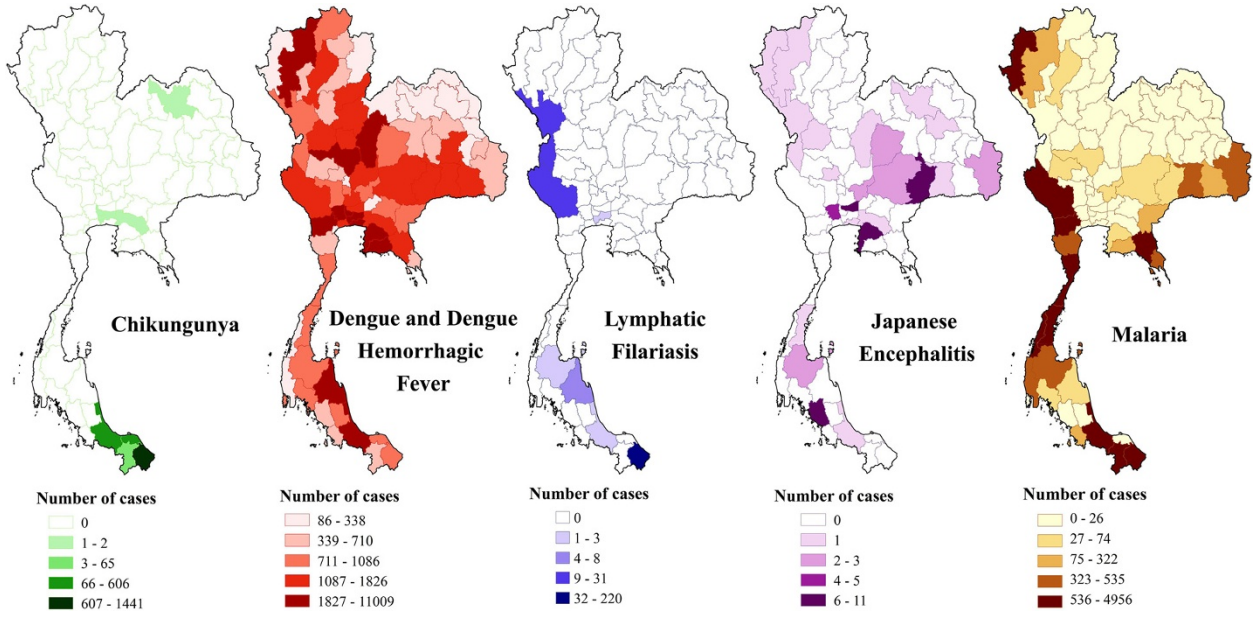


Figure 2 Distribution of the principle mosquito-borne diseases in Thailand (2012).

and rapidly spread to other islands of the Indian Ocean reaching India in 2006 where more than 1 million suspected cases were reported [48]. In the succeeding years, Sri Lanka, Indonesia, Singapore and Malaysia were affected, including reports of significant outbreaks of chikungunya in the south of Thailand in which 49,069 infections were reported with a notable increase of neurologic complications [36,49].

Japanese encephalitis (JE), a *Flavivirus*, is the leading cause of viral encephalitis in Asian countries with approximately 30,000–50,000 cases reported annually, with case fatality rates between 0.3% and 60%, depending on the age at time of infection and severity of symptoms [50]. In SE Asia, JE is rarely reported among travelers to countries where JE is endemic [51]. The number of overt cases has shown a sharp decline after mass immunization programs in the region. Transmission appears to be declining mostly in China, Japan, and Korea, but cases continue to be reported from Bangladesh, Myanmar, India, Nepal, Sri Lanka, Vietnam and Thailand. Japanese encephalitis was first recognized in Japan in the 1920s and is believed to have spread from India throughout Asia, Indonesia and more recently northern Australia and New Guinea Island [52,53]. Typically, JE occurs as outbreaks in the extensive rice growing areas of Asia associated with *Culex tritaeniorhynchus, Culex gelidus,* and *Culex vishnui* group mosquitoes [54,55], especially in Thailand [36]. Even through developing symptomatic illnesses, humans, cattle and horses are regarded as dead-end hosts and not involved in the natural transmission cycle that involves a bird-mosquito maintenance cycle and various species of aquatic wading birds (e.g., ardeid birds) and pigs as principal amplifying hosts.

Lymphatic filariasis (LF) is caused by several species of nematodes that reside in the lymphatic system of the vertebrate host and is estimated to collectively infect more than 120 million people worldwide [56,57]. This disease is known for its debilitating and disfiguring outcome in the more unfortunate, albeit relatively rare, cases. Filariasis remains a major public health threat in many Southeast Asian countries where it is endemic in 9 of 11 countries. In Thailand, both *Wuchereria bancrofti* and *Brugia malayi* are presently and widely distributed, particularly along the international borders. *Wuchereria bancrofti* has been found along the western Thai-Myanmar border, including the provinces of Ranong, Ratchaburi, Kanchanaburi, Tak and Mae Hongsorn [58]. The most common filarial parasite strain in Thailand is the nocturnal sub-periodic form with a distinct peak of

Table 2 List of *Anopheles* populations resistant to DDT in Thailand using the WHO standard contact assay (2000–2010)

Species	Insecticide	Location (province-district)	Geographic coordinates (DMS)	Published sources
Anopheles annularis	DDT	Chiang Mai-Chiang Dao	19°32'N 98°54'E	Prapanthadara *et al.* 2000 [96]
Anopheles annularis	DDT	Mae Hongsorn	19°9'N 98°1'E	
Anopheles minimus (A)*	DDT	Phrae	18°6'N 100°16'E	

**An. minimus* sensu strict. Note that resistance to DDT was also seen in non-vector anophelines of Thailand *before* 2000, *Anopheles aconitus*, *Anopheles culicifacies*, *Anopheles nivipes* and *Anopheles philippinensis* [8].

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011)

Insecticides	Location (province-district)	Geographic coordinates (DMS)	Published sources
Cyfluthrin	Nonthaburi-Mueang	13°51′44″N 100°30′48″E	Paeporn *et al.* 2010 [99]
	Saraburi-Mueang *	14°31′38″N 100°54′35″E	
	Singburi-Mueang	14°53′18″N 100°24′17″E	
	Phitsanulok-Mueang	16°49′29″N 100°15′34″E	
	Phichit-Mueang	16°26′18″N 100°21′0″E	
	Sukhothai-Mueang	17°0′28″N 99°49′23″E	
	Uttaradit-Mueang	17°37′33″N 100°5′48″E	
	Lamphun-Mueang	18°34′42″N 99°1′6″E	
	Chiang Mai-Mueang	18°47′25″N 98°59′4″E	
	Chiang Rai-Mueang	19°54′31″N 99°49′57″E	
	Khon Kean-Mueang	16°26′18″N 102°50′20″E	
	Prachinburi-Mueang	14°3′2″N 101°22′0″E	
	Sra Kaeo-Mueang	13°48′52″N 102°4′20″E	Satimai 2010 [100]
	Rayong-Mueang	12°43′3″N 101°23′31″E	
	Chanthaburi-Mueang	12°36′40″N 102°6′15″E	
	Trat-Mueang *	12°13′54″N 102°30′48″E	
Cypermethrin	Chiang Mai-Mae Tang	19°10′N 98°54′E	Chareonviriyaphap *et al.* 2006 [101]
	Kanchanaburi-Sai Yok	14°17′N 99°11′E	
DDT	Chiang Mai-Mae Tang	19°8′N 98°51′E	Prapanthadara *et al.* 2002 [102]
	Chiang Mai-Mae Tang	19°11′N 98°54′E	Somboon *et al.* 2003 [93]
	Chiang Mai-Mueang	18°46′N 98°57′E	
	Lampang-Mueang	18°23′N 99°31′E	
	Nan-Mueang	18°47′N 100°43′E	
	Chiang Mai-Mae Tang	19°9′N 98°47′E	Lumjuan *et al.* 2005 [103]
	Chiang Mai- Mae Tang	19°8′N 98°51′E	Prapanthadara *et al.* 2005 [104]
	Bangkok-Bang Khen	13°52′N 100°35′E	Yaicharoen *et al.* 2005 [105]
	Chiang Mai-Mae Tang	19°9′N 98°52′E	Sathantriphop *et al.* 2006 [106]
	Chonburi	13°22′0″N 100°58′60″E	Rajatileka *et al.* 2008 [107]
	Phang Nga	8°28′0″N 98°31′60″E	
	Phang Nga-Thap Pud	8°31′0″N 98°37′60″E	
	Chiang Mai-Mae Tang	19°14′N 98°59′E	Thanispong *et al.* 2008 [14]
	Pathum Thani-Lad Lumkeaw	14°02′N 100°24′E	
	Chiang Mai-Mueang	18°47′N 99°00′E	
	Kanchanaburi-Sai Yok	14°20′N 98°59′E	
	Nonthaburi-Mueang	13°53′N 100°29′E	
	Songkhla-Mueang	7°11′N 100°35′E	
	Satun-Mueang	6°37′N 100°03′E	
	Bangkok-Chatuchak	13°50′N 100°34′E	
	Lampang-Mueang	18°17′N 99°29′E	
	Tak-Mae Sot	16°46′N 98°34′E	
	Khon Kean-Mueang	16°25′N 102°50′E	
	Surat Thani-Mueang	9°08′N 99°20′E	
	Nakhon Sawan-Mueang	15°42′N 100°08′E	

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

	Kamphaeng Phet	16°28′0"N 99°30′0"E	
	Phang Nga-Takua Pa	8°52′0"N 98°20′60"E	
	Phuket	7°52′60"N 98°24′0"E	
Deltamethrin	Chiang Mai-Mueang	18°46′N 98°57′E	Somboon *et al*. 2003 [93]
	Nan-Muang	18°47′N 100°43′E	
	Ratchaburi-Pongsawai	13°32′43"N 99°51′7"E	Paeporn *et al*. 2004 [34]
	Ratchaburi-KhuBua	13°28′53"N 99°49′21"E	
	Chiang Mai-Mae Tang	19°10′N 98°54′E	Chareonviriyaphap *et al*. 2006 [101]
	Bangkok-Bang Khen	13°52′26"N 100°35′47"E	Yaicharoen *et al*. 2005 [105]
	Bangkok-Hauykwang	13°4′47.4"N 100°3′ 52.3"E	Jirakanjanakit *et al*. 2007 [13]
	Bangkok-Laksi	13°5′28.2"N 100° 3′ 41.2"E	
	Bangkok-Ladkrabang	13°4′47.5"N 100°4′23.6"E	
	Bangkok-Rasburana	13°3′59.2"N 100°3′58.8"E	
	Chonburi-Panusnikom	13°2′2.9"N 101°1′5.3"E	
	Chonburi-Banglamung	12°5′42.2"N 100°5′32.2"E	
	Nakhon Sawan-Taklee	15°1′53"N 100°1′48.2"E	
	Chanthaburi-Mueang	13°19′N 100°55′E	
	Nakhon Ratchasima-Kornburi	14°3′50"N 102°1′45.5"E	
	Nakhon Ratchasima-Senagsang	14°2′35.2"N 102°2′45.6"	
	Nakhon Sawan-Mae Pern	15°3′21"N 99°2′48"E	
	Nakhon Sawan-Mae Wong	15°4′35"N 99°3′7.4"E	
	Nakhon Ratchasrima-Kornburi	14°31′24"N 102°14′54"E	
	Bangkok-Hauykwang	13°45′54"N 100°34′39"E	Pethuan *et al*. 2007 [108]
	Bangkok-Laksi	13°53′15"N 100°34′44"E	
	Chonburi-Panusnikom	13°27′6"N 101°10′36"E	
	Chonburi-Banglamung	12°58′36"N 100°54′48"E	
	Nakhon Sawan-Taklee	15°15′47"N 100°20′37"E	
	Nakhon Ratchasrima-Serngsang	14°25′34"N 102°27′38"E	
	Nakhon Sawan-Mae Pern	15°39′28"N 99°28′9"E	
	Nakhon Sawan-Mae Wong	15°46′52"N 99°31′9"E	
	Chanthaburi-Makham *	12°40′25"N 102°11′48"E	Narksuwan *et al.* 2008 [109]
	Phetchaburi-Tha Yang *	12°58′24"N 99°53′16"E	
	Buri Ram-Cham ni *	14°47′18"N 102°50′30"E	
	Loei-Nong Hin *	17°7′24"N 101°51′30"E	
	Trang-Sikao *	07°34′18"N 99°20′42"E	
	Chum phon-Lang Suan	09°56′42"N 99°4′42"E	
	Phetchabun-Wichian Buri *	15°39′26"N 101°6′24"E	
	Kalasin-Yangtalat *	16°24′8"N 103°22′23"E	
	Lampang-Thoen *	17°36′42"N 99°12′57"E	
	Uthai Thani-Thap Than*	15°29′12"N 99°49′54"E	
	Suphanburi-Bang plama *	14°24′8"N 100°9′16"E	
	Ratchaburi-Damneon Saduak	13°31′6"N 99°57′18"E	
	Ayutthaya-Phak Hai *	14°27′30"N 100°22′12"E	
	Nonthaburi-Mueang	13°51′44"N 100°30′48"E	Paeporn *et al.* 2010 [99]

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

	Nakhon Pathom-Mueang	13°49′11″N 100°3′57″E	
	Sra Kaeo-Mueang *	13°48′53″N 102°4′19″E	
	Saraburi-Mueang *	14°31′38″N 100°54′35″E	
	Lopburi-Mueang	14°47′53″N 100°39′13″E	
	Suphanburi-Mueang	14°29′4″N 100°7′25″E	
	Angthong-Mueang	14°35′19″N 100°27′12″E	
	Singburi-Mueang	14°53′18″N 100°24′17″E	
	Kanchanaburi-Mueang	14°0′12″N 99°33′0″E	
	Phitsanulok-Mueang	16°49′29″N 100°15′34″E	
	Phichit-Mueang	16°26′18″N 100°21′0″E	
	Sukhothai-Mueang	17°0′28″N 99°49′23″E	
	Uttaradit-Mueang	17°37′33″N 100°5′48″E	
	Lamphun-Mueang	18°34′42″N 99°1′6″E	
	Uthaithani-Mueang *	15°22′46″N 100°1′29″E	
	Chiang Mai-Mueang	18°47′25″N 98°59′4″E	
	Chiang Rai-Mueang *	19°54′31″N 99°49′57″E	
	Khon Kean-Mueang	16°26′18″N 102°50′20″E	
	Udonthani-Mueang	17°24′54″N 102°47′12″E	
	Chai yaphum-Mueang	15°48′35″N 102°1′13″E	
	Nakhon Nayok-Mueang*	14°12′13″N 101°13′2″E	
	Rayong-Mueang *	12°40′6″N 101°16′30″E	
	Prachinburi-Mueang	14°3′2″N 101°22′0″E	
	Chonburi-Mueang	13°21′43″N 100°58′45″E	
	Sra Kaeo-Mueang *	13°48′52″N 102°4′20″E	Satimai 2010 [100]
	Rayong-Mueang	12°43′3″N 101°23′31″E	
	Chanthaburi-Mueang	12°36′40″N 102°6′15″E	
	Trat-Mueang *	12°13′54″N 102°30′48″E	
	Bangkok-Kannayaw	13°50′N 100°40′E	Chuaycharoensuk *et al.* 2011 [35]
	Chanthaburi-Mueang	12°39′N 102°7′E	
	Chonburi-Mueang	13°19′N 100°55′E	
	Khon Kean-Mueang	16°19′N 102°47′E	
	Udonthani-Wungsammor	15°54′N 103°28′E	
	Nakhon Sawan-Mueang	15°40′N 100°05′E	
	Tak-Mae Sot	16°43′N 98°34′E	
	Chumphon-Mueang	10°30′N 99°07′E	
	Prachuap Khiri Khan-Hua Hin	12°33′N 99°53′E	
	Songkhla-Namom	06°54′N 100°32′E	
	Songkhla-Sadao	06°45′N 100°24′E	
	Songkhla-Had Yai	07°00′N 100°27′E	
	Surat Thani-Mueang	09°02′N 99°22′E	
Etofenprox	Nan-Tha Wang Pha	19°7′N 100°43′E	Somboon *et al.* 2003 [93]
Fenitrothion	Nakhon Sawan-Mueang	15°4′47″N 100°34.7″E	Jirakanjanakit *et al.* 2007 [13]
	Nakhon Sawan-Krok Pra	15°3′12″N 100°33.7″E	
	Nakhon Ratchasima-Prathai	15°3′56″N 102°3′45.7″E	

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

	Nakhon Ratchasima-Kagsanamnang	15°4′14″N 102°1′36.9″E	
	Nakhon Ratchasima-Seekhew	14°5′19.5″N 101°4′28.8″E	
	Nakhon Ratchasima-Senagsang	14°2′35.2″N 102°2′45.6″E	
	Nakhon Sawan-Mae Pern	15°3′21″N 99°2′48″E	
	Nakhon Sawan-Mae Wong	15°4′35″N 99°3′7.4″E	
	Nakhon Ratchasrima-Seekhew	14°53′30″N 101°43′24″E	Pethuan *et al.* 2007 [108]
	Nakhon Ratchasrima-Prathai	15°32′0″N 102°43′22″E	
	Nakhon Ratchasrima-Kangsanamnang	15°45′0″N 102°15′17″E	
	Nakhon Sawan-Mueang	15°44′15″N 100°5′21″E	
	Nakhon Sawan-Krok Pra	15°34′48″N 100°0′13″E	
	Nakhon Ratchasrima-Serngsang	14°25′34″N 102°27′38″E	
	Nakhon Sawan-Mae Pern	15°39′28″N 99°28′9″E	
	Nakhon Sawan-Mae Wong	15°46′52″N 99°31′9″E	
	Nonthaburi -Mueang	13°51′44″N 100°30′48″E	Paeporn *et al.* 2010 [99]
	Sra Kaeo-Mueang *	13°48′53″N 102°4′19″E	
	Saraburi-Mueang *	14°31′38″N 100°54′35″E	
	Lopburi-Mueang	14°47′53″N 100°39′13″	
	Suphanburi-Mueang	14°29′4″N 100°7′25″E	
	Angthong-Mueang	14°35′19″N 100°27′12″E	
	Singburi-Mueang *	14°53′18″N 100°24′17″E	
	Phitsanulok-Mueang *	16°49′29″N 100°15′34″E	
	Phichit-Mueang	16°26′18″N 100°21′0″E	
	Sukhothai-Mueang	17°0′28″N 99°49′23″E	
	Uttaradit-Mueang*	17°37′33″N 100°5′48″E	
	Chiang Mai-Mueang	18°47′25″N 98°59′4″E	
	Khon Kean-Mueang	16°26′18″N 102°50′20″E	
	Udonthani-Mueang *	17°24′54″N 102°47′12″E	
	Rayong-Mueang*	12°40′6″N 101°16′30″E	
Lambdacyhalothrin	Sra Kaeo-Mueang	13°48′52″N 102°4′20″E	Satimai 2010 [100]
	Rayong-Mueang	12°43′3″N 101°23′31″E	
	Chanthaburi-Mueang	12°36′40″N 102°6′15″E	
	Trat-Mueang	12°13′54″N 102°30′48″E	
Malathion	Tak-Mae Sot	16°46′N 98°34′E	Thanispong *et al.* 2008 [14]
	Khon Kean-Mueang	16°25′N 102°50′E	
	Surat Thani-Mueang	9°08′N 99°20′E	
	Nakhon Sawan-Mueang	15°42′N 100°08′E	
	Nonthaburi-Mueang *	13°51′44″N 100°30′48″E	Paeporn *et al.* 2010 [99]
	Sra Kaeo-Mueang	13°48′53″N 102°4′19″E	
	Lopburi-Mueang	14°47′53″N 100°39′13″E	
	Suphanburi -Mueang *	14°29′4″N 100°7′25″E	
	Angthong-Mueang	14°35′19″N 100°27′12″E	
	Singburi-Mueang	14°53′18″N 100°24′17″E	
	Phichit-Mueang	16°26′18″N 100°21′0″E	
	Sukhothai-Mueang *	17°0′28″N 99°49′23″E	

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

	Uttaradit-Mueang *	17°37′33″N 100°5′48″E	
	Uthaithani-Mueang	15°22′46″N 100°1′29″E	
	Khon Kean-Mueang *	16°26′18″N 102°50′20″E	
	Udonthani-Mueang *	17°24′54″N 102°47′12″E	
Permethrin	Chiang Mai- Mae Tang	19°8′N 98°51′E	Prapanthadara *et al.* 2002 [102]
	Chiang Mai-Mueang	18°46′N 98°57′E	Somboon *et al.* 2003 [93]
	Nan-Mueang	18°47′N 100°43′E	
	Lampang-Mueang	19°11′N 98°54′E	
	Ratchaburi- Pongsawai	13°32′43″N 99°51′7″E	Paeporn *et al.* 2004 [34]
	Ratchaburi- KhuBua	13°28′53″N 99°49′21″E	
	Chiang Mai-Mae Tang	19°9′N 98°47′E	Lumjuan *et al.* 2005 [103]
	Tak-Mae Pa	16°45′N 98°33′E	Ponlawat *et al.* 2005 [98]
	Tak-Mae Pa	16°45′N 98°34′E	
	Nakhon Sawan-Phayuhakhiri	15°29′N 100°8′E	
	Surat Thani-Tha Chana	9°34′N 99°07′E	
	Phatthalung-Mueang	7°30′N 100°03′E	
	Nakhon Ratchasima-Kham Thale So	15°05′N 101°54′E	
	Chiang Mai- Mae Tang	19°8′N 98°51′E	Prapanthadara *et al.* 2005 [104]
	Chiang Mai-Mae Tang	19°9′N 98°52′E	Sathantriphop *et al.* 2006 [106]
	Nonthaburi-Baan Suan	13°51′N 100°29′E	
	Bangkok-Bangkoknoi	13°45′40″N 100°2′1.9″E	Jirakanjanakit *et al.* 2007 [13]
	Chonburi-Sriracha	13°19″N 101°11.8″E	
	Songkhla-Mueang	07°1′41.8″N 100°3′54.6″E	
	Kanchanaburi-Tamaka	13°55′15″N 99°45′56″E	
	Bangkok-Hauykwang	13°4′47.4″N 100°3′52.3″E	
	Bangkok-Laksi	13°5′28.2″N 100°3′41.2″E	
	Bangkok-Ladkrabang	13°4′47.5″N 100°4′23.6″E	
	Bangkok-Rasburana	13°3′59.2″N 100°3′58.8″E	
	Bangkok-Panusnikom	13°2′2.9″N 101°1′5.3″E	
	Bangkok-Banglamung	12°5′42.2″N 100°5′32.2″E	
	Nakhon Sawan-Taklee	15°1′53″N 100°1′48.2″E	
	Nakhon Sawan-Muang	15°4′47″N 100°34.7″E	
	Nakhon Sawan-Krok Pra	15°3′12″N 100°33.7″E	
	Nakhon Ratchasima-Senagsang	14°2′35.2″N 102°2′45.6″E	
	Nakhon Sawan-Mae Pern	15°3′21″N 99°2′48″E	
	Nakhon Sawan-Mae Wong	15°4′35″N 99°3′7.4″E	
	Bangkok-Hauykwang	13°45′54″N 100°34′39″	Pethuan *et al.* 2007 [108]
	Bangkok-Laksi	13°53′15″N 100°34′44″E	
	Chonburi-Panusnikom	13°27′6″N 101°10′36″E	
	Chonburi-Banglamung	12°58′36″N 100°54′48″E	
	Nakhon Sawan	15°15′47″N 100°20′37″E	
	Nakhon Sawan-Mueang	15°44′15″N 100°5′21″E	
	Nakhon Sawan-Krok Pra	15°34′48″N 100°0′13″E	
	Nakhon Ratchasrima-Serngsang	14°25′34″N 102°27′38″E	

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

Nakhon Sawan-Mae Pern	15°39′28″N 99°28′9″E	
Nakhon Sawan-Mae Wong	15°46′52″N 99°31′9″E	
Chonburi	13°22′0″N 100°58′60″E	Rajatileka *et al.* 2008 [107]
Phang Nga	8°28′0″N 98°31′60″E	
Phang Nga-Thap Pud	8°31′0″N 98°37′60″E	
Chiang Mai-Mueang	18°47′N 99°00′E	Thanispong *et al.* 2008 [14]
Kanchanaburi-Sai Yok	14°20′N 98°59′E	
Nonthaburi-Mueang	13°53′N 100°29′E	
Songkhla-Mueang	7°11′N 100°35′E	
Satun-Mueang	6°37′N 100°03′E	
Bangkok-Chatuchak	13°50′N 100°34′E	
Lampang-Mueang	18°17′N 99°29′E	
Tak-Mae Sot	16°46′N 98°34′E	
Khon Kean-Mueang	16°25′N 102°50′E	
Surat Thani-Mueang	9°08′N 99°20′E	
Nakhon Sawan-Mueang	15°42′N 100°08′E	
Nonthaburi-Mueang	13°51′44″N 100°30′48″E	Paeporn *et al.* 2010 [99]
Nakhon Pathom-Mueang	13°49′11″N 100°3′57″	
Sra Kaeo-Mueang	13°48′53″N 102°4′19″E	
Saraburi-Mueang	14°31′38″N 100°54′35″E	
Lopburi-Mueang	14°47′53″N 100°39′13″E	
Suphanburi-Mueang	14°29′4″N 100°7′25″E	
Angthong-Mueang	14°35′19″N 100°27′12″E	
Singburi-Mueang	14°53′18″N 100°24′17″E	
Kanchanaburi-Mueang	14°0′12″N 99°33′0″E	
Phitsanulok-Mueang	16°49′29″N 100°15′34″E	
Phichit-Mueang	16°26′18″N 100°21′0″E	
Sukhothai-Mueang	17°0′28″N 99°49′23″E	
Uttaradit-Mueang	17°37′33″N 100°5′48″E	
Lamphun-Mueang	18°34′42″N 99°1′6″E	
Uthaithani-Mueang	15°22′46″N 100°1′29″E	
Chiang Mai-Mueang	18°47′25″N 98°59′4″E	
Chiang Rai-Mueang	19°54′31″N 99°49′57″E	
Khon Kean-Mueang	16°26′18″N 102°50′20″E	
Udonthani-Mueang	17°24′54″N 102°47′12″E	
Chai yaphum-Mueang	15°48′35″N 102°1′13″E	
Nongkhai-Mueang	17°52′48″N 102°44′30″E	
Nakhon Nayok-Mueang	14°12′13″N 101°13′2″E	
Rayong-Mueang	12°40′6″N 101°16′30″E	
Prachinburi-Mueang	14°3′2″N 101°22′0″E	
Chonburi-Mueang	13°21′43″N 100°58′45″E	
Sra Kaeo-Mueang	13°48′52″N 102°4′20″E	Satimai 2010 [100]
Rayong-Mueang	12°43′3″N 101°23′31″E	
Chanthaburi-Mueang	12°36′40″N 102°6′15″E	

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

	Trat-Mueang	12°13′54″N 102°30′48″E	
	Bangkok-Kannayaw	13°50′N 100°40′E	Chuaycharoensuk *et al.* 2011[35]
	Chanthaburi-Mueang	12°39′N 102°7′E	
	Chonburi-Mueang	13°19′N 100°55′E	
	Prachinburi-Mueang	14°7′N 101°21′E	
	Buri Rum-Lam Plai Mat	15°00′N 102°50′E	
	Kalasin-Huaypung	16°40′N 103°53′E	
	Prachinburi-Mueang	14°6′N 101°19′E	
	Khon Kean-Mueang	16°19′N 102°47′E	
	Nakhon Ratchasima-WangNam Kheow	14°26′N 101°47′E	
	Srisaket-Uthumpronpisai	15°08′N 104°12′E	
	Surin-Dontoom	15°14′N 103°30′E	
	Roi Et-Suwannaphum	15°40′N 103°49′E	
	Nakhon Ratchasima-WangNam Kheow	14°24′N 101°51′E	
	Udonthani-Wungsammor	17°3′N 103°26′E	
	Chiang Mai-Mueang	18°46′N 98°56′E	
	Chiang Rai-Mae Chun	20°08′N 99°51′E	
	Kampaeng Phet-Kanuworralukburi	16°00′N 99°48′E	
	Lampang-Mueang	18°14′N 99°26′E	
	Lamphun-Mueang	18°37′N 99°00′E	
	Nakhon Sawan-Mueang	15°40′N 100°05′E	
	Phrae-Mueang	18°05′N 100°12′E	
	Tak-Mae Sot	16°43′N 98°34′E	
	Uthaithani-Ban Rai	15°12′N 99°41′E	
	Chumphon-Mueang	10°30′N 99° 07′E	
	Phang Nga-Takuaytung	08°12′N 98°17′E	
	Phatthalung-Pa Bon	07°16′N 100°09′E	
	Phuket-Mueang	07°53′N 98°23′E	
	Prachuap Khiri Khan-Hua Hin	12°33′N 99°53′E	
	Songkhla-Namom	06°54′N 100°32′E	
	Songkhla-Ranode	07°52′N 100°18′E	
	Songkhla-Sadao	06°45′N 100°24′E	
	Songkhla-Had Yai	07°00′N 100°27′E	
	Surat Thani-Mueang	09°02′N 99°22′E	
Propoxur	Nakhon Sawan-Mae Wong	15°4′35″N 99°3′7.4″E	Jirakanjanakit *et al.* 2007 [13]
	Nakhon Sawan-Mae Wong	15°46′52″N 99°31′9″E	Pethuan *et al.* 2007 [108]
	Surat Thani-Mueang	9°08′N 99°20′E	Thanispong *et al.* 2008 [14]
	Nakhon Sawan-Muang	15°42′N 100°08′E	
Temephos	Tak-Mae Pa	16°45′N 98°33′E	Ponlawat *et al.* 2005 [98]
	Tak-Mae Pa	16°45′N 98°34′E	
	Nakhon Sawan-Phayuhakhiri	15°29′N 100°8′E	
	Surat Thani-Tha Chana	9°34′N 99°07′E	
	Phatthalung-Mueang	7°30′N 100°03′E	
	Roi Et-Mueang	16°03′N 103°39′E	Saelim *et al.* 2005 [110]

Table 3 Locations in Thailand with *Aedes aegypti* populations tested against insecticides using the WHO standard contact assay (2000–2011) *(Continued)*

Bangkok- Hauykwang	13°4′47.4″N 100°3′52.3″E	Jirakanjanakit *et al.* 2007 [111]
Nakhon Sawan-Mae Wong	15°4′35″N 99°3′7.4″E	
Nakhon Ratchasrima- Prathai	15°3′56″N 102°3′45.7″E	
Nonthaburi-Mueang *	13°51′44″N 100°30′48″E	Paeporn *et al.* 2010 [99]
Suphanburi-Mueang *	14°29′4″N 100°7′25″E	
Angthong-Mueang	14°35′19″N 100°27′12″E	
Lopburi-Mueang	14°47′53″N 100°39′13″E	
Chiang Mai-Mueang *	18°47′25″N 98°59′4″E	
Uthaithani-Mueang	15°22′46″N 100°1′29″E	
Nongkhai-Mueang *	17°52′48″N 102°44′30″E	
Khon Kean-Mueang *	16°26′18″N 102°50′20″E	
Chanthaburi-Mueang *	12°36′38″N 102°6′15″E	
Chanthaburi-Mueang *	12°36′40″N 102°6′15″E	Satimai 2010 [100]

*Evidence of low-grade insecticide resistance.

circulating microfilaria in the peripheral blood occurring between 18.00 and 20.00 h [59,60].

Malaria vectors

Of the approximately 73 *Anopheles* species found in Thailand, select members of the Leucosphyrus Group (Neomyzomyia Series), Maculatus Group (Neocellia Series), and Minimus Subgroup (Myzomyia Series), as the most important malaria vectors in the country [38]. Five species within these 3 assemblages are incriminated as primary malaria vectors in Thailand, including *Anopheles baimaii* (previously *An. dirus* D) [61], *Anopheles dirus* [61,62], *Anopheles minimus* (previously species A) [63], *Anopheles pseudowillmori* [61] and *Anopheles aconitus* [61,64,65]. Manguin *et al*. [38] provides a current review of the vectorial capacity and bionomics of malaria vectors in the SEA region.

Several other potential vectors of malaria in Thailand that have a close association with humans, included *An. maculatus*, *Anopheles epiroticus* (= *An. sundaicus* A), *Anopheles karwari, Anopheles philippinensis,* and *Anopheles tessellatus*. Additionally, some members of the Barbirostris Group, subgenus *Anopheles* (*Anopheles barbirostris* and *Anopheles campestris*) exhibit malaria vector potential in Thailand [66].

Dengue vectors

Only two species of *Aedes* mosquitoes, *Aedes aegypti* and *Aedes albopictus* are considered as primary vectors of dengue viruses in Thailand [67]. *Aedes aegypti* is highly anthropophilic and often propagates in and around human dwellings with a high propensity for resting inside houses. Larval habitats are typically artificial containers holding fresh water such as discarded tires, flower pots, drums, refuse bottles and cans, and other water storage devices [67,68]. *Aedes albopictus* is believed to be a native to Southeast Asia [69]. *Aedes albopictus* prefers to breed in natural habitats like tree holes, bamboo stumps and other natural containers but will also utilize outdoor man-made habitats that typically contain a higher amount of organic matter than tolerated by *Ae. aegypti.* Predominantly a 'rural' species in Thailand, *Ae. albopictus* has been reported invading residential areas of larger urban zones, especially in the vicinity of Bangkok [70].

Female *Ae. albopictus* also displays a greater preference to rest and feed outdoors [71,72]. Similar to *Ae. aegypti*, it is a daytime feeder and can be found resting in shady areas in shrubs nearer ground level [73]. Likewise, *Ae. albopictus* blood feeding activity peaks in the early morning and late afternoon and is considered an aggressive biter with a wider host feeding range (human, domestic and wild animals) than *Ae. aegypti* [74]. This species is also known to be a competent vector of several other viral human pathogens, including Chikungunya virus [75,76] and Eastern equine encephalitis virus [77].

Lymphatic filariasis vectors

In Asia, at least 36 mosquito species belonging to six genera have been incriminated as either primary or secondary vectors of *W. bancrofti*, with the majority being *Anopheles* species (24) followed by aedine mosquitoes (7 spp.), *Culex* (4 spp.) and two *Mansonia* (*M. dives*, *M. uniformis*) [38]. In Thailand, two species of *Mansonia* and five *Anopheles* are vectors of *Brugia malayi* [78]. Larval habitats for many of these vector species are commonly marshes/swamps found in close proximity to villages. The NSP form of the parasite has been experimentally and naturally transmitted by several mosquito genera, including *Aedes*, *Mansonia*, *Anopheles* and *Downsiomyia* [58,60]. In Thailand, lymphatic

filariasis and malaria parasites can naturally share the same vector species, in particular *Anopheles dirus* and *Anopheles minimus* complexes, the *Anopheles maculatus* group, *Anopheles aconitus* and *Anopheles vagus* [38]. Active cases of filariasis in Thailand have declined over the past 20 years. Transmission is now found primarily with at-risk Thai migrants who enter endemic areas where vectors are common, especially along the Thai-Myanmar border. Increases in temporary migrant workers along the border have been associated with increases in disease transmission in the region. The prevalence of filariasis among a group of migrant workers in Tak Province, western Thailand, was approximately 4.4% and 2.4% in Prachuab Kiri Khan Province, southern Thailand [79,80].

Japanese encephalitis vectors

In Thailand, JE virus is maintained enzootically within the rice field–breeding mosquitoes *Cx. tritaeniorhynchus*, *Cx. fuscocephala*, and *Cx. gelidus* [81]. Burke and Leake [82] have also reported *Cx. pseudovishnui* and *Cx. vishnui* to be competent vectors of JE and more recently, the virus has also been isolated from *Culex quinquefasciatus* in Thailand [83]. These mosquitoes can serve as the 'bridging' vectors to humans from pigs that are the primary amplifying hosts in endemic areas and native wading birds associated with rice fields and natural wetlands as the natural virus reservoirs [84].

Chikungunya vectors

The virus can be transmitted to humans via several species of mosquitoes, most notably several *Aedes* species, but also *Culex annulirostris*, *Mansonia uniformis* and a few species of *Anopheles* [85-87]. In Asia, the same two species that transmit dengue viruses, *Ae. aegypti* and *Ae. albopictus*, are of prime importance in chikungunya transmission in both urban and rural settings [88,89]. *Aedes albopictus* has been shown to have a higher susceptibility and a greater propensity to transmit the chikungunya virus than *Ae. aegypti* [75,76,90,91]. Similar to *Ae. aegypti*, the normal flight range of *Ae. albopictus* is generally limited within a radius of 400–600 m from their original larval habitat [92]. Like dengue, the virus has also been shown to be vertically transmitted from an infected female mosquito to her eggs and prodigy. The rapid extension of strains of *Ae. albopictus* worldwide, especially in temperate areas, represents a serious potential threat of chikungunya transmission in areas where it has not been seen before.

Physiological resistance to insecticides

One of the primary methods of preventing vector-borne disease transmission is to disrupt human-vector contact using chemical means [12,13]. Synthetic chemicals of various classes have been used for many years in national public health vector control programs [1,2],

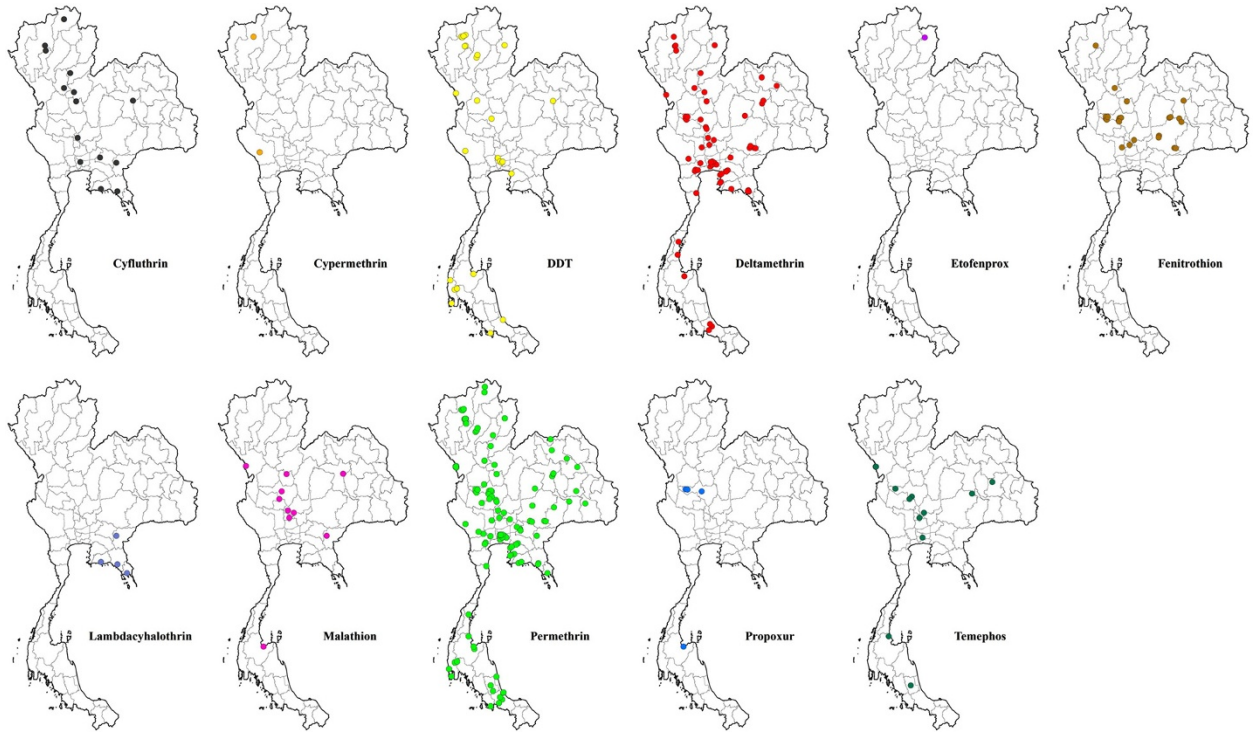


Figure 3 Distribution of *Aedes aegypti* with resistance to insecticides in Thailand (2000–2011).

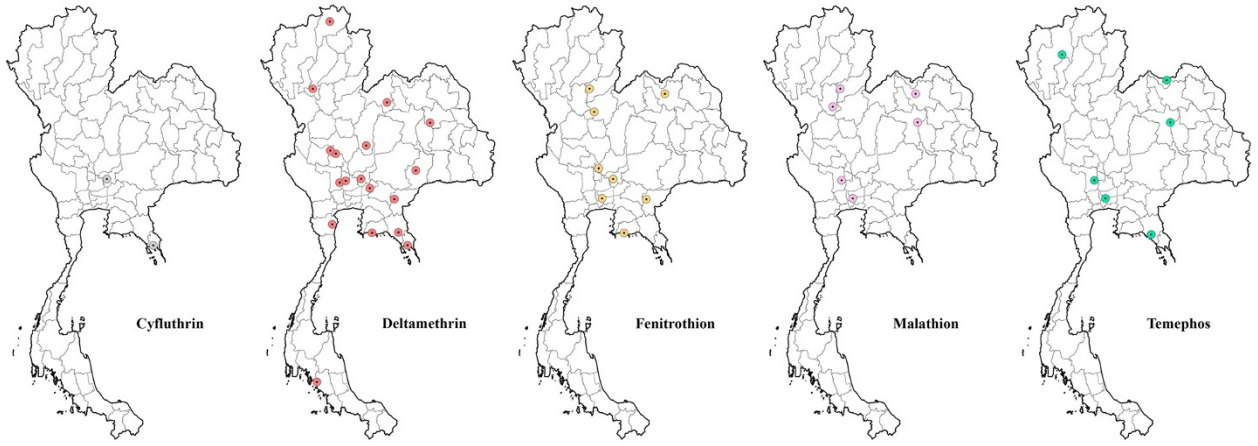


Figure 4 Distribution of *Aedes aegypti* with resistance to incipient insecticides in Thailand (2000–2011).

currently the majority of which are pyrethroid-based formulations. In Thailand, combinations of different pyrethroids are commercially available to home owners to control mosquitoes and other indoor/outdoor arthropod pests. Pyrethroids have become relatively inexpensive, provide quick knockdown and are relatively safe compounds to use near humans to control common house-frequenting mosquitoes [8,93]. Since 1994, deltamethrin has been intensively used in organized vector control programs in Thailand to interrupt dengue transmission, generally in response to an outbreak. Numerous pyrethroid-based formulations (e.g., aerosols, coils, spray and gels), that include one or more of 12 different active ingredients, are available commercially to the general public [8,35,94]. A survey of 23 household products in public markets in metropolitan Bangkok found 11 containing varying low concentrations of permethrin, 11 with tetramethrin, 4 with bioallethrin and prallethrin, many with a synergist (piperonyl butoxide) added. Only three products had a mixture of deltamethrin (Table 1). One carbamate (propoxur) and one organophosphate (dichlorvos) are also available as an approved mixture in household pest control products. Frequent exposure of mosquito populations to sub-lethal concentrations of these chemicals may result in, or contribute to, the development of insecticide resistance with a direct operational impact on the effective management and prevention of vector-borne diseases [95].

In Thailand, resistance to DDT has been documented in only 2 species of anophelines (*Anopheles annularis* and *Anopheles minimus*) (Table 2), located in the northwestern part of the country [96]. In spite of decades of organized control activities using chemicals against malaria vectors, particularly IRS, there has been no published data reporting resistance to any other chemical class or active ingredient (mainly deltamethrin) used for vector control. This interesting finding contrasts significantly with the resistance patterns seen in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in the country and could be explained by the predominant exophilic/exophagic behavior of major malaria vectors in the region that would limit the exposure time of mosquitoes with residual insecticides present inside houses (e.g., IRS and bednets).

Table 4 Locations in Thailand with *Aedes albopictus* populations tested against insecticides using the WHO standard contact assay (2000–2011)

Insecticide	Location (province-district)	Geographic coordinates (DMS)	Published sources
DDT	Chiang Mai-Mae Tang	19°11′N 98°54′E	Somboon *et al.* 2003 [93]
Permethrin	Phatthalung-Mueang	7°30′N 100°03′E	Ponlawat *et al.* 2005 [98]
	Tak-Mae Sot	16°45′N 98°33′E	
	Tak-Mae Sot	16°45′N 98°34′E	
	Chumphon-Mueang	10°30′N 99°07′E	Chuaycharoensuk *et al.* 2011 [35]
	Prachuap Khiri Khan-Hua Hin	12°33′N 99°53′E	
	Songkhla-Namom	6°54′N 100°32′E	
	Songkhla-Sadao	6°45′N 100°24′E	
	Surat Thani-Mueang	9°02′N 99°22′E	

Resistance or incipient (tolerance) resistance to temephos, a common organophosphate used to control mosquito larvae, has been observed in *Ae. aegypti* in Thailand [13,97,98]. Of 19 populations tested, 12 were found resistant to temephos while 7 populations demonstrated tolerance. The degree of susceptibility to temephos also appeared to vary depending upon the history of chemical usage/exposure in the area. Data on the susceptibility to malathion and fenitrothion (organophosphates) and propoxur (carbamate) in adult *Ae. aegypti* has been compiled from 2000–2011 based on WHO susceptibility tests (Table 3). The degree of susceptibility to these two chemical groups also varied according to the geographic settings and previous chemical exposure. For example, strong resistance to malathion and fenitrothion in *Ae. aegypti* has been reported in the central, northern and northeastern areas of Thailand; whereas only moderate to low grades of resistance have been seen in the far north of the country [13,97]. Only one locality in southern Thailand has reported significant malathion resistance in *Ae. aegypti* populations [14].

Thanispong *et al.* [14], reported *Ae. aegypti* resistant to six different synthetic pyrethroids, namely (cyfluthrin, cypermethrin, deltamethrin, etofenprox, lambda-cyhalothrin, and permethrin) (Table 3). Permethrin resistance is widely distributed in the country, while deltamethrin, lambda-cyhalothrin and cyfluthrin resistance has so far shown a much narrower spatial distribution (Figure 3). DDT resistance has been found throughout the country, whereas fenitrothion (OP) was restricted to the central and northern regions of Thailand (Figure 3). In addition, incipient resistance has been reported for three major groups of insecticides, with the majority being synthetic pyrethroids, especially permethrin and deltamethrin (Figure 4). Although data is limited, temephos resistance (OP) in *Ae. aegypti* appears more prevalent than resistance to propoxur (carbamate) (Figure 3). For *Aedes albopictus*, few reports on physiological resistance patterns are available; however, most of the *Ae. albopictus* populations sampled have demonstrated resistance to permethrin and one population showed resistance to DDT (Table 4 and Figure 5).

More recent data on pyrethroid resistance in *Ae. aegypti* and *Ae. albopictus* populations from Thailand have been restricted in specific geographical areas [8,13,14,34,105,106,112]. Physiological resistance to three different pyrethroids among 32 *Ae. aegypti* strains collected throughout Thailand and five strains of *Ae. albopictus* from southern Thailand were investigated [35]. The frequency of resistance to permethrin in *Ae. aegypti* populations varied between 4% and 56.4%. All 32 strains of *Ae. aegypti* were found to have evidence of incipient resistance (62.5%) or levels of permethrin post-exposure survival deemed clearly resistant (37.5%). However, the majority of *Ae. aegypti* strains were found to be susceptible (> 98% mortality) to deltamethrin. Four strains of *Ae. albopictus* showed evidence of incipient resistance to lambda-cyhalothrin and one showed high resistance to permethrin [35].

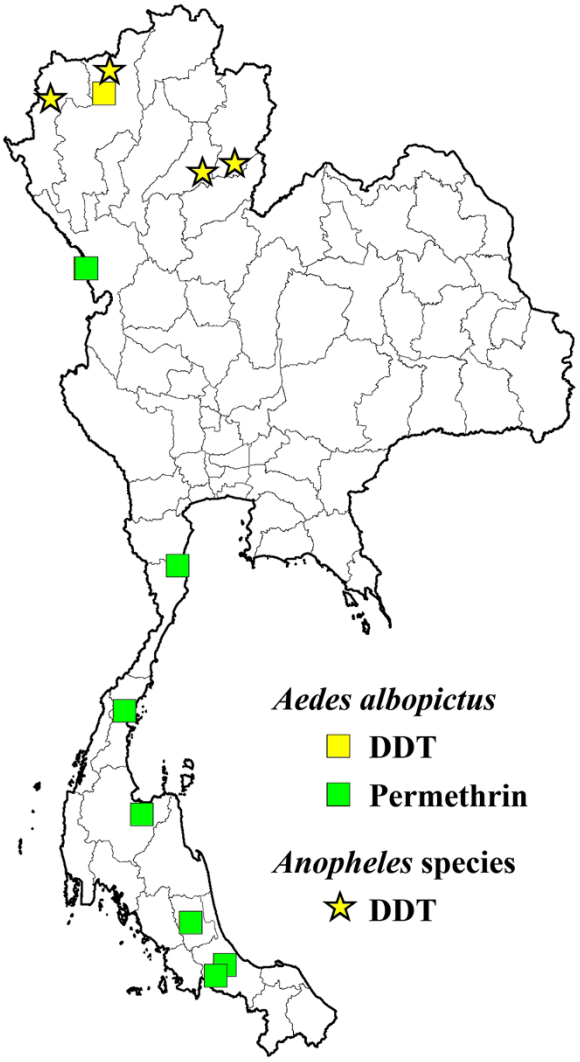


Figure 5 Distribution of *Aedes albopictus* and *Anopheles* species with resistance to DDT and permethrin, and DDT, respectively, in Thailand (2000–2011).

In *Cx. quinquefasciatus*, there were high levels of resistance to DDT, permethrin, deltamethrin and propoxur, whereas susceptibility to malathion and fenitrothion (Table 5, Figure 6) was maintained [93,113]. However, the limited number of sites and populations tested render it difficult to estimate the country-wide importance and impact of insecticide resistance in this mosquito species.

Behavioral responses to insecticides

Behavioral responses of mosquitoes to insecticides can be observed using various laboratory devices and field 'excito-repellency' assay systems. For laboratory assays,

Table 5 Locations in Thailand with *Culex quinquefasciatus* populations tested against insecticides using the WHO standard contact assay (2000–2011)

Insecticide	Location (province-district)	Geographic coordinates (DMS)	Sources
DDT	Chiang Mai- Mueang	18°47′N 99°1′E	Prapanthadara *et al.* 2000 [96]
	Chiang Mai-Mueang	18°46′N 98°57′E	Somboon *et al.* 2003 [93]
	Chiang Mai-San Kampaeng	18°44′43″N 99°7′13″E	
	Lampang-Mueang	18°17′31″N 99°30′16″E	
	Nan-Tha Wang Pha	19°7′35″N 100°48′53″E	
	Nonthaburi-Baan Suan	13°51′N 100°29′E	Sathantriphop *et al.* 2006 [113]
	Bangkok-Phom Pabsatupai	13°44′N 100°29′E	Thanispong *et al.* 2008 [14]
	Nonthaburi-Mueang	13°50′N 100°31′E	
	Pathum Thani-Lad Lumkeaw	14°02′N 100°24′E	
Deltamethrin	Phang-Nga- Keuk-kak	8°41′47.2″N 98°15′28.6″E	Komalamisra *et al.* 2006 [114]
	Nonthaburi-Baan Suan	13°51′N 100°29′E	Sathantriphop *et al.* 2006 [106]
	Nonthaburi-Muenng	13°50′N 100°29″E	Sathantriphop *et al.* 2006 [113]
	Tak-Mae Sot	16°47′N 98°36′E	
	Bangkok-Pom Prab Satru Phai	13°45′N 100°30′E	
	(laboratory strain) in 1978		
Etofenprox	Chiang Mai-Mueang	13°19′N 100°55′E	Somboon *et al.* 2003 [93]
	Nan-Tha Wang Pha	19°7′35″N 100°48′53″E	
Fenitrothion	Nonthaburi-Baan Suan	13°51′N 100°29′E	Sathantriphop *et al.* 2006 [106]
Malathion	Nan-Tha Wang Pha	19°7′35″N 100°48′53″E	Somboon *et al.* 2003 [93]
Permethrin	Phang-Nga Keuk-kak	8°41′47.2″N 98°15′28.6″E	Komalamisra *et al.* 2006 [114]
	Nonthaburi-Baan Suan	13°51′N 100°29′E	Sathantriphop *et al.* 2006 [106]
	Nonthaburi-Mueang	13°50′N 100°31′E	Thanispong *et al.* 2008 [14]
	Pathum Thani-Lad Lumkeaw	14°02′N 100°24′E	
Propoxur	Nonthaburi-Baan Suan	13°51′N 100°29′E	Sathantriphop *et al.* 2006 [106]
	Nonthaburi-Mueang	13°50′30″N 100°29′45″E	Sathantriphop *et al.* 2006 [113]

many of the assay variations have been reviewed [12]. The World Health Organization developed the first test box to evaluate the excitation ("irritability") of exposed mosquitoes following physical contact with insecticides [115]. This system was subsequently referred to as an "excito-repellency" test box [116]. The test system was further modified by other investigators interested in behavioral avoidance responses to DDT and some of the early synthetic pyrethroids [12,117-121]. A light-proof test chamber was designed to study the irritant response of *Anopheles gambiae*, an important malaria vector in Africa, to several chemical compounds [122]. One limitation associated with all of these earlier test systems was the procedural difficulty for introducing and removing test specimens with the assay designs. Other concerns were the ability to evaluate various physiological states found in wild-caught mosquitoes and selecting the ideal range of concentrations for chemical evaluation. Moreover, at the time no universal analysis or set of statistical methods for interpretation of data had been fully accepted nor had any test system been designed to more clearly discriminate between contact excitation and noncontact (spatial) repellency responses [12]. An improved excito-repellency test device that was able to better differentiate between excitation and spatial repellency was developed and first tested against field populations of *Anopheles albimanus* in Central America [12,19]. Unfortunately, this fixed prototype was cumbersome to handle and required considerable time for attaching the insecticide-treated test papers inside the chamber. Eventually, a more field deployable test system was designed that was collapsible and easily transportable [123,124]. This system has been extensively used to investigate the behavioral responses of various mosquito species in Thailand and elsewhere in Asia [125-128]. Additionally, a more compact, modular assay system called the

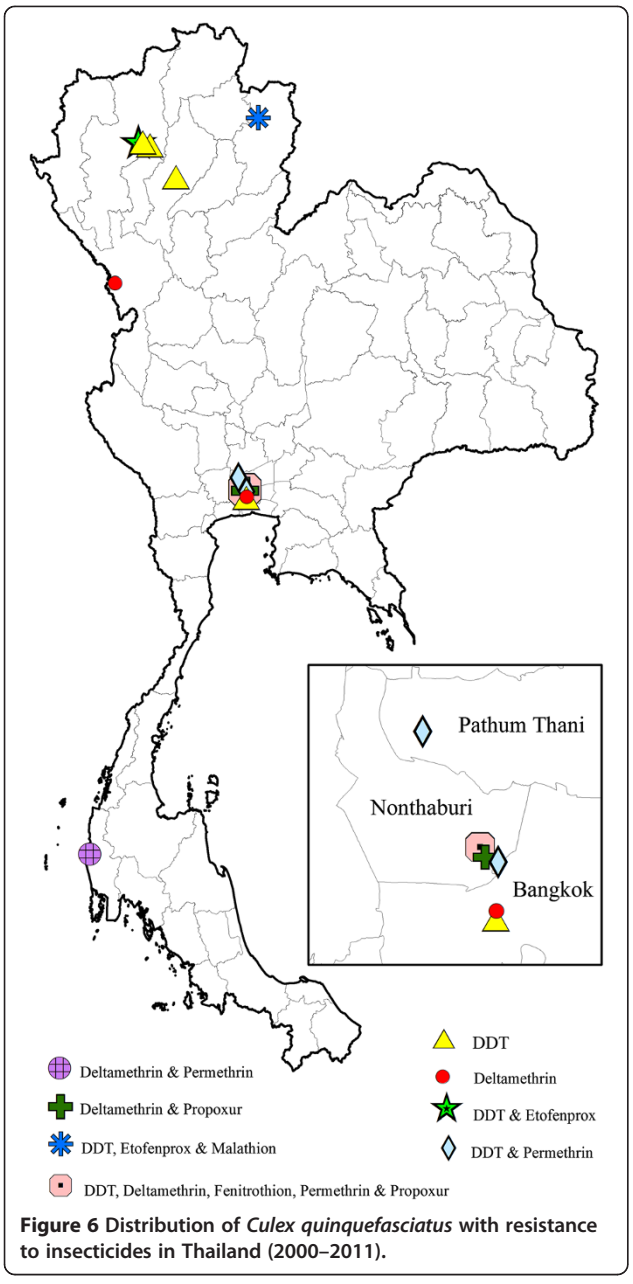


Figure 6 Distribution of *Culex quinquefasciatus* with resistance to insecticides in Thailand (2000–2011).

High Through-put Screening System (HITSS) was developed for mass screening of chemicals and adult mosquito responses; including contact irritancy, spatial repellency, and toxicity depending on the specific design set-up [129]. This modular system is of a reduced size compared to previous excito-repellency box devices and greatly minimizes the treated surface area required thereby reducing the amount of chemical required for handling and testing. This system is now approved by WHOPES for testing efficacy of new active ingredients intended for spatial repellent [45].

To better approximate insect behaviors in natural field settings, numerous experiments have been made over many decades using specially constructed experimental huts [4,31,121,130-136]. Most experimental hut studies have been conducted to observe the behavior of *Anopheles* mosquitoes; however, Grieco *et al*. [4] successfully demonstrated all 3 chemical actions could be observed in experimental huts using *Ae. aegypti* as a model system. The results obtained from both laboratory and field studies can help facilitate the choice of the most effective chemicals and measures to control house-frequenting adult mosquitoes. The behavioral responses of *Anopheles* species to various compounds are provided in Table 6 and those for *Aedes aegypti* and *Culex quinquefasciatus* in Table 7.

Behavioral avoidance to insecticides

DDT

Unlike physiological resistance, accurately measuring behavioral responses remains elusive and difficult to detect. Some agricultural and medically important insects, including malaria vectors, have allegedly demonstrated what has been termed “behavioral resistance” following repeated exposure to sub-lethal concentrations of DDT [30]. However, “behavioral avoidance” is preferred terminology as it is an innate, involuntary response to an external stimuli rather than a permanent, genetically-based shift in behavior as the development of apparent fixed behavioral changes because of insecticide selective pressure has not been sufficiently documented to occur under natural conditions [143]. The early observations on behavioral responses of mosquitoes focused on DDT and have been investigated using experimental huts and excito-repellency box (ERB) test systems. The first study on the irritant effect of DDT residual deposits was conducted using *Anopheles quadrimaculatus* where females were found to be irritated shortly after making contact with the treated surfaces resulting in a rapid escape response from a treated house before taking a blood meal [144]. Subsequent observations found that *An. quadrimaculatus* often received a lethal dose from this brief contact and perished within 24 hours [145]. Unfortunately, these studies were conducted without having a matched control (untreated houses) for comparison. Furthermore, the high mortality seen with *An. quadrimaculatus* may have been caused by further contact with toxic ingredients while attempting to leave a treated house through the small portals of the experimental hut [143]. In studies with *Anopheles albimanus* in Panama, Trapido [146] concluded that wild-caught mosquitoes lacking re-exposure to DDT for a long period of time, showed the same susceptibility levels to DDT as those from a laboratory colony with no history of previous exposure. Malaria vectors in some countries (e.g., Brazil, Thailand) have apparently never

developed resistance to DDT [125,130] despite years of extensive use inside houses, suggesting that the particular mosquito populations avoid making direct physical contact with the chemical, thereby precluding any selection for resistance. Table 6 lists *Anopheles* species and levels of behavioral responses to DDT and synthetic pyrethroids in Thailand.

In Thailand, the irritant and repellent effects of DDT were demonstrated against two populations of *Anopheles minimus* (sensu lato) using an ERB test system [125]. A study comparing 2 members of the Minimus Complex; *An. minimus* (species A) and *Anopheles harrisoni* (species C) found that DDT produced a rapid and striking irritant response in both species, while repellency was more pronounced in *An. minimus* [127]. With *Ae. aegypti*, it has been suggested an association exists between increased levels of physiological resistance to DDT and a greater suppression of both excitation and repellency responses, yet the overall avoidance behavior remains a significant response compared to non-exposed controls [140].

Pyrethroids

Numerous studies have found that synthetic pyrethroids clearly elicit a range of excitation and repellent effects on many insect species that also typically result in mosquitoes moving away ('avoidance') from insecticide sprayed surfaces [30,147,148]. The extensive and continuing use of pyrethroids should serve as an incentive to intensify investigations on the operational significance of pyrethroid-induced avoidance behavior in mosquito vectors and other arthropods. Given the important role of indoor residual spraying of homes as a means of reducing risk of malaria transmission, the consequence of excitation and repellent actions should be well defined for specific malaria vectors in defined locations before and after beginning any large scale control program. Following the refinement of the ERB test system and development of the HITSS assay, both that allow the discrimination of 2 primary types of behavioral actions [12], a series of important findings on excito-repellency behavior in *Anopheles* mosquitoes have been reported in Thailand [19,70,125,127,137,138,142]. In general, synthetic pyrethroids consistently result in significantly stronger contact irritant responses in *Anopheles* compared to repellency (Table 6). For example, lambda-cyhalothrin and deltamethrin act as strong excitatory agents on test populations of *An. minimus* while showing relatively weak repellent activity [125]. Pothikasikorn *et al*. [127] also confirmed that *An. minimus* and *An. harrisoni* showed a rapid escape response to contact with lambda-cyhalothrin and deltamethrin, while repellency remained significant but lagged in intensity. Chareonviriyaphap *et al*. [70] described the excito-repellent actions of deltamethrin in four different *Anopheles* species, all representing important malaria vectors in Thailand, showing deltamethrin can produce a pronounced irritant response relative to a slower, weaker spatial repellency. Although repellency was less outstanding an escape response compared to irritancy, the repellent action and escape responses were still statistically significant compared to the matched paired controls (minus active ingredient).

Numerous behavioral responses of *Ae. aegypti* populations exposed to a series of pyrethroids (deltamethrin, permethrin, alpha-cypermethrin, cyphenothrin, d-tetramethrin and tetramethrin) have been investigated in depth in Thailand (Table 7). In general, all tested populations have exhibited moderate to strong irritancy when compared to repellency [97,101,129,140,142]. Far fewer numbers of populations of *Cx. quinquefasciatus* have been tested against the 3 principal classes of insecticides used in vector control; pyrethroids (deltamethrin), organophosphates (fenitrothion) and carbamates (propoxur), yet again, contact excitation appears stronger than repellency against all three compounds (Table 7). However, marked differences in behavioral responses have been seen between different mosquito populations, active ingredients and concentrations. Prominent irritant responses were observed in a long-established colony population exposed to deltamethrin and fenitrothion as compared to two field populations of the same species [106], demonstrating the importance of testing field populations as opposed to long-colonized material.

The behavioral responses to insecticides by mosquitoes are important components of a chemical's overall effectiveness and value in reducing human-vector contact and disease transmission. To date, there is no convincing evidence of fixed behavioral 'resistance' developing in mosquito species to continuous or intermittent exposure to insecticides [149]. Rather, the majority of data supports that a mosquito's actions are part of an innate involuntary behavioral repertoire involving reflex stimulus–response mechanisms. Behavioral responses can be split into 2 distinct categories, stimulus-dependent and stimulus-independent actions [150]. The term *avoidance behavior* is generally used to describe actions that are stimulus-dependent by some combination of excitation and repellency [131].

A stimulus-dependent action requires sensory stimulation of the insect in order for an avoidance behavior to occur. In general, this form of avoidance enables the insect to detect a chemical after making direct contact but before acquiring a lethal dose [143] or detection of vapor-phase molecules of the active ingredient in the air that initiates repellency (deterrence). On the other hand, a stimulus-independent response does not require direct sensory stimulation of the insect for avoidance to occur but rather involves other natural behavioral components such as exophily (outside resting) or zoophily (non-

Table 6 Evidence of behavioral avoidance responses to insecticides in *Anopheles* populations in Thailand (2000–2011)

Species	Field/Lab	Dose	Insecticide	Location (province-district)	Published source
Anopheles minimus A*	Lab	2.00 g/m^2	DDT	Phrae-Rong Klang	Chareonviriyaphap *et al.* 2001 [125]
		0.0625 g/m^2	Deltamethrin	Phrae-Rong Klang	
		0.0369 g/m^2	Lambdacyhalothrin	Phrae-Rong Klang	
Anopheles minimus complex	Field	2.00 g/m^2	DDT	Kanchanaburi-Pu Teuy	
		0.0625 g/m^2	Deltamethrin	Kanchanaburi-Pu Teuy	
		0.0369 g/m^2	Lambdacyhalothrin	Kanchanaburi-Pu Teuy	
Anopheles minimus A*	Lab	0.02 g/m^2	Deltamethrin	Phrae-Rong Klang	Chareonviriyaphap *et al.* 2004 [70]
Anopheles dirus B†	Lab	0.02 g/m^2	Deltamethrin	Chantaburi-Ban Paung	
Anopheles minimus complex	Field	0.02 g/m^2	Deltamethrin	Kanchanaburi-Pu Teuy	
Anopheles maculatus B‡	Lab	0.02 g/m^2	Deltamethrin	Tak-Mae Sot	
Anopheles swadwongporni	Field	0.02 g/m^2	Deltamethrin	Kanchanaburi-Pu Teuy	
Anopheles dirus complex	Field	0.02 g/m^2	Deltamethrin	Kanchanaburi-Pu Teuy	
Anopheles minimus A*	Field	2.00 g/m^2	DDT	Tak-Mae-Sot	Potikasikorn *et al.* 2005 [127]
		0.02 g/m^2	Deltamethrin	Tak-Mae-Sot	
		0.03 g/m^2	Lambdacyhalothrin	Tak-Mae-Sot	
Anopheles minimus C§	Field	2.00 g/m^2	DDT	Kanchanaburi-Pu Teuy	
		0.02 g/m^2	Deltamethrin	Kanchanaburi-Pu Teuy	
		0.03 g/m^2	Lambdacyhalothrin	Kanchanaburi-Pu Teuy	
Anopheles maculatus	Field	2.00 g/m^2	DDT	Kanchanaburi-Pu Teuy	Muenworn *et al.* 2006 [137]
		0.5 g/m^2	Permethrin	Kanchanaburi-Pu Teuy	
Anopheles swadwongporni	Field	2.00 g/m^2	DDT	Kanchanaburi-Pu Teuy	
		0.5 g/m^2	Permethrin	Kanchanaburi-Pu Teuy	
Anopheles minimus A*	Field	0.04 g/m^2	Cypermethrin	Kanchanaburi-Pu Teuy	Pothikasikorn *et al.* 2007 [138]
		0.04 g/m^2	Carbaryl	Kanchanaburi-Pu Teuy	
		0.19 g/m^2	Malathion	Kanchanaburi-Pu Teuy	
Anopheles minimus C§	Field	0.04 g/m^2	Cypermethrin	Kanchanaburi-Pu Teuy	
		0.04 g/m^2	Carbaryl	Kanchanaburi-Pu Teuy	
		0.19 g/m^2	Malathion	Kanchanaburi-Pu Teuy	
Anopheles harrisoni	Field	0.03 g/m^2	Bifenthrin	Kanchanaburi-Pu Teuy	Tisgratog *et al.* 2011 [139]
Anopheles minimus	Field	0.03 g/m^2	Bifenthrin	Tak-Mae Sot	

With the exception of one population (*An. minimus* s.s. in Tak Province/Mae Sot District to DDT) all species show contact excitation (irritancy) as the predominant response versus noncontact spatial repellency.
*Published as *Anopheles minimus* A, now formally named *Anopheles minimus* s.s.; †*Anopheles dirus* B now as *Anopheles crascens*; ‡*Anopheles maculatus* B as *Anopheles maculatus* s.s. [66] and § *Anopheles minimus* C as Anopheles harrisoni.

human blood preference) in which an insect avoids the exposure to a chemical by preferentially utilizing habitats without insecticides present [151]. This second type of response has also been included as innate, genetically-driven phenotypic and genotypic behaviors [152]. Stimulus-dependent behavioral responses include the avoidance behaviors discussed in this review.

Conclusions

A number of synthetic pyrethroids, i.e. allethrin, deltamethrin, permethrin, cypermethrin, alpha-cypermethrin, and cyfluthrin, are commonly used by home owners, private business and in the public sector to control both household pests and medically important insect vectors. In the past, the prevailing practice has been to classify chemicals simply based on their toxicity (killing) profile of insects. We have preferred the use of the term "chemical" in place of "insecticide" as it is more appropriate for recognizing the multiple actions these active ingredients have on mosquitoes via toxicity alone. This also recognizes that chemicals protect humans from the mosquito blood feeding by at least 3 different primary

Table 7 Evidence of behavioral avoidance responses in *Aedes aegypti* and *Culex quinquefasciatus* to insecticides in Thailand (2000 – 2011)

Species	Field/Lab	Dose	Insecticide	Location	Published source
Aedes aegypti	Field	0.02 g/m^2	Deltamethrin	Bangkok	Kongmee *et al.* 2004 [126]
Aedes aegypti	Field	0.02 g/m^2	Deltamethrin	Pathum Thani	
Aedes aegypti	Field	0.02 g/m^2	Deltamethrin	Nonthaburi	
Aedes aegypti	Lab	0.02 g/m^2	Deltamethrin	Ayutthaya	
Aedes aegypti	Lab	0.02 g/m^2	Deltamethrin	Bangkok	
Aedes aegypti	Lab	0.25 g/m^2	Permethrin	Nonthaburi	Paeporn *et al.* 2007 [97]
Aedes aegypti	Field	0.025 g/m^2	Alphacypermethrin	Kanchanaburi	Thanispong *et al.* 2009 [140]
		2.00 g/m^2	DDT	Kanchanaburi	
Aedes aegypti	Field	0.025 g/m^2	Alphacypermethrin	Chiang Mai	
		2.00 g/m^2	DDT	Chiang Mai	
Aedes aegypti	Lab	0.025 g/m^2	Alphacypermethrin	USDA	
		2.00 g/m^2	DDT	USDA	
Aedes aegypti	Field	0.010%	Deltamethirn	Kanchanaburi	Mongkalagoon et al. 2009 [141]
		0.0113%	Cyphenothrin	Kanchanaburi	
		2.091%	d-Tetramethrin	Kanchanaburi	
		2.377%	Tetramethrin	Kanchanaburi	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Chiang Mai	Thanispong *et al.* 2010 [142]
		0.05%	Deltamethrin	Chiang Mai	
		0.25%	Permethrin	Chiang Mai	
		4.00%	DDT	Chiang Mai	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Kanchanaburi	
		0.05%	Deltamethrin	Kanchanaburi	
		0.25%	Permethrin	Kanchanaburi	
		0.25%	DDT	Kanchanaburi	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Khon Kean	
		0.05%	Deltamethrin	Khon Kean	
		0.25%	Permethrin	Khon Kean	
		4.00%	DDT	Khon Kean	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Nonthaburi	
		0.05%	Deltamethrin	Nonthaburi	
		0.25%	Permethrin	Nonthaburi	
		4.00%	DDT	Nonthaburi	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Songkhla	
		0.05%	Deltamethrin	Songkhla	
		0.25%	Permethrin	Songkhla	
		4.00%	DDT	Songkhla	
Aedes aegypti	Field	0.05%	Alphacypermethrin	Satun	
		0.05%	Deltamethrin	Satun	
		0.25%	Permethrin	Satun	
		4.00%	DDT	Satun	

Table 7 Evidence of behavioral avoidance responses in *Aedes aegypti* and *Culex quinquefasciatus* to insecticides in Thailand (2000 – 2011) *(Continued)*

Culex quinquefasciatus	Field	0.02 g/m^2	Deltamethrin	Nonthaburi	
		0.20 g/m^2	Fenitrothion	Nonthaburi	
		0.20 g/m^2	Propoxur	Nonthaburi	Sathantriphop *et al.* 2006 [113]
Culex quinquefasciatus	Field	0.02 g/m^2	Deltamethrin	Tak	
		0.20 g/m^2	Fenitrothion	Tak	
		0.20 g/m^2	Propoxur	Tak	
Culex quinquefasciatus	Lab	0.02 g/m^2	Deltamethrin	Nonthaburi	
		0.20 g/m^2	Fenitrothion	Nonthaburi	
		0.20 g/m^2	Propoxur	Nonthaburi	

All species show contact excitation (irritancy) as the predominant response versus noncontact spatial repellency.

actions: excitation ('irritancy'), repellency and toxicity [4,32]. Historically, the vast majority of studies have focused on the direct toxicological (mortality) responses (susceptibility and resistance patterns) of chemicals on mosquito populations; whereas very little emphasis was placed on the vector's behavior in response to sub-lethal exposures to various compounds. Knowledge of a mosquito's normal and chemical-induced behavioral responses under varying conditions (e.g., age, physiological state, parity, etc.) is important in the prioritization and design of appropriate vector prevention and control strategies. The development of insecticide resistance in insect pests and disease vectors worldwide is increasing on an alarming scale. However, patterns of resistance is not uniform across all areas, with some vector populations having had low instances of resistance in spite of long-term use of chemicals used to control them, suggesting that behavioral responses may likely be playing a significant role influencing how certain chemicals perform to interrupt human-vector contact while reducing the selection pressure on target insects for developing physiological resistance [32]. In Thailand, populations of *Ae. aegypti* showing patterns of low-grade resistance to pyrethroids may indicate that resistance is increasing in a particular population; it is also possible that resistance may be declining subsequent to diminution of selective pressure. We postulate that behavioral avoidance may result in a third alternative - a low level equilibrium of physiological resistance.

Avoidance responses in which normal host-seeking and biting activities are disrupted pose a challenge to wild adult mosquitoes on a tight energy budget to fly longer and greater distances than would have otherwise occurred. This might have an impact on its survival or fecundity thereby impacting both subsequent vector population densities and disease transmission. Behavioral avoidance of insecticides may have similar effects, should insecticide coverage be sufficiently high. Recent evidence of apparent decreasing densities of vectors and reduction of malaria in Africa poses questions as to the reasons or mechanisms behind the population reductions [153]. The debate whether repellency or toxicity is the more favored attribute of an insecticide [32,33,154], may likely depend on the specific vector species being targeted for control. As IRS and ITN chemical treatments gradually reach sublethal doses what are the implications on the biology of mosquito vectors, their populations and the diseases they transmit? Is behavioral avoidance a liability or an asset in the control of new infections? For example, ITNs have been implicated in the massive reduction in *An. darlingi* in Suriname [155], whether due to direct killing effects or deterrence. On the other hand, deterrence may impact transmission dynamics that possibly exacerbate control efforts to suppress transmission [156,157]. One example of attempting to expolit contact irritant responses of *Ae. aegypti* has been the evaulation of sublethal concentrations and focal application of pyrethroid chemicals for the distruption of normal blood feeding and resting behavior [25].

Whether a chemical is acting by direct contact with a mosquito or spatially from a distance, both behavioral responses are stimulus–response actions that result in distinct movement away from an area where the chemical is present. We now have various types of excito-repellency test systems (e.g., ERB and HITSS assays) that can accurately measure the behavioral responses of mosquitoes to chemicals. WHOPES has very recently recommended a battery of test procedures to better address the efficacy and mode of action of active ingredients intended for "spatial repellent" applications [158]. However, the "proof of principle" that spatial repellency can effectively reduce malaria or dengue transmission, both indoors and out, merits further investigation.

To conclude, we believe that a better understanding and evaluation of physiological and behavioral responses by mosquitoes to chemicals are an important, if not critical, components of any disease control operation dependent on

this abatement strategy. We recommend that in addition to establishing a country-wide network of routine resistance monitoring and behavioral response evaluations where vector control is taking place, a greater emphasis is needed to investigate the biochemical and genetic mechanisms responsible for resistance in vector populations. There should also be an expansion of the number of different vector species examined. For example, there have been virtually no investigations on the response to insecticides by the primary culicine vectors of Japanese encephalitis virus or Mansonia species responsible for lymphatic filariasis. Lastly, because of the recent governmental re-organization in Thailand allowing each municipality decision-making authority for how local vector control operations are conducted, including the purchasing insecticides, there is concern that both ineffectual and inefficient vector control practices might result. Such an unstructured and independently managed system might also contribute to an escalation of insecticide resistance. It would be advisable, that clear and enforceable policies that are evidence-based for insecticide selection be directed from the MOPH, Department of Disease Control.

Knowledge of vector/pest susceptibility to pesticides, changing patterns of resistance and their operational implications, will continue to be basic requirements to help guide chemical use in evidence-based, vector-borne disease and pest control programs. Although not an exhaustive compilation, this review has attempted to summarize the current knowledge of how chemicals affect various vector species and populations in Thailand. With this background, it is hoped that operational and research endeavors will benefit from this overview and focus on areas where critical information is lacking.

Abbreviations

IRS: Indoor residual spraying; ITN: Insecticide-treated netting (bednets); DEET: *N*, *N*-diethyl-*meta*-toluamide; PCO: Pest control operators; MFO: Mixed function oxidases (P-450 mediated monooxygenases); GST: Glutathione *S*-transferases; ERB: Excito-repellency box; HITSS: High throughput screening system; WHOPES: World Health Organization Pesticide Evaluation Scheme; MOPH: Ministry of Public Health (Thailand).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TC reviewed the literature and formulated the review. TC wrote the paper with assistance from MJB, WS, MK, VC, and RNK. All authors read and approved the final manuscript.

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Review

Challenges and prospects for dengue and malaria control in Thailand, Southeast Asia

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Despite significant advances in the search for potential dengue vaccines and new therapeutic schemes for malaria, the control of these diseases remains difficult. In Thailand, malaria incidence is falling whereas that of dengue is rising, with an increase in the proportion of reported severe cases. In the absence of antiviral therapeutic options for acute dengue, appropriate case management reduces mortality. However, the interruption of transmission still relies on vector control measures that are currently insufficient to curtail the cycle of epidemics. Drug resistance in malaria parasites is increasing, compromising malaria control and elimination. Deficiencies in our knowledge of vector biology and vectorial capacity also hinder public health efforts for vector control. Challenges to dengue and malaria control are discussed, and research priorities identified.

Dengue and malaria burden in Southeast Asia

Despite recent advances in the understanding of vectorial systems (see Glossary), development of potential vaccines, and new therapeutic schemes, the control of dengue and malaria remains extremely difficult [1,2]. Dengue fever is the fastest emerging arboviral infection transmitted by *Aedes* mosquitoes. This has major public health consequences in over 100 tropical and subtropical countries in Southeast Asia (SEA), the Western Pacific (WP), and South and Central America [3]. The four antigenically distinct dengue viruses (DENV-1 to -4) cause a broad spectrum of clinical manifestations comprising asymptomatic infection, undifferentiated fever (UF), dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). The World Health Organization (WHO)–SEA region together with the WP region bears nearly 75% (approximately 40 million cases) of the current global disease burden. The incidence of dengue fluctuates periodically but is globally increasing both in terms of the number of reported cases (63 672 reported cases in the SEA region in 2000 to 355 314 cases in 2010) (http://www.searo.who.int/en/) and the number of countries in which the disease is emerging or re-emerging [4]. The reason for the increase in reported cases is multifactorial, with global trade (transport of the mosquitoes), increasing international travel (movement of viremic people), urban crowding (favoring human vector contact), and ineffective vector control strategies all supporting the spread of the disease [2]. Modeling exercises also suggest that climate change may lead to an expansion in the area of land with a climate suitable for dengue transmission, hence increasing the proportion of the human population at risk [5,6].

Malaria remains a public health problem in the SEA region with approximately 22 million cases reported per year, accounting for 14% of the total malaria burden [7]. According to the WHO, approximately 93% of those who are at a moderate to high risk of malaria infection are living in Bangladesh, India, Indonesia, Myanmar, Thailand, and Timor Leste, and are contributing more than 95% of confirmed malaria cases and deaths (http://www.searo.who.int/en/). Human malaria is caused by five

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Glossary

8-Aminoquinoline: family of drugs belonging to aminoquinoline with an amine at the 8-position of quinoline. This family contains three members, primaquine, tafenoquine, and pamaquine, and is used in the treatment of malaria. They may be used to eradicate malaria hypnozoites from the liver and have both been used for malaria prophylaxis. The 8-aminoquinoline drugs must not be given to patients with G6PD deficiency because they cause potentially fatal hemolysis in these patients.

Annual Parasite Index: is defined as the number of confirmed malaria cases during 1 year/total population × 1000.

Artemisinin-based combination therapy (ACT): the combination of artemisinin or one of its derivatives (e.g., artesunate, arthemeter, etc.) with an antimalarial or antimalarials of a different class (e.g., mefloquine, lumefantrine, amodiaquine, piperaquine, and pyronaridine).

Dengue fever: refers to dengue infection with acute febrile illness and sometimes biphasic fever associated with symptoms such as headache, myalgia, arthralgia, rashes, leucopenia, and thrombocytopenia with no evidence of plasma leakage.

Dengue hemorrhagic fever (DHF): dengue infection with high fever, hemorrhagic phenomena, and hepatomegaly. There are common hemorrhagic diatheses such as positive tourniquet test (TT), thrombocytopenia, petechiae, easy bruising, and/or gastrointestinal hemorrhage with evidence of plasma leakage, such as increased hematocrit in severe cases.

Dengue shock syndrome: dengue infection presenting all of the criteria of DHF plus circulatory disturbance and shock as evidenced by rapid and weak pulse or age-specific hypotension and cold, clammy skin, and restlessness.

Drug resistance: refers to the resistance to antimalarials as the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a medicine given in doses equal to or higher than those usually recommended but within the tolerance of the subject, provided drug exposure at the site of action is adequate.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency: a genetically inheritable disease characterized by abnormally low levels of G6PD, a metabolic enzyme involved in red blood cell metabolism. In severe G6PD deficiency, primaquine is contraindicated for the treatment of *Plasmodium vivax* infection.

Insecticide resistance: defined as the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations.

Knockdown (Kdr)-type mechanism: refers to changes in the sensitivity of the voltage-gated sodium channels due to non-silent point mutations that confer resistance to the organochlorine DDT and pyrethroids.

Malaria control: defined as the reduction of the malaria disease burden to a level at which it is no longer a public health problem.

Malaria elimination: defined by the WHO as the reduction to zero of the incidence of infection caused by human malaria parasites in a defined geographical area as a result of deliberate efforts. Continued measures to prevent re-establishment of transmission are required.

Malaria pre-elimination: defined as reduction of malaria burden at an annual malaria incidence of less than 1 case per 1000 people at risk. This requires setting up high quality-controlled systems for reaching the elimination phase.

Relapse: the recurrence of asexual parasitemia in *P. vivax* malaria derived from persisting liver stages. Relapse occurs when the blood stage infection has been eliminated, but hypnozoites persist in the liver and mature to form hepatic schizonts. After variable intervals of weeks to months, the hepatic schizonts burst and liberate merozoites into the bloodstream.

Release of insects carrying a dominant lethal (RIDL): refers to an insect control method based on the release of transgenic individuals carrying a dominant, repressible, female-specific lethal gene.

Spatial repellent (SR): refers to any molecules (e.g., insecticide or repellent) that prevent an arthropod from entering a space occupied by a potential human host. SRs are expected to reduce encounters between humans and vectors, thereby eliminating or reducing the risk of pathogen transmission.

Targeted chemo-elimination (TCE): refers to the administration of antimalarial treatment to entire populations in a selected area with the goal of eliminating malaria.

Vectorial capacity: is defined as the number of new infections the population of a given vector would distribute per case per day at a given place and time, assuming conditions of nonimmunity.

Vectorial system: refers to all genetically competent insect species and populations that play an active role in the transmission of an infectious agent/pathogen in an ecosystem (defined in time and space).

Plasmodium species that are transmitted by anopheles mosquitoes including *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*, as well as *Plasmodium knowlesi*, which is gaining widespread recognition as a human pathogen [8]. Implementation of integrated national malaria control programs has resulted in significant reduction of malaria infections since 2000, although foci and hot spots for malaria transmission are still present, especially in forested and rural areas, and along the country borders from where it can spread to areas which are currently malaria-free [9,10]. The malaria situation in SEA remains highly dynamic and is likely to be further aggravated by climate change and environmental modifications [11].

To achieve a better understanding of the situation of dengue and malaria in SEA, we address the biodiversity, ecology, environmental, and social factors that influence dengue and malaria transmission and discuss challenges and prospects for their control. We focus mainly on Thailand as an excellent case study of the implementation of a national control strategy based on the integrated Roll Back Malaria/WHO strategy (http://www.rollbackmalaria.org/forumV/docs/gsp_en.pdf) and Asia-Pacific Dengue Strategic Plan (http://www.wpro.who.int/mvp/Dengue_Strategic_Plan.pdf).

Dengue and malaria in Thailand: a public health problem?

Dengue burden

Dengue is described as hyperendemic in Thailand where the four serotypes of DENV co-circulate in both urban and rural areas. The prevailing serotype varies over time, and a large epidemic was associated with DENV-3 in the 1990s [12]; whereas, DENV-1 and -2 were more predominant in the past decade [13]. Using historical data the mean prevalence of serotypes from 1973 to 2009 were estimated to be 9.8%, 14.6%, 7.5%, and 5.2% for DENV-1 to DENV-4, respectively [14]. Differences in severity and clinical manifestations between DENV serotypes have been reported (DENV-2 was most likely to cause DHF) [15], but factors besides serotype such as sequences of primary and secondary infections are known to influence disease severity [16]. Dengue remains a major public health problem and represents the foremost cause of hospitalization, mainly in children, with the highest incidence in 9–14 year olds [17]. However, there is considerable heterogeneity in dengue incidence within the country (Figure 1). Fortunately, due to appropriate case management the mortality rate is low [18]. The overall burden of the disease, however, remains considerable and has a significant social and economic impact; patients and their families suffer, healthcare organizations are disrupted during epidemics, and there are direct and indirect costs due to loss of productivity [19].

Malaria burden

Similar to other countries in SEA, malaria displays significant geographical heterogeneity in Thailand, exemplified by the 'border malaria' type (Figure 2), with most of the malaria cases concentrated along the borders with Myanmar and Cambodia [10,20]. Malaria transmission along the border is difficult to control due to the presence of efficient vectors and extensive population movement across the border, especially foreign workers and refugees who make a disproportionate contribution to the regional malaria burden [9,21]. The total number of confirmed cases of malaria was 28 190 in 2012 with an Annual Parasite

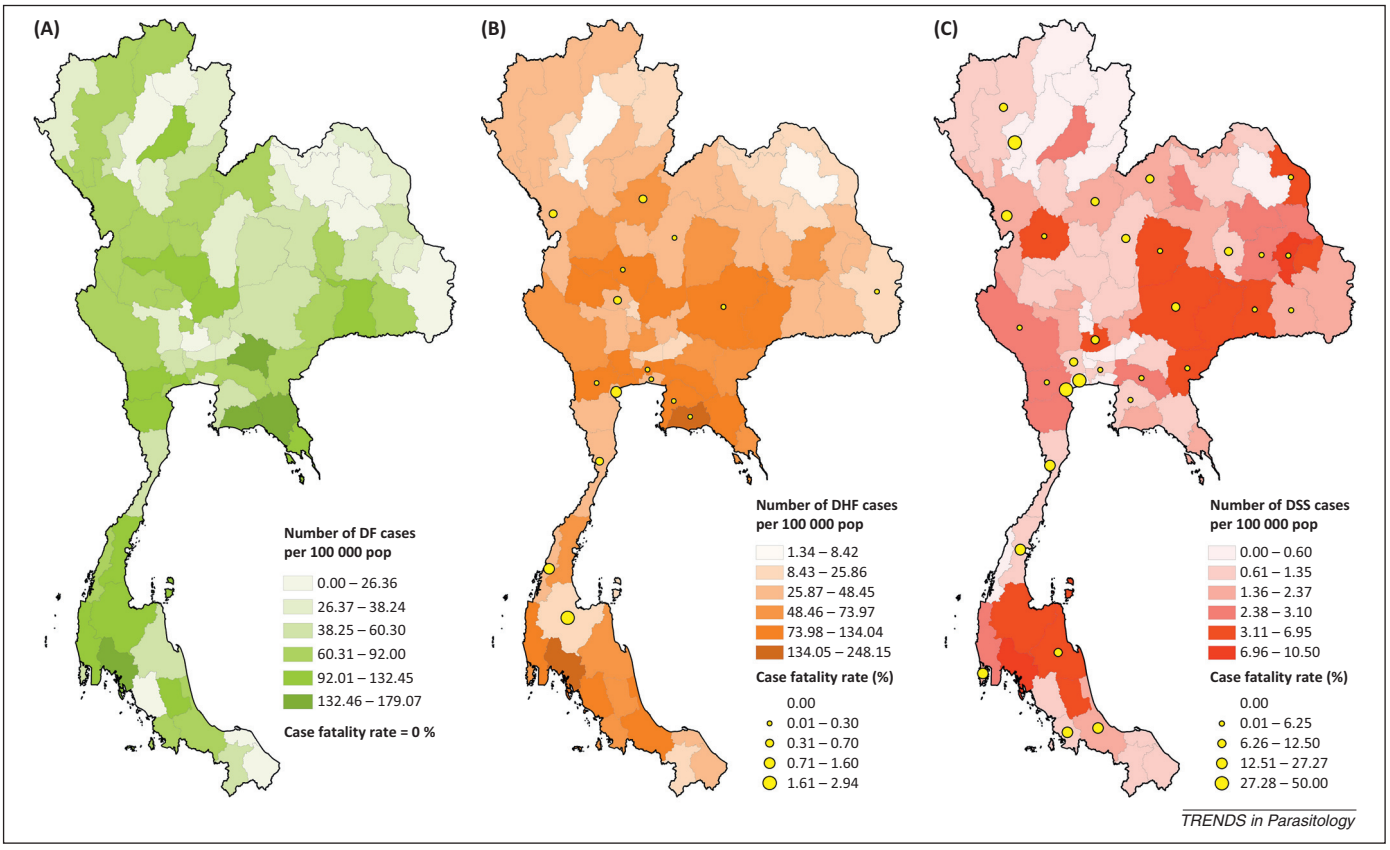


Figure 1. Dengue incidence in Thailand. Maps show the distribution of **(A)** dengue fever (DF), **(B)** dengue hemorrhagic fever (DHF), and **(C)** and dengue shock syndrome (DSS) in 2012 (Source: Bureau of Epidemiology, Ministry of Public Health, Thailand, www.boe.moph.go.th). The total number of DF, DHF, and DSS cases were 39 392 (61.5 cases per 100 000 people), 37 798 (59/100 000), and 1321 (2.1/100 000), respectively. Case fatality rates were 0% for DF, 0.07% for DHF, and 4% for DSS. Spatial heterogeneity in dengue incidence was observed within the country; the five provinces at higher risk of DF were Bangkok, Surin, Nakhon Ratchasima, Chang Mai, and Songkhla.

Index of 0.429 per 1000 population (source: Bureau of Vector Borne Diseases, Ministry of Public Health, Thailand, www.thaivbd.org). The predominant parasite species are *P. falciparum* and *P. vivax*. These have had nearly equal prevalence since 1997, although *P. vivax* has become more prevalent (60%) than *P. vivax* (40%) since the turn of the century [22] (Figure 2). Widespread use of artemisinin combination therapies (ACTs) is a plausible explanation for the shift in parasite species incidence in the country [10]. Sporadic zoonotic infections by the monkey parasite *P. knowlesi* have also been reported in southern Thailand [23].

Dengue and malaria vectors in Thailand

Vectors of dengue

Only two species of *Aedes* mosquitoes, *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), are known to be important DENV vectors in Thailand. According to Strickman and Kittayapong [24], the risk of dengue transmission is greatest during the months of May and June (i.e., beginning of the rainy season). Both vector species predominantly bite during the daytime, preferring the early morning and late afternoon. Nevertheless, many exceptions have been recorded depending on the season, region, host availability, and the nature of the human habitat [25,26]. *Ae. aegypti* is the primary vector of the four serotypes of DENV in Thailand [24]. Unlike *Ae. albopictus*, this species rests predominately indoors and prefers the clean water found in various domestic containers inside and around the human dwellings of urban areas [27]. Important genetic differentiation was noted among 19 *Ae. aegypti* populations from Thailand, and vector control efforts are suspected to be one of the main causes of the genetic variance observed [28]. Knowledge of the distribution, behavior, and ecological preferences of *Ae. aegypti* and *Ae. albopictus* remains incomplete, especially where the environment has been strongly modified by intense urbanization, agricultural development, reforestation, or climatic disruption (e.g., flooding). Clearly, more work on *Aedes* genetic adaptation to new environments (using population genetics and geographic information systems) is required to provide further insight into understanding dengue epidemiology in the country.

Vectors of malaria

The main malaria vector species belong to the specific members of the Leucosphyrus group (Neomyzomyia series), the Maculatus group (Neocellia series), and the Minimus subgroup (Myzomyia series) [29]. Several other potential vectors of malaria are present in Thailand including *Anopheles epiroticus* (=*sundaicus* A), *Anopheles maculatus*, *Anopheles karwari*, *Anopheles philippinensis*, and *Anopheles tessellatus* [30,31]. In addition, some members of the Barbirostris group are considered to exhibit a high vector potential for malaria [32]. By definition, members of species complexes or groups cannot be

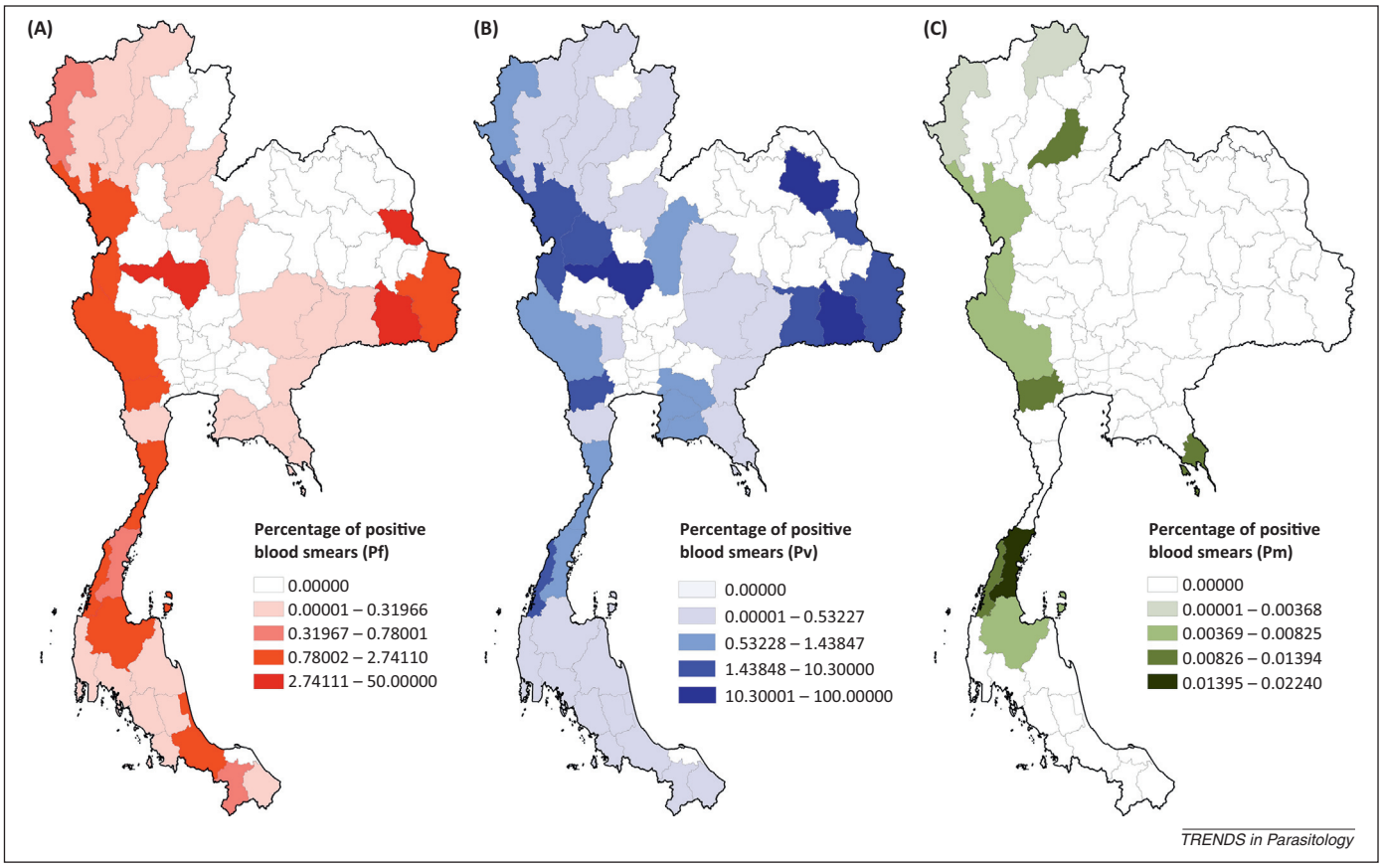


Figure 2. Malaria incidence in Thailand. Maps show the distribution of malaria infections by **(A)** *Plasmodium falciparum*, **(B)** *Plasmodium vivax*, and **(C)** *Plasmodium malariae* in Thai and migrant populations in 2012 (Source: Bureau of Epidemiology, Ministry of Public Health, Thailand, www.boe.moph.go.th). The percentage of positive blood smears with *P. vivax* (Pv), *P. falciparum* (Pf), and *P. malariae* (Pm) were 1% ($n = 16\,471$ positive blood smears), 0.7% ($n = 11\,553$), and 0.003% ($n = 57$), respectively (total number of blood smears inspected was 1 716 811). Mixed infections (Pf/Pv) represent barely 0.5% of the total malaria cases. The five provinces showing the highest prevalence of malaria cases were Tak, Kanchanaburi, Mae Hong Son, Ranong, and Songkla.

distinguished morphologically, but these sibling species may show very different behavior and vectorial capacity (reviewed in [21]). Efficient vectors are present in the higher malaria risk areas of southern Thailand and the forested region along the Thai–Myanmar border, particularly selected members of the Dirus, Maculatus, and Minimus complexes [21,33,34]. Climatic, sociodemographic, and environmental factors have been shown to strongly influence the distribution of malaria vector species and hence transmission patterns in the country [35,36]. For example, the development of rubber plantations in Thailand offer optimal conditions and suitable habitats for anopheline species [35] and increase the likelihood of re-emergence of malaria in the country. It is therefore essential to conduct site-specific studies to evaluate the biology and ecology of anopheline mosquitoes as far as they relate to malaria transmission. A better understanding of mosquito behavior related to hosts and patterns of feeding activity should facilitate development of more effective vector control strategies.

Challenges for dengue and malaria control

Dengue control and limitations in Thailand

Until now control of dengue and DHF has proven extremely difficult with both vector and virus remaining entrenched and expanding in many districts within the country. Surveillance of dengue is mainly based on clinical criteria and is biased towards hospitalized cases, thus the actual disease burden is underestimated [4]. Indeed, several long-term cohort studies have brought additional information, such as the under-reporting factor (estimated to be more than eight fold) [17], the extent of asymptomatic and mildly symptomatic infections [37], the spatiotemporal variations demonstrating highly focal transmission [38,39], and the variation of predominant serotypes over the years [40]. Appropriate case management, in which healthcare providers are taught to recognize the warning signs of severity and to manage fluid replacement, can reduce mortality. Thailand has been very successful with this approach and mortality from dengue has been maintained at an extremely low level. However, the transmission of the dengue viruses is difficult to interrupt because the vector is ubiquitous. The viral carriage is typically short in symptomatic cases, but asymptomatic and mildly symptomatic infections that are essential for maintaining the transmission are not treated.

In the absence of antiviral drugs for the treatment of dengue, integrated vector control management using biological and chemical control agents, source reduction, environmental management, and community participation remain critical for controlling dengue transmission [41]. Unfortunately, control strategies relying on source reduction and/or the periodic application of insecticides to water sources have failed to durably control dengue vectors due

to their capacity to colonize broad types of larval habitats in urban and suburban environments. Furthermore, control of adult mosquitoes using space spray applications of a variety of chemicals is fraught with complications including high cost, slow operational response, low community buy-in, ineffective timing of application, and rather low efficacy and/or residual effect [42,43]. The development of insecticide resistance in the *Aedes* vector is also challenging the public health system for dengue vector control [44]. In Thailand, strong levels of pyrethroid resistance were detected in adults of *Ae. aegypti*, whereas larvae showed susceptibility to the organophosphate temephos, except in some specific foci (Figure 3). *kdr*-type mechanisms and metabolic detoxification through elevation of cytochrome P450 monooxygenases (CYPs) and esterase activities are suspected to be the primary cause of pyrethroid resistance [45,46]. The gene *CYP9J32* encoding for a monooxygenase that is capable of metabolizing both these pyrethroids [47] has increased transcription compared with wild type in deltamethrin- and permethrin-resistant populations in Thailand [48], hence representing a good marker for further insecticide-resistance monitoring. Extensive efforts are needed, however, to identify the causal mutations associated with target site and metabolic resistance to pyrethroids in the dengue vectors.

Malaria control and limitations in Thailand

The implementation of WHO recommended preventive and curative strategies, including early diagnosis and prompt access to treatment, ACTs, universal coverage of long-lasting insecticidal treated mosquito nets (LLINs), and indoor residual spraying (IRS) in areas at risk have played a key role in the reduction of malaria. The country is now entering the pre-elimination phase in order to achieve the Millennium Development Goals for malaria control. Unfortunately, malaria control remains difficult in foci located in forested areas and along the country borders due to complexities in scaling up malaria control measures and effective interventions in all villages where malaria is endemic, as well as building the capacity of local health personnel in malaria prevention [10]. Moreover, the emergence of artemisinin-resistant strains of *P. falciparum* in Western Cambodia [49] and on the Thai–Myanmar border [50] represent a major obstacle to the elimination of *P. falciparum* malaria in the region (Figure 4). This phenotype is characterized by a slower parasite clearance from the circulation following treatment with an artemisinin derivative. The precise mechanism of resistance is unknown but is thought to involve changes in the gene expression at the ring stage of the parasite cycle [51]. Whether or not this phenomenon is related to dormancy is unknown [52]. Initially, the efficacy of the ACTs used in the region (mostly artesunate combined with mefloquine or di-hydro-artemisinin with piperaquine) was not compromised; however, recent reports suggest that the cure rate obtained with these treatments is declining [10]. Another challenge in the region is also related to drug resistance; there is increasing evidence that *P. vivax* is becoming more resistant to chloroquine [53,54], the main drug used to treat this infection. Furthermore, the liver stages of *P. vivax* and relapse of parasites in the bloodstream makes it very difficult to eliminate (reviewed in [55]). The only drug available to treat *P. vivax* liver stages, primaquine, requires a 14-day treatment and is associated with hemolysis in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a genetically inherited disorder found in 4–15% of the population. Finally, the huge population fluxes in SEA pose a formidable challenge to malaria control (and for any communicable diseases) in the region. This is illustrated by the fact that drug resistance is most severe in the border regions, where the movement of people is greatest, and their access to healthcare is difficult [10].

Integrated vector control programs have been implemented in various settings and shown to be a strategy with great promise for malaria control [56]. Unfortunately, the implementation of integrated vector management requires a needs assessment of vector control capability at a national level and remains costly and difficult to implement, especially in low income countries. In addition, existing vector control methods do not take into account the biology, ecology, and behavior of malaria vectors. This is all the more important because most malaria vector populations preferentially bite outdoors and in the early evening [57], and are not properly targeted by conventional vector control tools (e.g., LLINs or IRS). Several studies showed that secondary or minor malaria vectors can significantly contribute to malaria transmission following environmental changes (e.g., irrigation, land development, deforestation), which could shift their feeding preference towards humans and enhance their survival, hence increasing their vectorial capacity [21]. In diverse environments, modulation of vectorial capacity can result in variable patterns of disease transmission and changes in the relative epidemiological importance of various components of the vector system. In addition, resistance of mosquitoes to insecticide can impact on the vectorial capacity by increasing the fitness of mosquitoes in the presence of insecticide treatments. Fortunately, most *Anopheles* species in Thailand remain susceptible to insecticides (Figure 5), but pyrethroid resistance has been found in northern Thailand (i.e., Chang Mai) where significant amounts of pesticides are used for agricultural pest control [58,59]. The concern is that resistance may rapidly spread through migration of resistant mosquitoes from neighboring countries (i.e., Cambodia, Vietnam) where resistance is present [60,61]. Substantial and careful attention to insecticide practices and land use changes, along with climatic and environmental changes may help prevent or delay the re-emergence of malaria in these areas.

Prospects for dengue and malaria control

Dengue

Integrated epidemiological and entomological surveillance represents a promising approach to rapidly detect the focal epidemic peaks, implement vector control tools, and measure their effectiveness. It can also investigate the impact of changes in serotypes and genotypes and risk of transmission as a function of vector presence and abundance. Epidemiological surveillance is limited, however, by the lack of rapid tests for the detection of milder viremic cases and insensitivity to unreported, undifferentiated fevers that may be important sources of transmission [40,62].

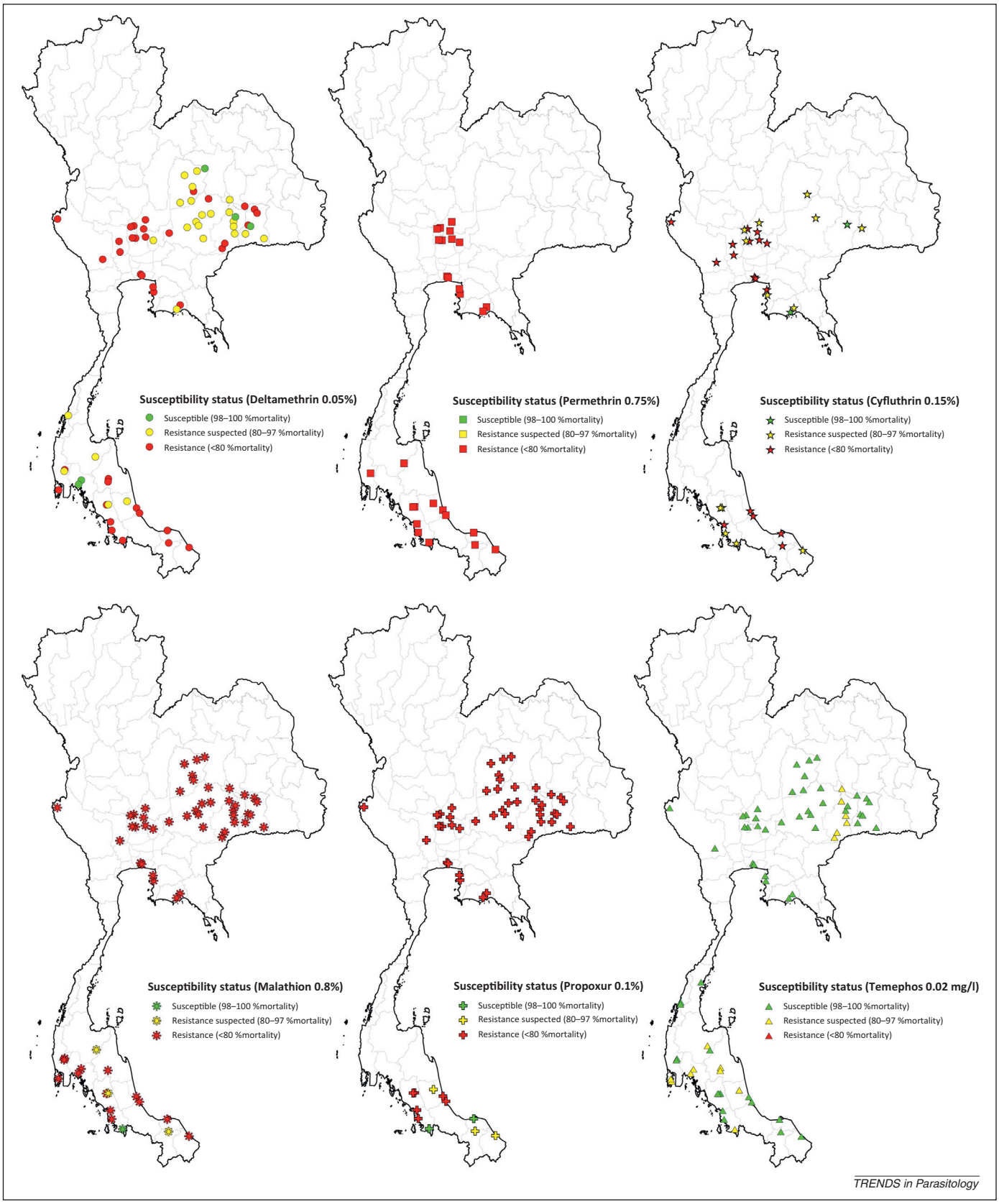


Figure 3. Insecticide resistance in the main dengue vector *Aedes aegypti* in Thailand. The maps were built using data collected from 2012 to 2013. Sites were located using a database from the Bureau of Vector Borne Diseases, Ministry of Public Health, Thailand, www.thaivbd.org and spatialized using ArcGIS 9.2 software (ESRI, Inc., Redlands, CA, USA). Resistance was measured at the larval stage for temephos and at the adult stage for other chemicals using cylinder tubes according to the standard World Health Organization (WHO) criteria [94]. The maps show that pyrethroid resistance in *Ae. aegypti* is widespread in the country, whereas temephos tolerance is present in specific foci in the Southern and Northeastern areas of Thailand. All tests were carried out with insecticides at WHO diagnostic concentrations [94].

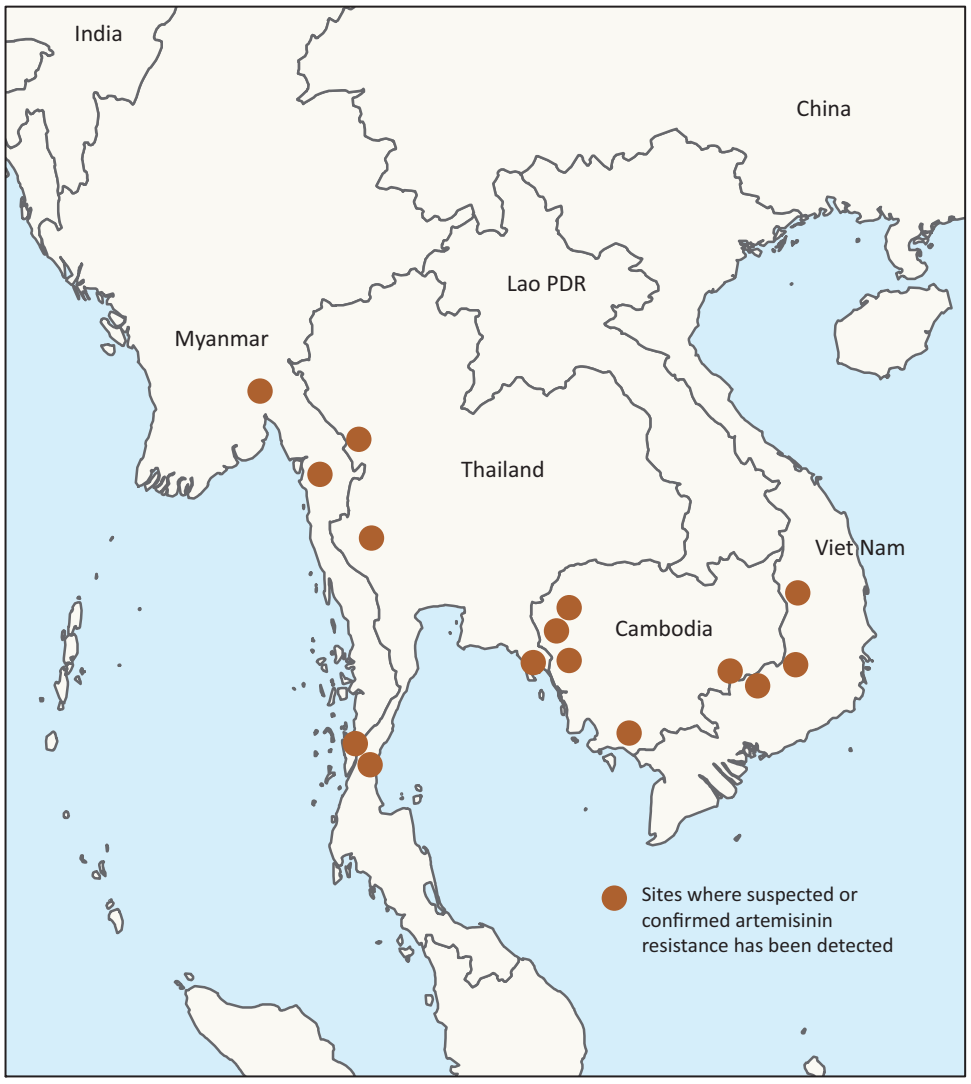


Figure 4. Artemisinin resistance in the Mekong subregion. The map shows sites where suspected or confirmed artemisinin resistance has been detected in therapeutic efficacy studies in the Mekong subregion from 2007 to 2012 (Source: see [7]; Source of data: World Health Organization Global Database on Antimalarial Drug Efficacy, as of November, 2012). To date, *Plasmodium falciparum* resistance to artemisinin has been detected in four countries in the Greater Mekong subregion including Cambodia, Myanmar, Thailand, and Vietnam.

Furthermore, mild and moderate cases cannot be differentiated from numerous other causes of febrile illness in the region (Chikungunya, scrub typhus, leptospiroses, etc.), biasing estimates of true dengue infections [63,64]. A shift from reactive interventions to planning and long-term strategies was recommended at the first Association of Southeast Asian Nations dengue day in 2011 [65]. To do so, there is a need for easy tools to predict the risk and target interventions in a timely manner and in appropriate areas. Several models have been proposed, including recent ones to predict dengue outbreaks several weeks in advance. These models rely on meteorological data, either alone [66] or combined with clinical, climatic, and socioeconomic data [67]. However, these models are heavily based on specific local assumptions and need further prospective evaluation, because factors influencing dengue transmission are complex and likely to change over time.

To date, no antiviral candidate has reached clinical development [68] and case management still focuses on symptomatic treatment (i.e., fever and plasma leakage in severe cases). Consequently, the development of an effective vaccine represents the most promising hope for the control of dengue worldwide. Several candidate vaccines are in clinical or preclinical development [69,70]. In a recent clinical trial conducted in 4000 Thai children aged 4–11 years, the most advanced candidate in its clinical development was a recombinant, live, attenuated, tetravalent dengue vaccine, which showed for the first time that a safe, efficacious vaccine against dengue was possible [71]. There were no vaccine-related severe adverse events after 2 years of active follow-up of more than 2600 vaccinated children, in particular any sign of enhanced disease in breakthrough cases after vaccination. This candidate vaccine protected against three of the four serotypes (DENV-1, -3, and -4). However, no protection was observed against the serotype 2, affecting the overall efficacy. These results were obtained from a single setting, and large-scale Phase III studies are being conducted in various epidemiological settings to provide additional pivotal data for this candidate. The introduction of vaccines in the coming years may

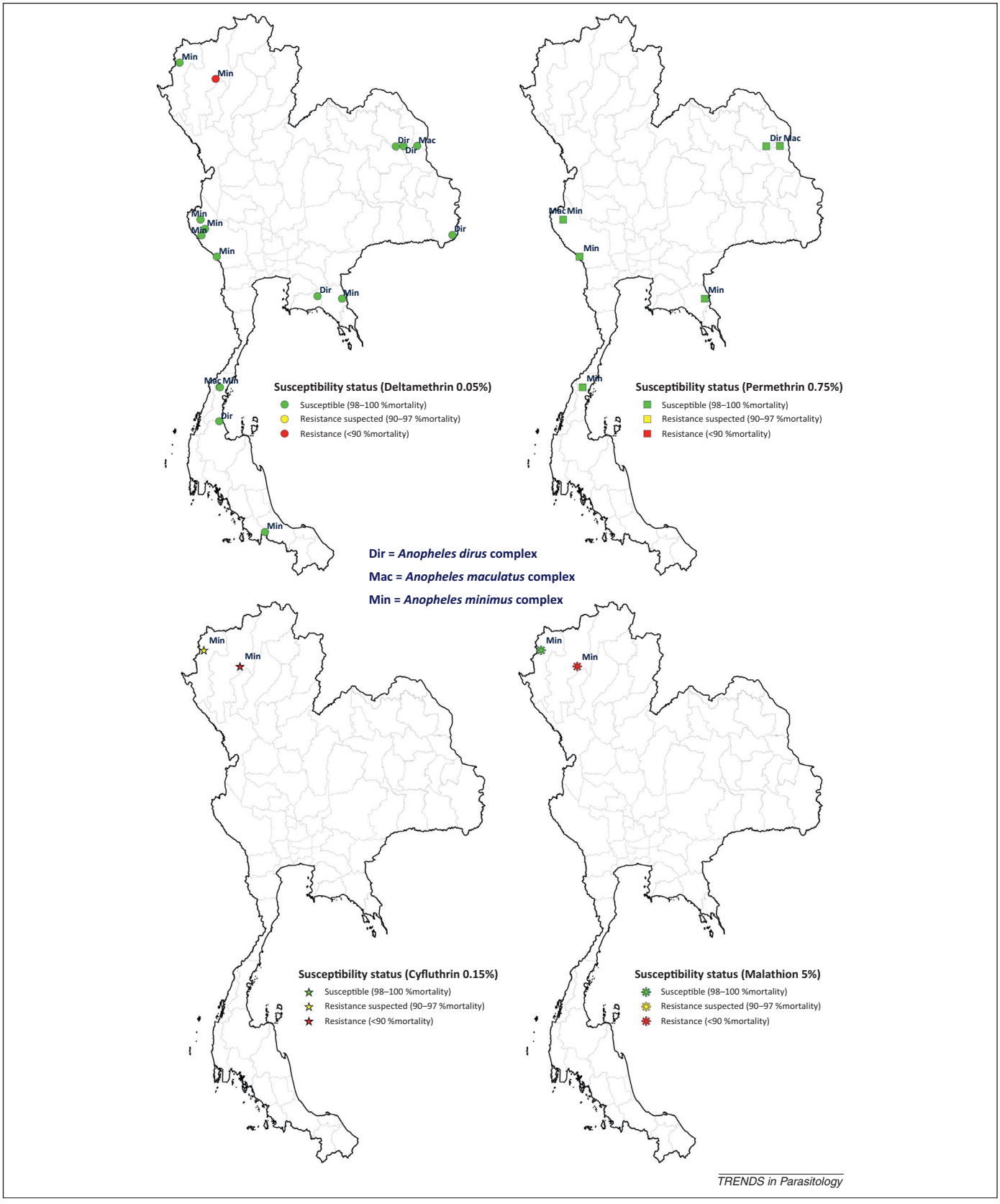


Figure 5. Insecticide resistance in the main malaria vectors in Thailand. The maps were built using data collected from 2012 to 2013. Sites were located using a database from the Bureau of Vector Borne Diseases, Ministry of Public Health, Thailand, www.thaivbd.org and spatialized using ArcGIS 9.2 software (ESRI, Inc., Redlands, CA, USA). Resistance was measured for the main malaria vector species *Anopheles dirus* (Dir), *Anopheles minimus* (Min), *Anopheles maculatus* (Mac) using World Health Organization (WHO) cylinder tubes according to new WHO criteria [95], in line with the recommendations of the Global Plan for Insecticide Resistance Management in Malaria Vectors [96]. The maps show that adult *Anopheles* mosquitoes are mostly susceptible to insecticides, except in the northern part of the country where deltamethrin, cyfluthrin, and malathion resistance was detected in *An. minimus* (i.e., Chang Mai province). All tests were carried out with insecticides at WHO diagnostic concentrations [95].

become part of an integrated approach to control the accelerated spread of dengue [14].

Regarding the vectors, the risk of emergence of dengue (and Chikungunya as they both share same vectors) in new ecological settings/habitats has been underevaluated and requires much more attention. Indeed, the presence of *Aedes* vectors in rubber tree plantations [72], brackish waters [73], and/or xenobiotic-polluted habitats [74] is worrying because these vector populations may create new foci of transmission. Understanding the contribution of ecological interactions on the origin and maintenance of species diversity and on the geographical range of organisms is a fundamental challenge for designing more effective vector control strategies. A better understanding of the complex interplay between diverse molecular and metabolic factors involved in insecticide resistance is also required for promoting successful dengue vector management. Alternative vector control strategies for managing insecticide resistance are a priority because recent studies conducted in the Caribbean showed that pyrethroid resistance in *Ae. aegypti* can strongly reduce the knockdown effect and mortality caused by pyrethroid-thermal fogging sprays [75,76]. The concept of exploiting adult mosquitoes as the 'vehicle' of insecticide transfer to disseminate specific molecules between resting and oviposition sites [77] offers an interesting prospect to reduce the density of immature stage *Aedes* mosquitoes but has never been tested at an operational level. Genetic control represents another option to better control *Aedes* populations without using chemicals. An open field trial conducted in the Cayman Islands showed that genetically modified male mosquitoes carrying a dominant lethal genetic system (RIDL) mated successfully with wild females and fertilized their eggs [78]. The survival and dispersal of the released males (OXITEC strain) was, however, not investigated, and the cost-effectiveness of genetic control over other conventional control methods needs further evaluation. Currently, the most exciting hope comes from the capacity to suppress or block transmission of dengue virus in *Aedes* mosquitoes by *Wolbachia* infection [79]. A recent field trial demonstrated successful invasion of *Wolbachia* in *Ae. aegypti* at two sites in northern Australia and relatively small fitness costs in mosquitoes, indicating that *Wolbachia*-based strategies can be deployed as a practical approach to dengue suppression with potential for area-wide implementation [80].

Malaria

There is at present no effective vaccine for malaria, therefore control of the disease and its elimination rely mainly on early diagnosis of cases and treatment with antimalarials, combined with vector control measures adapted to the local vectors [1]. For *P. falciparum*, new compounds are in Phase IIb studies; OZ439 [81], a synthetic artemisinin-like molecule, KAF156, and KAE609 are new entities developed by Novartis [82]. Nevertheless, their complete development and combination with a suitable partner drug will take a few years, and it is feared that by then, resistance to artemisinin will have become more severe and may have spread to neighboring countries and beyond. The race has started to try to eliminate *P. falciparum* malaria before this happens, as the consequence of letting resistance reach the African continent will be that millions will die [83]. Where foci of high prevalence are found, targeted chemo-elimination (TCE) of *P. falciparum* may have to be considered. The treatment of *P. vivax* will probably have to be changed, and some advocate for a common treatment with *P. falciparum*, that is, with ACTs [84]. For hypnozoites, the drug tafenoquine (amino-8-quinolines) is the only candidate in the final phase of clinical development [85] and presents the advantage of being administered in a single dose (rather than 14 days for primaquine). However, it carries the same liability with regards to the hemolytic risk in G6PD-deficient patients. The development of a rapid test to detect G6PD-deficient patients is welcome because it should facilitate the routine use of the amino-8-quinolines at point-of-care.

With regards to the vector, control strategies will have to be adapted to the local ecology in order to ensure effective personal protection for exposed subjects, for example, impregnated hammocks [86] and mosquito repellents [87]. The modification of vector behavior through the use of 'spatial repellent' (SR) is also a promising option to create a vector-free space, thereby preventing contact between human and vector and hence reducing disease transmission [88]. Although SRs represent a pragmatic approach for delaying the onset of resistance and targeting both exophagic and endophagic mosquitoes [89], their efficacy as a means of vector-borne disease prevention merits further investigation. Innovation of more specific and sensitive tools for the evaluation of vector control interventions should also be encouraged. Recent findings in Africa showed that human antibody response to mosquito salivary proteins, especially against one salivary peptide (gSG6-P1 peptide) present only in *Anopheles* spp., is a quantitative and specific biomarker to measure the level of individual exposure to anopheles bites [90,91] and evaluate the protective efficacy of vector control interventions [92,93]. The use of such biomarkers could be particularly promising in assessing the efficacy of LNs and predict the risk of *Plasmodium* transmission in low malaria transmission settings such as the SEA region.

Concluding remarks

Dengue and malaria remain important public health problems in Thailand and the SEA region and are exacerbated by inter-country movement of people that renders their control difficult. Drug resistance and the absence of a malaria vaccine, combined with the lack of an antiviral therapeutic treatment for dengue represent important challenges for the control of these illnesses. Vector control through the use of physical, biological, chemical, or, less frequently, genetic methods is an important component of dengue and malaria prevention. However, these methods face several obstacles, in particular the development of insecticide resistance in the vectors, the drastic reduction of chemicals available for public health, and the lack of reliable indicators to evaluate the treatment efficacy. Furthermore, the existing methods do not take into account the biology, ecology, and behavior of vectors that strongly impact on the efficacy of vector control tools, especially LLINs. National and international efforts are still needed

to strengthen the training and research capacity of academic institutes and public health authorities in vector-borne disease control in the SEA region.

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Parasites & Vectors

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Effect of *Aedes aegypti* exposure to spatial repellent chemicals on BG-Sentinel™ trap catches

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Abstract

Background: An integrated approach to reduce densities of adult *Aedes aegypti* inside homes is currently being evaluated under experimentally controlled field conditions. The strategy combines a spatial repellent (SR) treatment (applied indoors) with the Biogents Sentinel™ (BGS) mosquito trap positioned in the outdoor environment. In essence, when combined, the goal is to create a push-pull mechanism that will reduce the probability of human-vector contact. The current study measured BGS recapture rates of *Ae. aegypti* test cohorts that were exposed to either SR or control (chemical-free) treatments within experimental huts. The objective was to define what, if any, negative impact SR may have on BGS trap efficacy (i.e., reduced BGS collection).

Methods: *Aedes aegypti* females were exposed to SR compounds within experimental huts in the form of either treated fabric (DDT and transfluthrin) or mosquito coil (metofluthrin). Test cohorts were released within individual screen house cubicles, each containing 4 BGS traps, following SR exposure according to treatment. Two separate test cohorts were evaluated: (i) immediate release (IR) exposed from 06:00–12:00 hours and released at 12:00 hours and (ii) delayed release (DR) exposed from12:00–18:00 hours and released at 05:30 hours the following day. BGS recapture was monitored at 09:30, 13:30 and 15:30 hours and the cumulative recapture by time point quantified.

Results: Exposure of *Ae. aegypti* females to either DDT or metofluthrin did not significantly impact BGS capture as compared to cohorts of non-exposed females. This was true for both IR and DR exposure populations. IR cohorts exposed to transfluthrin resulted in significantly lower BGS recapture compared to matched controls but this effect was primarily due to high mosquito mortality during transfluthrin trials.

Conclusion: Our data indicate no more than minor and short-lived impacts (i.e., reduced attraction) on BGS trap catches following exposure to the pyrethroid compounds transfluthrin and metofluthrin and no change in recapture densities using DDT as compared to matched controls. These findings suggest a combined SR and BGS approach to vector control could function as a push-pull strategy to reduce *Ae. aegypti* adults in and around homes.

Keyword: *Aedes aegypti*, Spatial repellents, Screen house, Experimental huts, BG-Sentinel™ trap, Push-pull strategy, Thailand

Background

Dengue and dengue hemorrhagic fever occur in the tropics and subtropics with an estimated 2.5 billion people residing in areas where dengue is endemic [1]. Dengue viruses are transmitted primarily by *Aedes aegypti*, a day-biting mosquito that feeds and rests indoors and preferentially bites humans [2-5]. Despite years of public health efforts and research progress, an effective vaccine against dengue virus is not yet available. For this reason, disease prevention remains dependent on vector management and control strategies [1,4]. However, controlling *Ae. aegypti* has proven difficult due to its strong association with domestic and peridomestic human environments that harbor and sustain development sites (artificial containers) for the immatures. Furthermore, control of *Ae. aegypti* adults is commonly based on indoor and outdoor spraying of insecticides to reduce mosquito abundance and disrupt dengue virus

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transmission during outbreaks [6-8]. This is complicated by the worldwide rise and increasing impact of resistance of *Ae. aegypti* to commonly used insecticides, including in Thailand [9-12]. New approaches are urgently needed to improve our capacity to control this mosquito, especially targeting the adult stage in and around the home.

Push-pull strategies, combining a repellent with an attractant, have been effective in the control of some agricultural pests [13,14]. The mechanism underlying a push-pull system includes: (i) behavioral manipulation of the target species to repel or deter (push) them away from a resource (i.e., a crop) using stimuli that renders the resource unsuitable or unattractive and (ii) a device, for example a trap, through which the target species are removed from the environment (pull) [13-15]. Such an approach may also prove effective in the control of pathogen-transmitting mosquitoes, especially in and around the home where many vector-borne pathogens are predominantly transmitted in the developing world. One clear benefit of a push-pull system is that it can be effective in settings where insecticide resistance occurs. This is because the chemical doses that elicit sublethal behavioral responses, such as spatial repellency, are below that required for toxicity, thereby reducing insecticide resistance selection pressure while continuing to prevent human-vector contact [1,16-18].

Spatial repellents are defined as chemicals that function in the vapor phase to affect biting insects at a distance from the treatment source and can inhibit the ability of vectors to locate and track a host [19]. The vapor plume formed by the source of a spatial repellent creates a protective barrier extending to a certain radius from the source of the repellent chemical [20]. This has potential for protection of entire households. Moreover, continuous day and night protection can be provided through formulations that allow slow and continuous evaporation of the repellent substance. Continuous use of spatial repellents is expected to result in prevention of vectors from entering the treated space thereby prolonging times for the mosquito to locate hosts and/or resting places, thus increasing the likelihood of adverse environmental conditions, predation or other causes inducing mortality [21].

In the specific case of *Ae. aegypti* control, a trap or pull component may pose the greatest challenge within a push-pull system. Several trap designs have been commonly used for adult mosquito surveillance purposes [22,23]. However, many of these have not been satisfactory for *Ae. aegypti* [24-26]. The development of new, improved traps, such as the BG-Sentinel™ (BGS) and Zumba™ traps, provides an opportunity for improved entomological surveillance and possibly also control of *Ae. aegypti* [27-31] and *Ae. albopictus* [32,33]. The BGS trap targets the most important elements of *Ae. aegypti* host-seeking behavior by combining an olfactory cue (BGS Lure) with a visual cue (black and white contrast) to attract the mosquito. This trap has proven to be an effective tool for surveillance of *Ae. aegypti* adults, outperforming other collection devices such as the CDC backpack aspirator, the Fay-Prince trap, the Encephalitis Virus Surveillance trap and the Mosquito Magnet Liberty™ trap [29,30].

Based on these findings, the BGS trap was selected for evaluation in a push-pull *Ae. aegypti* control strategy currently under experimental evaluation. The push component uses spatial repellent chemicals that have been shown to deter Thai *Ae. aegypti* from entering homes (Ojo *et al.*, unpublished data). The BGS trap provides the pull component to remove repelled *Ae. aegypti* from the peridomestic environment thereby further reducing human-vector contact. Previous studies have confirmed that the BGS trap effectively removes *Ae. aegypti* from a controlled environment [34]. However, the effects of exposure to repellents, or sublethal doses of insecticides, on BGS trap collections have not been previously evaluated. For the pull component to be most effective, previous exposure to the spatial repellent being used to push vectors from entering homes should not substantially decrease the likelihood of the mosquito being trapped outdoors.

The objective of the current study was to define the effect of previous exposure of *Ae. aegypti* to spatial repellents in experimental huts on BGS trap efficacy. This information is important to define potential limitations in strategy success when both tools are used in combination. In addition, as a critical debate in the use of spatial repellents for vector control includes potential diversion or movement of repelled vectors to unprotected human hosts, findings will also provide insight as to how SR exposure may interfere with the host-seeking (i.e. attraction) response.

Methods

Study area and experimental huts

Studies were conducted near Pu Teuy (14°17′N, 99° 11′ E), which is a small agricultural village (<1,500 inhabitants) located 150 km northwest of Bangkok in Sai Yok District, Kanchanaburi Province, Thailand. The village is situated in a mountainous area (420 m above sea level) and completely surrounded by dense primary forest, orchards and vegetable plantations. *Aedes aegypti* is prevalent in Pu Teuy village. The abundance of immatures in artificial water-holding containers is surveyed weekly, by the Thongpaphum District Clinic, and mosquito control interventions include distribution of organophosphate larvicide (temephos). Our experimental site is located >800 m from the closest indigenous home, creating a distance buffer for mark-release-recapture mosquito behavioral studies

which exceeds the normal flight range (< 100 m) of *Ae. aegypti* [5,35]. The experimental huts used in the study have been previously described [36]. The huts mimic indigenous Thai homes in materials and dimensions, and are also used within the larger research program to evaluate *Ae. aegypti* entering and exiting behaviors as part of the development of the push-pull strategy [37].

Mosquitoes

Immatures of *Ae. aegypti* were collected weekly from Pu Teuy village and reared to adults at the on-site field insectary. Female, nulliparous, 3–5 d old sugar-starved (i.e. reflecting a host-seeking physiological status) were used for the experimental trials. This age range and starvation treatment increased the probability that mosquitoes would respond to human host and BGS trap cues during evaluations. Mosquito test cohorts (control/treatment) were marked with unique colored fluorescent powder following previous dusting protocols [38].

One day pre-trial, cohorts (n = 50) were placed into individual 'exposure cages', mesh screen cages (26 × 26 × 30 cm), to: 1) facilitate transfer between huts and the screen house and 2) to prevent contact with treated surfaces so that chemical exposure would be based entirely on vapor phase particles. For each repellent treatment, two separate exposure cohorts were used: (i) an Immediate Release (IR) cohort exposed during 06:00–12:00 hours and then released into the screen house containing BGS traps at 12:00 hours and (ii) a Delayed Release (DR) cohort exposed during 12:00–18:00 hours and then released at 05:30 hours the following day, thus having a recovery period of nearly 12 h (with access to water soaked cotton pads). Individual screened cages were placed in the center of experimental huts according to exposure time. A matched control (i.e., chemical-free hut) was used simultaneously for each exposure trial.

BG-Sentinel™ (BGS) trap

All BGS traps were baited with the BG-Lure and operated according to the manufacturer's instructions. The trap consists of a collapsible container made of white plastic sack material. The top of the container is covered with white gauze cloth surrounding a black plastic funnel. This funnel is connected to a mesh catch bag that collects trapped mosquitoes. A 12 volt suction fan below the base of the funnel creates downward suction after connection to an external power source. The air is then pushed upwards passing through the gauze cover creating convection currents [27]. The contrasting black and white colors of the trap provide visual attraction. The accompanying BG-Lure consists of lactic acid, ammonia and caproic acid, compounds that are found in human sweat [39-41]. When the trap fan is operating, the air current carries the lure volatiles out through the gauze cloth cover into the surrounding environment. The BG-Lures were used within 4 months after opening per manufacturer's recommendation.

Chemical exposure

Two persons were present inside each of the experimental huts during trials to monitor coil burning and conduct collections for push-pull evaluations (Ojo *et al.* unpublished data). All test chemicals are USEPA registered and as such, have passed mammalian toxicology thresholds for human safety. Informed consent was conducted according to corresponding Uniformed Services University of the Health Sciences and Kasetsart University scientific and ethical review committee approvals.

The following repellent chemicals were evaluated in separate trials: 1) the organochlorine, DDT –1,1 Bis(4-chlorophenyl)-2,2,2- trichloroethane, (CAS 50-29-3,Sigma-Aldrich), 2) the synthetic pyrethroid, transfluthrin-2,3,5,6-tetrafluorobenzy(1R,3S)-3-(2,2-dichlorovinyl) 2,2dimethy lcyclopropanecarboxylate (CAS118712-89-3, Bayer, AG) and 3) another synthetic pyrethroid, metofluthrin- 2,3,5,6-Tetrafluoro-4-(methoxymethyl)benzyl2,2-dimethyl-3-(prop-1-en-1-yl) cyclopropanecarboxylate (S.C. Johnson & Son, Inc). These chemicals represent standards in household mosquito control products (i.e., mosquito coils) or use within organized vector control campaigns (i.e. indoor residual spraying) and have been reported to have spatial repellent characteristics [16-18,21,42-55]. Although DDT has been prohibited for use in mosquito control programs in some areas, it was used here based on evidence of effectively controlling pests and mosquitoes transmitting malaria parasites and dengue virus [42,43,55].

Repellent treatments consisted of either chemical-treated fabric panels (DDT and transfluthrin trials) or a standard mosquito coil (metofluthrin trials). Matched control huts contained either chemical-free fabric (solvent only) or blank coil (coil without active ingredient). DDT was applied to fabric to mimic previous experimental hut studies evaluating its spatial repellent characteristics [17] and because this chemical is typically applied as an indoor residual spray to interior walls of houses [44]. Transfluthrin was similarly applied to fabric to match conditions employed for push-pull trials within the larger research project. Metofluthrin exposure was evaluated using a coil as this is a typical delivery format for the compound and volatile insecticides in general [45].

The preparation, treatment and positioning of DDT or transfluthrin-treated fabric inside experimental huts followed procedures previously described [17]. DDT and transfluthrin were applied to fabric panels 48 h pretesting using solvent solution. The panels were air-dried under a chemical fume hood prior to storage at 4°C until used. Control fabric panels were treated with solvent

alone and processed following the same procedure. Fabric panels were fitted onto metal mesh frames positioned along the interior walls of each hut using magnets [17,36]. One set of treated fabric panels was used for each experimental trial. DDT exposure was conducted using field application rate (FAR; 2 g *ai*/m^2) at 75%, 50% and 25% surface area coverage (SAC). Evaluations were also performed using 25% SAC against 0.5 FAR (1 g *ai*/m^2). Transfluthrin evaluations included 1.0 (40 μg *ai*/cm^2), 0.5 (20 μg *ai*/cm^2), 0.125 (5 μg *ai*/cm^2), and 0.062 FAR (2.5 μg *ai*/cm^2) using 25% SAC. The selected coverage during transfluthrin trials was based on data from experiments evaluating the push-pull system that showed 25% coverage to be as effective as coverage at 50% and 100% (Achee *et al.* unpublished data).

Metofluthrin coils representing high (0.0065% *ai*) and low (0.003% *ai*) doses were burned according to manufacturer's recommendation within a metal dish positioned in the center of the hut. Coils were lit at 05:30 h, or 30 min before exposure cages were introduced, and were replaced at 12:00 h to ensure burning continued until 18:00 h, which represents the typical time range of expected *Ae. aegypti* biting period. The lag time following initial lighting allowed vaporization of the chemical and thereby distribution within interior air space. Both negative (no coil) and positive (coil without active ingredient) controls were used simultaneously in metofluthrin trials.

On each trial day, one mosquito exposure cage (i.e. one test cohort) was placed in the center of each experimental hut (approximately 2 m from treated fabric panels and 1 m from burning coils) representing either control (chemical-free) or treatment (with spatial repellent) conditions (Figure 1). For trials with mosquito coils, an additional hut containing no coil was used as a negative control. A total of four replicates were performed for each chemical treatment.

BGS capture evaluation

Post-exposure BGS captures were evaluated under semi-field screen house conditions. The screen house measures 4 m (width) × 3.5 m (height) × 40 m (length) and is located on-site with the experimental huts [34]. The screen house is subdivided into four 10 m long cubicles using metal partitions, each with a space volume of 140 m^3 (Figure 1). This is similar to the volume inside and within 2 m outdoors of the experimental huts used for vector behavior studies at the field site [36] and is the expected approximate space volume within which *Ae. aegypti* primarily would make contact with an outdoor trap at a typical home in a dengue-endemic environment in Thailand [3,5].

Screen house cubicles were designated as control or treatment for evaluation of unexposed cohorts (chemical-free huts) and repellent-exposed cohorts, respectively. Within each cubicle, 4 BGS traps were operated simultaneously. The traps were monitored for mosquitoes based on sampling periods established from previous studies [35]. Those used for IR cohorts included: 13:30 and 17:30 hours Day of exposure (Day 1) and 05:30, 09:30, 13:30 and 17:30 hours Day following exposure (Day 2). Those used for DR cohorts included: 09:30, 13:30 and 17:30 hours Day 2 and 05:30, 09:30, 13:30 and 17:30 hours two days following exposure (Day 3).

Environmental parameters (temperature, relative humidity and light intensity) were recorded inside each cubicle using HOBO data loggers (HOBO U12-012 Model, Onset Computer Corporation, Bourne, MA). Baseline experiments were conducted to measure environmental variables among cubicles to ensure comparability before exposure studies were performed [34].

Data analyses

Cumulative percentage BGS recapture of each test cohort were computed after correcting for the number of knocked down mosquitoes following exposure and prior to release. A Kruskal-Wallis statistical test was used to compare percentage recapture between treatment and control cohorts. The Mann–Whitney statistical test was used to compare cumulative BGS densities between IR and DR exposure cohorts. For all analyses, a p-value of 0.05 or less was considered statistically significant. Statistical analyses were performed in STATA 11.2 using the ranksum and kwallis syntax for Mann–Whitney test and Kruskal Wallis tests, respectively.

Results

DDT exposure

Cumulative BGS recapture, across application dose and SAC, ranged from 86-94% for DDT-exposed mosquitoes and 85-95% for control cohorts (Figure 2, Table 1). There was no significant difference in BGS recapture between DDT-exposed and control cohorts for either IR or DR populations (Figure 2, Table 1). This was true for evaluations using 75%, 50% and 25% SAC 1.0 FAR (2 g/m^2) exposure conditions. Similar results were seen for trials using 25% SAC at 0.5 FAR (1 g/m^2).

Metofluthrin exposure

There were no significant differences in BGS recapture rates among metofluthrin-exposed and both positive (blank coil) and negative control (no coil) cohorts for either IR or DR populations. This was true using both high (0.0065%) and low dose (0.003%) coils (Figure 3A-D, Tables 2). Cumulative recaptures ranged from 77-93% for negative controls, 73-90% for positive controls and a combined range of 80-93% for cohorts

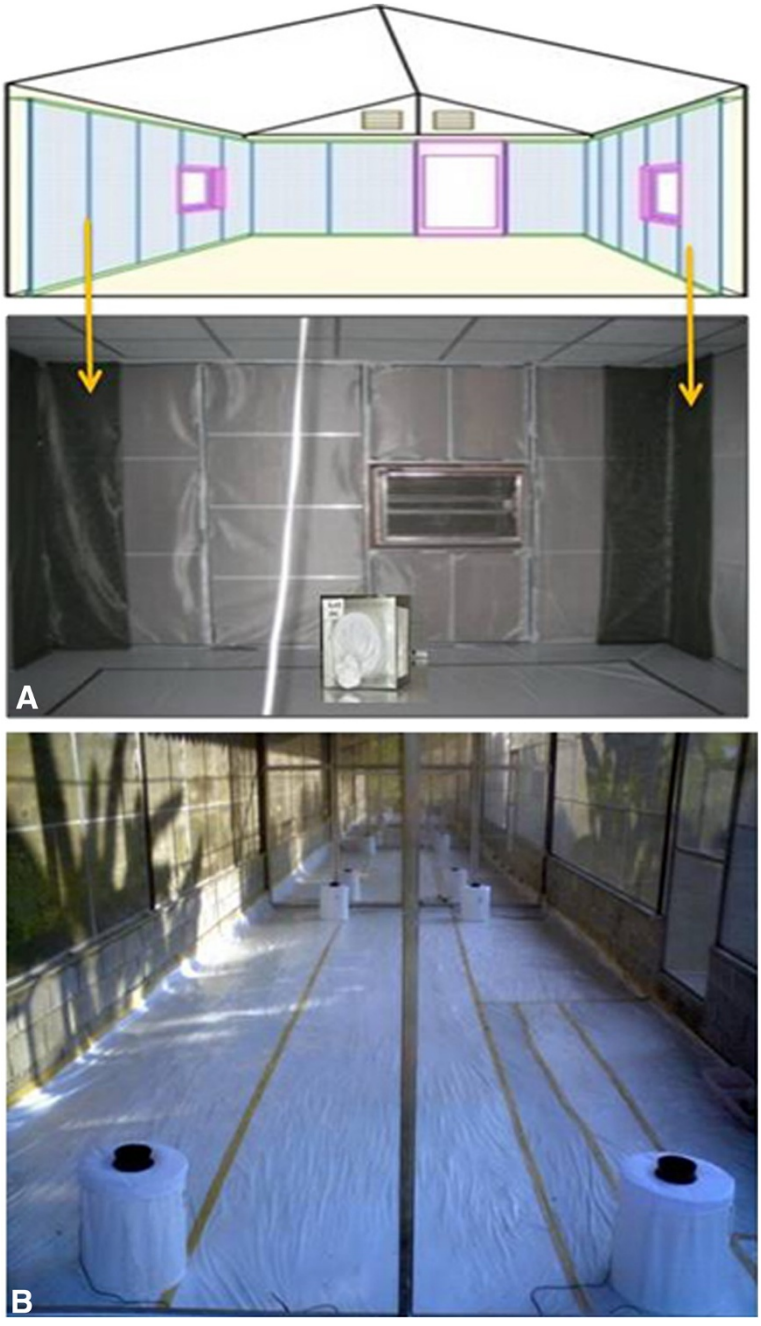


Figure 1 (A) Positioning of treated material and exposure of *Aedes aegypti* in huts; (B) Mosquito trapping with BG-Sentinel™ traps in the screen house.

exposed to both low or high dose coils (Figure 3A-B, Table 2).

Transfluthrin exposure

Exposure to transfluthrin at 1.0 FAR (40 μg *ai*/cm^2) using 100, 50 or 25% SAC resulted in high mortality rates of *Ae. aegypti* cohorts. Mortality ranged from 95-100% and prevented BGS trap evaluations (Figure 4, Table 3). Trials using 0.125 FAR (5 μg *ai*/cm^2) and 0.062 FAR (2.5 μg *ai*/cm^2) at 25% SAC, however, did not have the same killing effect (23-69% mortality). Exposure to these treatment conditions resulted in significantly reduced BGS recapture for IR cohorts as compared to control; in contrast, there was no significant difference between BGS recapture of DR and control cohorts (Figure 4A-B, Table 3). Overall, BGS recapture was

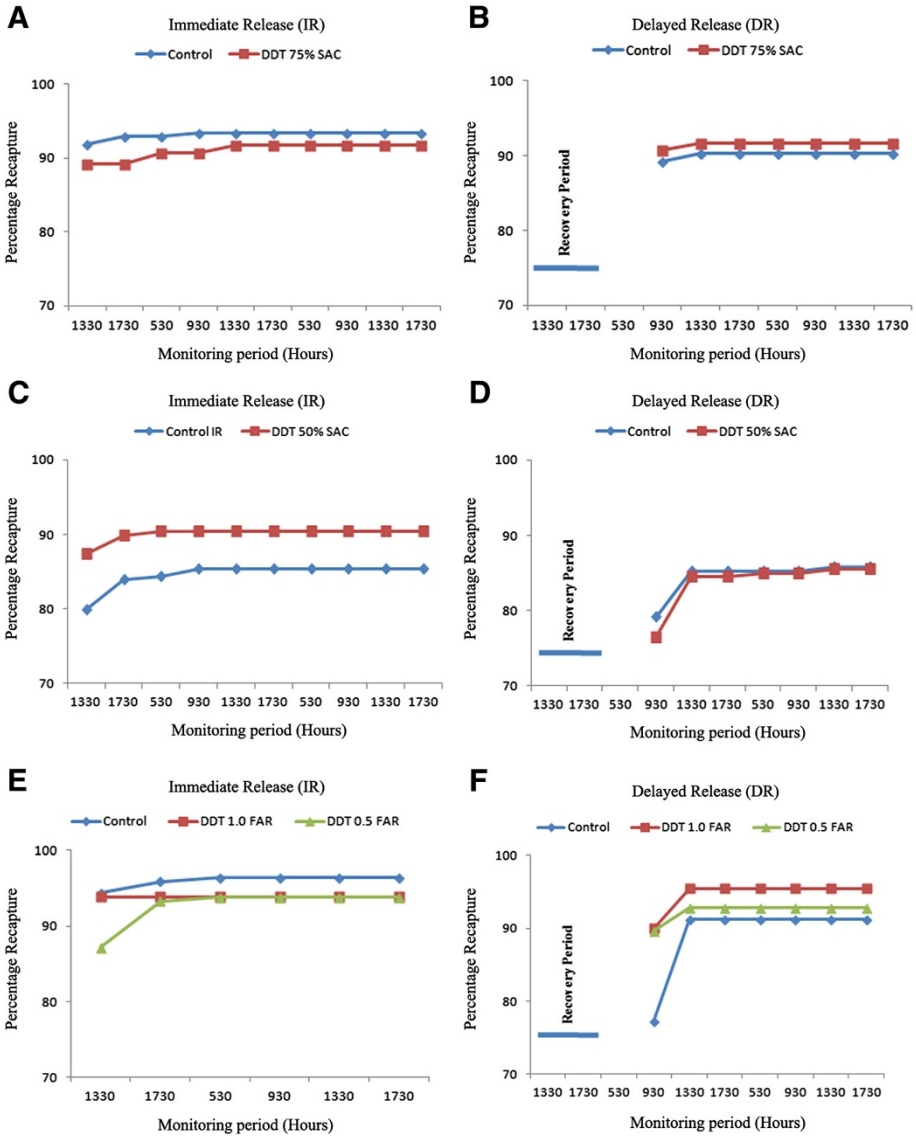


Figure 2 Cumulative BG-Sentinel™ trap recaptures for *Ae. aegypti* females in trials using immediate release or delayed release of mosquitoes previously exposed to DDT (2 g ai/m^2) A and B - 75% SAC; C and D - 50% SAC) or DDT (2 g and 1 *ai*/m^2) E and F - 25% SAC exposed populations.

higher for DR as compared to IR cohorts (Figure 4A-B, Table 3). BGS trap catches for IR were reduced following exposure to the higher transfluthrin concentration (0.125 FAR), where only 45% of released females were recaptured as compared to 76% using 0.0625 FAR ($p = 0.01$).

Environmental parameters

No significant differences ($p > 0.05$) were found among mean daily temperature, relative humidity or light intensity variables measured within screen house cubicles designated as either control (for release of unexposed cohorts) or treatment (for release of repellent-exposed cohorts) for all trials (Tables 1, 2, 3).

Discussion

The primary objective of the current study was to quantify the effects of exposure of *Ae. aegypti* to spatial repellent compounds on catch rates from a validated adult mosquito trap – the Biogents Sentinel™ (BGS) . The purpose was to generate critical information regarding how a spatial repellent may interfere with the efficacy of the BGS when the two tools are used in combination as a push-pull strategy. We specifically sought to determine: (i) if *Ae. aegypti* females exposed to spatial repellent chemicals (DDT, metofluthrin and transfluthrin) have a reduced likelihood of being captured with BGS traps (i.e., effect host-attraction) and (ii)

Table 1 Cumulative BG-Sentinel™ trap catches for immediate release (IR)[1] and delayed release (DR)[2] trials with *Ae. aegypti*[3] exposed to DDT-treated fabrics

Surface area coverage	Release[5]/ Treatments	Cumulative mean percentage (±SD) of released *Ae. aegypti* recaptured by time point[4]													
		←----Day 1------→		←----------Day 2----------→				←----------Day 3----------→					Mean day-time (12 hr) conditions		
		13:30 h	17:30 h	05:30 h	09:30 h	13:30 h	17:30 h	05:30 h	09:30 h	13:30 h	17:30 h	N[6]	Temp (°C)	RH (%)	Light intensity (lx/ft^2)
75%	**IRa**														
	Control	91.9a (±4.5)	92.9a (±3.5)	92.9a (±3.5)	93.4a (±3.5)	93.4a (±3.5)	93.4a (±3.5)	-	-	-	-	185/198	33.4	25.9	382.1
	DDT (2 g *ai*/m^2)	89.2a (±5.3)	89.2a (±5.3)	90.7a (±4.1)	90.7a (±4.1)	91.8a (±3.8)	91.8a (±3.8)	-	-	-	-	178/194	31.9	28.6	331.6
	DRa														
	Control	-	-	-	89.3a (±4.8)	90.3a (±3.7)	90.3a (±3.7)	90.3a (±3.7)	90.3a (±3.7)	90.3a (±3.7)	90.3a (±3.7)	177/196	33.5	25.9	371.7
	DDT (2 g *ai*/m^2)	-	-	-	90.7a (±5.2)	91.8a (±3.8)	91.8a (±3.8)	91.8a (±3.8)	91.8a (±3.8)	91.8a (±3.8)	91.8a (±3.8)	178/194	31.9	28.4	318.8
50%	**IRa**														
	Control	79.9a (±10.6)	83.9a (±4.9)	84.4a (±5.9)	85.4a (±5.5)	85.4a (±5.5)	85.4a (±5.5)					170/199	28.7	31.9	479.0
	DDT (2 g *ai*/m^2)	87.4a (±9.6)	89.9a (±7.7)	90.4a (±7.7)	90.4a (±7.7)	90.4a (±7.7)	90.4a (±7.7)	-	-	-	-	178/198	29.7	69.1	454.5
	DRa														
	Control				79.2a (±10.8)	85.3a (±5.9)	85.3a (±5.9)	85.3a (±5.9)	85.3a (±5.9)	85.8a (±6.3)	85.8a (±6.3)	169/197	30.8	66.7	494.2
	DDT (2 g *ai*/m^2)				76.5a (±11.1)	84.5a (±3.4)	84.5a (±3.4)	85.0a (±3.5)	85.0a (±3.5)	85.5a (±3.5)	85.5a (±3.5)	171/200	29.2	71.8	501.2
25%	**IRa**														
	Control	94.4a (±4.7)	95.9a (±4.5)	96.4a (±4.6)	96.4a (±4.6)	96.4a (±4.6)	96.4a (±4.6)	-	-	-	-	189/196	28.1	24.4	192.16
	DDT (2 g *ai*/m^2)	93.9a (±3.0)	93.9a (±3.0)	93.9a (±3.0)	93.91a (±2.95)	93.9a (±3.0)	93.9a (±3.0)	-	-	-	-	185/197	27.4	26.0	126.2
	DDT (1 g *ai*/m^2)	87.2a (±14.6)	93.3a (±3.9)	93.9a (±3.6)	93.9a (±3.6)	93.9a (±3.6)	93.9a (±3.6)					183/195	27.1	31.7	76.4
	DRa														
	Control	-	-	-	77.2a (±8.9)	91.2a (±3.7)	91.2a (±3.7)	91.2a (±3.7)	91.2a (±3.7)	91.2a (±3.7)	91.2a (±3.7)	176/193	28.4	25.3	205.5
	DDT (2 g *ai*/m^2)	-	-	-	90.0a (±5.4)	95.5a (±2.6)	95.5a (±2.6)	95.5a (±2.6)	95.5a (±2.6)	95.5a (±2.6)	95.5a (±2.6)	190/199	27.8	26.6	135.2
	DDT (1 g *ai*/m^2)	-	-	-	89.6a (±4.7)	92.8a (±4.11)	92.8a (±4.1)	92.8a (±4.1)	92.8a (±4.1)	92.8a (±4.1)	92.8a (±4.1)	181/195	27.5	32.8	85.6

[1] Cohort exposed from 0600–1200 hours and released immediately afterwards.
[2] Cohort exposed from 1200–1800 hours but released only after a holding period of 12 h.
[3] 3–5 day old starved females.
[4] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females within release trial types (IR or DR; Kruskal-Wallis 95% confidence limit) or between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[5] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[6]Total recaptured /total released minus total knock down.

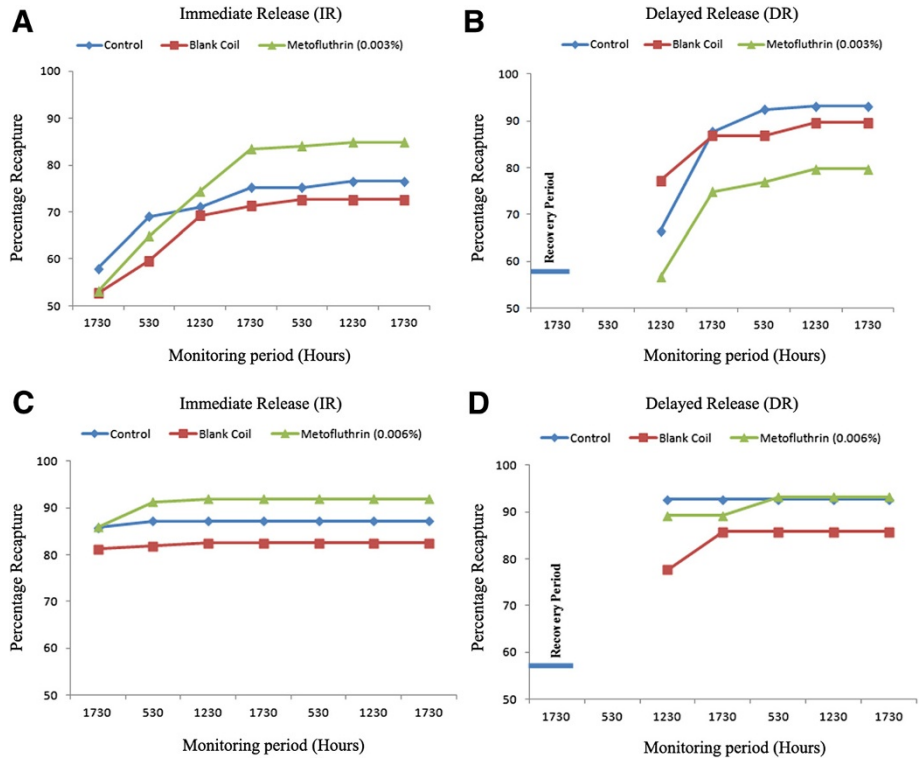


Figure 3 Cumulative BG-Sentinel™ trap recaptures for *Ae. aegypti* females in trials using immediate release or delayed release of mosquitoes previously exposed to metofluthrin: A and B - low dose (0.003%) and C and D - high dose (0.006%).

if such an effect, should it occur, is immediate but short-lived or potentially latent.

Metofluthrin has been previously evaluated for repellency against *Ae. aegypti* [46,47], *Culex quinquefasciatus* [48,49] and *An. balabacensis* [48,49] and metofluthrin coils have been reported to significantly reduce landing counts of *Ae. aegypti* [46]. Transfluthrin is a fast acting insecticide that exhibits high volatility and knock down activity at high concentrations [50]. It is used in household products against various pest insects, such as mosquitoes, flies and moths. Evaluations have been conducted using transfluthrin to repel *Cx. quinquefasciatus* [51,52], *An. arabiensis* [53] and *Ae. albopictus* [54,56]. DDT has spatial repellent qualities as indicated in previous experimental hut studies [17,42,43].

The BGS trap has previously been validated as an effective tool for the monitoring and surveillance of the dengue virus vector *Ae. aegypti* [26-31]. However, the efficacy of the BGS trap to attract and catch (or pull) chemically-repelled or insecticide-exposed mosquitoes is not known. Many chemicals can elicit repellent behavioral responses in *Ae. aegypti* at doses well below those required for toxic outcomes [1,16,17], but the effects of such exposures on the mosquito's host-seeking behavior are poorly understood. For another important dengue virus vector, *Ae. albopictus*, changes in both host-seeking and blood-feeding behaviors upon exposure to plant volatiles under laboratory conditions have been described [57]. *Aedes albopictus* females surviving exposure to geraniol, citral, eugenol, or anisaldehyde for 24 and 48 h all showed different degrees of reduction in host-seeking ability (e.g., increased times to reach a target location and to search for a suitable feeding site and insert the stylet). After 48 h of exposure to 0.250 μg/cm^3 of anisaldehyde, 100% of the mosquitoes showed loss of host-seeking ability, through impacts on the time to host-seeking activation, orientation, probing and engorgement compared to unexposed controls. In another study, *Ae. aegypti* females were exposed to sublethal levels (LD_{25}) of pyrethroid insecticides to evaluate the effects of the neurotoxicants 24 h post-exposure. A significant reduction in time of activation to flight was observed in mosquitoes exposed to deltamethrin and permethrin [58]. Similarly, excito-repellency studies using lower concentrations of deltamethrin showed that mated *Ae. aegypti* exhibited significant differences in escape responses with and without hosts present [59]. This type of knowledge is critical to define the expected efficacy of a BGS trap in a repellent focused push-pull strategy.

Holding female *Ae. aegypti* in experimental huts with DDT-treated fabric did not significantly impact

Table 2 Cumulative BG-Sentinel™ trap catches for immediate release (IR)[1] and delayed release (DR)[2] trials with *Ae. aegypti*[3] exposed to metofluthrin coils

		Cumulative mean percentage (±SD) of released *Ae. aegypti* recaptured by time point[4]										
Dose	Release[5]/Treatments	Day 1	←----------Day 2----------→			←----------Day 3----------→			N[6]	Mean day-time (12 hr) conditions		
		17:30 h	05:30 h	12:30 h	17:30 h	05:30 h	12:30 h	17:30 h		Temp (°C)	RH (%)	Light intensity (lx/ft^2)
Low dose (0.00312%)	**IRa**											
	Control	57.9a (±5.6)	69.0a (±6.5)	71.0a (±6.3)	75.2a (±7.9)	75.2a (±7.9)	76.6a (±7.9)	76.6a (±7.9)	111/145	24.9	60.2	167.6
	Blank Coil	52.7a (±7.9)	59.6a (±13.6)	69.2a (±18.7)	71.2a (±6.3)	72.6a (±8.7)	72.6a (±8.7)	72.6a (±8.7)	106/146	25.2	59.9	177.9
	Metofluthrin	53.1a (±11.0)	64.8a (±8.2)	74.2a (±8.9)	83.5a (±1.3)	84.2a (±0.3)	84.8a (±1.1)	84.8a (±1.1)	123/145	24.1	61.8	66.6
	DRa											
	Control	-	-	66.4a (±18.8)	87.7a (±9.5)	92.5a (±2.6)	93.2a (±3.4)	93.2a (±3.4)	136/146	26.2	63.0	178.5
	Blank Coil	-	-	77.2a (±14.6)	86.9a (±3.7)	86.9a (±3.7)	89.9a (±1.7)	89.9a (±1.7)	130/145	26.3	62.7	173.9
	Metofluthrin	-	-	56.6a (±7.4)	74.8a (±10.3)	76.9a (±11.5)	79.7a (±13.8)	79.7a (±13.9)	113/143	25.2	65.7	61.5
High Dose (0.00625%)	**IRa**											
	Control	85.8a(±5.3)	87.2a (±5.8)	87.2a (±5.8)	87.2a (±5.8)	87.2a (±5.8)	87.2a (±5.8)	87.2a (±5.8)	123/148	28.1	65.6	167.6
	Blank Coil	81.2a (±8.9)	81.9a (±7.9)	82.6a (±6.9)	82.55a (±7.0)	82.6a (±6.9)	82.6a (±6.9)	82.6a (±6.9)	123/149	28.0	65.8	185.1
	Metofluthrin	85.9a (±6.0)	91.3a (±1.1)	92.0a (±1.9)	92.0a (±1.9)	92.0a (±1.9)	92.0a (±1.9)	92.0a (±1.9)	137/149	26.7	69.2	63.7
	DRa											
	Control	-	-	92.7a (±4.2)	92.7a (±4.2)	92.7a (±4.2)	92.7a (±4.2)	92.7a (±4.2)	144/150	28.0	65.8	194.8
	Blank Coil	-	-	77.7a (±1.6)	85.8a (±5.4)	85.8a (±5.4)	85.8a (±5.4)	85.8a (±5.4)	126/148	29.7	65.9	186.8
	Metofluthrin	-	-	89.2a (±5.4)	89.2a (±5.4)	93.2a (±3.0)	93.2a (±3.0)	93.2a (±3.0)	138/148	26.7	69.4	61.7

[1] Cohort exposed from 0600–1200 hours and released immediately afterwards.
[2] Cohort exposed from 1200–1800 hours but released only after a holding period of 12 h.
[3] 3–5 day old starved females.
[4] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females within release trial types (IR or DR; Kruskal-Wallis 95% confidence limit) or between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[5] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[6]Total recaptured /total released minus total knock down.

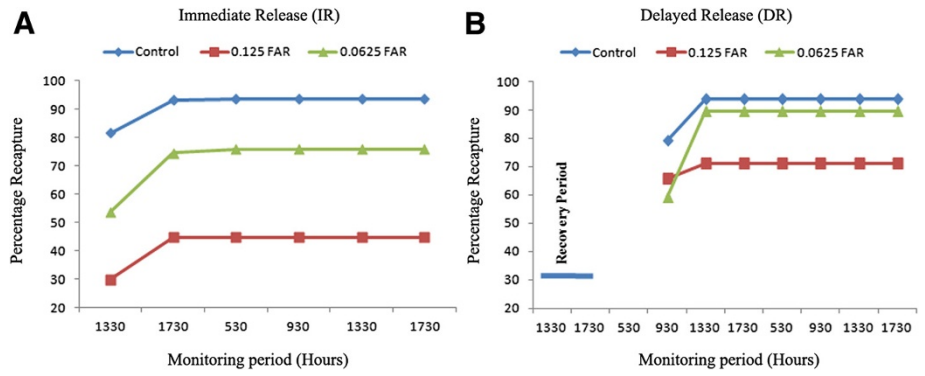


Figure 4 Cumulative BG-Sentinel™ trap recaptures for *Ae. aegypti* females in trials using immediate release (A) or delayed release (B) of mosquitoes previously exposed to transfluthrin.

subsequent BGS capture compared to non-exposed females, regardless of whether the exposed females were evaluated immediately following DDT exposure or following a 12 h recovery period in a repellent–free setting. Thus, there was no immediate or latent negative impact on host-seeking ability, as estimated by BGS trap catches, using this DDT exposure route. Similarly, mosquitoes exposed to metofluthrin coils with high (0.0065%) or low (0.003%) concentrations were as likely to be captured with the BGS traps as non-exposed control mosquitoes. Previous exposure to transfluthrin at 0.125 and 0.062 FAR resulted in significantly lower trap catches, compared to control mosquitoes, for mosquitoes released immediately following exposure but not for those allowed to recover for 12 h before BGS trap evaluation.

The comparison of results for mosquitoes released immediately following exposure versus those allowed to recover for 12 h before being released into the environment with the BGS traps indicate that this is a temporary phenomenon. This is suggestive of effects on sensory pathways used to detect host cues that resolve following the 12 h holding period. Similarly, Hao *et al.* [57] noted that a reduction in host-seeking ability in *Ae. albopictus* in the laboratory was reversible following recovery times that were dependent on chemical and concentration specific exposure conditions. However, we cannot rule out the possibility that the observed increase in host-seeking activity/trap catch rates for the delayed release females resulted, in part, from that they were only supplied with water during the recovery phase and thus were more motivated to locate a food source at the end of the recovery period.

Repellents have been shown to induce changes in responses of olfactory receptor neurons of female mosquitoes [60], specifically involving the grooved peg sensilla and sensilla trichodea, which are located on the mosquito antennae [61-64]. This neuronal activation disrupts the mosquito's ability to detect host-seeking kairomones, components of human sweat and presumably also the BGS trap lure (BG Lure). The decline in sensitivity of *Ae. aegypti* to human odor as a result of repellent exposure might be a mechanism for the temporary suppression of host-seeking behavior [65], as seen for the IR mosquitoes exposed to transfluthrin. Most evidence published to date on the basis of action of repellent compounds (e.g., DEET) are clearly conflicting and support either hypotheses indicating that repellents mask odors by blocking their receptors or act as true odorants that seem to be avoided by pests. However, none of these studies refer to insecticides similar to those tested in the present study. Several insecticides have been described that induce hyperactivity at sublethal doses and even promote the avoidance of impregnated areas. However, no clear evidence exists to date to link these effects to those of known repellents acting on insect chemoreceptors. The specific mechanism of action behind the observed change in BGS recapture rates over time following exposure to transfluthrin, could not be addressed in the current study, but highlights the need to integrate laboratory and field evaluations as a model for translational research.

There are several study design biases that could have influenced our results. This includes innate differences in spatial repellent actives, such as volatility, and the fact that trials were performed independently at various times of year under varying temperatures that could also affect chemical volatility. Although the two treatment formats varied (treated fabric vs. coils), each format represented the typical exposure method that target vector mosquitoes would experience under operational implementation for these interventions. This allowed for a more accurate assessment of exposure effects of the spatial repellents as would be expected under natural conditions. In addition, the exposure methods used – where mosquitoes were held in screened cages placed

Table 3 Cumulative BG-Sentinel™ trap catches for immediate release (IR)[1] and delayed release (DR)[2] trials with *Ae. aegypti*[3] exposed to transfluthrin-treated fabrics

	Cumulative mean percentage (±SD) of released *Ae. aegypti* recaptured by time point[4]													
Release[5]/ Treatments	←-----Day 1-----→		←----------Day 2----------→				←----------Day 3----------→					Mean day-time (12 hr) conditions		
	13:30 h	**17:30 h**	**05:30 h**	**09:30 h**	**13:30 h**	**17:30 h**	**05:30 h**	**09:30 h**	**13:30 h**	**17:30 h**	**N[6]**	**Temp (°C)**	**RH (%)**	**Light intensity (lx/ft²)**
IR_a Control	81.8a (±11.1)	93.3a (±8.6)	93.8a (±7.6)	93.8a (±7.6)	93.8a (±7.6)	93.8a (±7.6)	-	-	-	-	180 /192	28.7	77.1	314.7
Transfluthrin (5 µg *ai*/cm²)	29.9c (±11.8)	44.8c (±18.7)	44.8c (±18.7)	44.8c (±18.7)	44.8c (±18.7)	44.8c (±18.7)	-	-	-	-	39/87	27.2	83.4	97.1
Transfluthrin (2.5 µg *ai*/cm²)	53.9b (±16.0)	74.7b (±9.3)	76.0b (±9.3)	76.0b (±9.3)	76.0b (±9.3)	76.0b (±9.3)					117/154	26.1	37.1	120.3
DR_b														
Control	-	-	-	79.2a (±9.4)	93.9a (±4.3)	93.9a (±4.3)	93.9a (±4.3)	93.9a (±4.3)	93.9a (±4.3)	93.9a (±4.3)	185/197	28.0	78.6	308.3
Transfluthrin (5 µg *ai*/cm²)	-	-	-	65.6a (±18.3)	70.9a (±16.4)	70.9a (±16.4)	70.9a (±16.4)	70.9a (±16.4)	70.9a (±16.4)	70.9a (±16.4)	43/61	26.8	85.8	98.0
Transfluthrin (2.5 µg *ai*/cm²)	-	-	-	59.2a (±28.1)	89.6a (±4.6)	89.6a (±4.6)	89.6a (±4.6)	89.6a (±4.6)	89.6a (±4.6)	89.6a (±4.6)	92/138	25.7	37.7	126.8

[1] Cohort exposed from 0600–1200 hours and released immediately afterwards.
[2] Cohort exposed from 1200–1800 hours but released only after a holding period of 12 h.
[3] 3–5 day old starved females.
[4] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females within release trial types (IR or DR; Kruskal-Wallis 95% confidence limit) or between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[5] Different lowercase letters in the same column indicate significant differences between mean recapture percentages for released females between IR and DR trials (Mann-U Whitney Test, 95% confidence limit).
[6]Total recaptured /total released minus total knock down.

inside experimental huts containing spatial repellent treatments (treated fabrics or coils) mimicked expected exposure routes for airborne repellent molecules where direct contact with treated surfaces does not occur. Despite these potential study design biases, it is clear that mosquito behavior is an area of research that will continue to be of high importance, especially as development of novel vector control tools are necessary in order to combat diseases such as malaria and dengue [66].

Conclusion

Overall, data indicate that exposure of *Ae. aegypti* to the test repellents had no more than minor and short-lived impacts on BGS capture rates. This finding suggests the use of these repellents may not negatively impact the ability of the BGS to remove deterred *Ae. aegypti* adults from outdoor areas around treated homes. Deriving maximum benefits from an outdoor trap (pull) component within a repellent-focused combination push-pull system requires that previous exposure to a repellent chemical, used as the push component, will not substantially reduce trap efficacy to capture vectors from the peridomestic environment. We show here that exposure to DDT, metofluthrin or transfluthrin results in no more than minor and short-lived reductions in the efficacy of the Biogents Sentinel™ trap to recapture *Ae. aeygpti* females. However, using BGS recapture rates as a proxy for host-seeking, exposure to the highly volatile pyrethroid compound, transfluthrin, appeared to have some impact on test populations' attraction or movement towards the trap immediately following exposure, (i.e., IR populations) based on reduced recapture rates but this effect was absent following a 12 h recovery period (i.e., DR populations). This delay may have significant impact on disease reduction, as several models have shown that the time period required for the mosquito to regain its ability to seek a host after repellent exposure likely increases the probability of mortality due to adverse environmental factors or predation [55]. This study has begun to elucidate an understanding of: (i) the selection of spatial repellent chemicals best suited for inclusion in a combined intervention system in which traps are required to function in removing repelled vectors from the peridomestic environment and (ii) the estimation of recovery time a vector may require to respond to an attractant source following repellent exposure.

Competing interests

The authors declare no competing interests.

Authors' contributions

All the authors have contributed significantly to this study. FVS, NLA, JPG and TC contributed to conceptualization the study and wrote the manuscript. AP performed analysis the data. TAO, LE, CD and SP participated in its study and performed the coordination and helped the draft of the manuscript. All authors read and approved the final manuscript. Some of the authors are U. S. government employees. The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. Mention of specific commercial products does not constitute an endorsement or recommendation.

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RESEARCH ARTICLE OPEN ACCESS

Seasonality and daily flight activity of stable flies (Diptera: Muscidae) on dairy farms in Saraburi Province, Thailand

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Abstract – Knowledge of seasonal abundance and flight activity patterns are required to design effective management programs for insect pests of humans and livestock. In this study, the seasonality and daily flight activity of *Stomoxys* species were observed on two dairy farms in Saraburi Province, Thailand. Data were assessed throughout 1 year using Vavoua traps from September 2010 to August 2011. A total of 2,520 individuals belonging to four species were collected. Most *Stomoxys* species peaked in September (rainy season) and gradually decreased in number toward February (dry season); a second peak occurred between March and April (hot season). *Stomoxys calcitrans* was caught throughout the year and was the most abundant species in this study. The total number of males and females of *S. calcitrans* differed significantly among seasons and time intervals. The weather parameters of relative humidity and light intensity were significantly correlated with *S. calcitrans* abundance.

Key words: *Stomoxys*, daily flight activity, dairy cattle, seasonal abundance, stable flies, Thailand.

Résumé – Saisonnalité et activité de vol quotidienne des mouches d'étables (Diptera : Muscidae) dans des fermes laitières de la province de Saraburi, Thaïlande. La connaissance de l'abondance saisonnière et des patrons d'activité en vol est nécessaire pour établir des programmes efficaces de contrôle des insectes nuisibles a l'homme et au bétail. Dans cette étude, les variations saisonnières et l'activité en vol quotidienne des espèces de *Stomoxys* ont été observées dans deux fermes laitières de la province de Saraburi, Thaïlande. Les données ont éte obtenues pendant un an en utilisant des pièges Vavoua de septembre 2010 à août 2011. Un total de 2,520 individus appartenant à quatre espèces a été récolté. La plupart des espèces de *Stomoxys* avaient un pic d'abondance en septembre (saison humide) et leur nombre décroissait régulièrement jusqu'en février (saison sèche) ; un deuxième pic apparaissait entre mars et avril (saison chaude). *Stomoxys calcitrans* a été capturé toute l'année et était l'espèce la plus abondante dans cette étude. Le nombre total de mâles et de femelles de *S. calcitrans* différait significativement selon les saisons et les intervalles de temps. Les paramètres climatiques d'humidité relative et d'intensité lumineuse étaient corrélés de manière significative avec l'abondance de *S. calcitrans*.

Introduction

Stable flies belong to the subfamily Stomoxyinae in the family Muscidae (Diptera). Among 18 *Stomoxys* species described, six species are recorded from Thailand, of which one is cosmopolitan, *S. calcitrans* (Linnaeus, 1758) [9, 22, 24]. Stable flies resemble house flies but can be easily distinguished by their piercing-sucking mouthparts which are conspicuous, long and project straightforward from under the head. They are important and widely distributed insect pests of livestock, wildlife and sometimes humans. Adult stable flies of both sexes are blood-sucking flies and cause painful bites and significant blood loss in some animals. High populations of biting activity can reduce animal productivity and disturb feeding resulting in reduced weight gain and milk production [2–5, 23]. Moreover, they may act as both biological and mechanical vectors for pathogens such as trypanosomes [19]. Stable flies may also act as an intermediate host of the nematode *Habronema* [21]. In Thailand, little is known about the presence of different stomoxyine fly species, their distribution and their biology. However,

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Figure 1. Location of Muak Lek district (study area) in Saraburi Province, Thailand.

Masmeatathip *et al.* [11] and Muenworn *et al.* [13] described the seasonal abundance and daily activity of *Stomoxys* species in Thailand. Muenworn *et al.* [12, 13] conducted stable fly surveys and reported their distribution in Thailand. A better understanding of the seasonal and daily activity of the flies will facilitate and make fly control programs more effective. This study had the objective to evaluate the seasonal abundance and daily activity of *Stomoxys* species.

Materials and methods

Study sites

This study was conducted on two dairy farms in Muak Lek district, Saraburi Province (Figure 1). Saraburi is located in the central highlands of Thailand, an area of relatively high plains and plateaus about 140 km Northeast of Bangkok. The study sites are 355 and 460 m above sea level (a.s.l.). Thailand has three seasons: hot (March to May), rainy (June to October) and dry (November to February). At the time of the study, farm 1 (14° 48′ N, 101° 17′ E; 460 m a.s.l) had 10 cows and farm 2 (14° 47′ N, 101° 15′ E; 355 m a.s.l) had 57 cows in milk production.

Specimen collection

At each site, four Vavoua traps [8] were randomly installed around sample sites at least 5 m apart from each other. The traps were placed at 5–10 cm above the ground. Stable flies were observed monthly during a one-year study (September 2010 to August 2011). Flies were captured every hour between 0600 and 1800 h. After trapping, flies in the Vavoua traps were killed using ethanol spray and preserved in 80% ethanol. Air temperature, relative humidity and light intensity were also recorded every hour by using a digital thermometer and a digital light meter at each farm. The specimens were brought back to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand, for identification using the keys of Zumpt [24] and Tumrasvin & Shinonaga [22].

Data analysis

The total numbers of each sex of stable flies collected from traps at each collection site were analyzed using a one-way analysis of variance (ANOVA) and the time intervals were considered as treatment effects. Variations in the numbers of each sex between seasons were compared using the least significant difference (LSD) test. Stepwise multiple regression analysis was used to determine the relationship between the abundance of stable fly species and weather parameters. The data were analyzed using SPSS (Version 17, SPSS Inc., Chicago, IL, USA). All statistical significance levels were set at $p < 0.05$.

Results

In total, 2,520 individuals of *Stomoxys* species were collected during the one-year study, of which 1,622 were trapped

Table 1. Total numbers of *Stomoxys* spp. collected and their relative abundance (RA) on two dairy farms, Muak Lek district, Saraburi Province from September 2010 to August 2011.

Species	Sex	Farm 1	RA (%)	Farm 2	RA (%)
S. bengalensis Picard, 1908	Male	0	0	1	0.1
	Female	0	0	0	0
S. calcitrans (Linnaeus, 1758)	Male	1,206	74.4	535	59.6
	Female	355	21.9	268	29.8
S. indicus Picard, 1908	Male	19	1.2	12	1.3
	Female	34	2.1	25	2.8
S. sitiens Rondani, 1873	Male	5	0.3	24	2.7
	Female	3	0.2	33	3.7
Species/total number		3/1,622		4/898	

on farm 1 and 898 on farm 2 (Table 1). The following four species were captured: *S. bengalensis* Picard, 1908 [15], *S. calcitrans* (Linnaeus, 1758) [9], *S. indicus* Picard, 1908 [15] and *S. sitiens* Rondani, 1873 [17].

The highest temperature was recorded in May and the lowest temperature was in October. The average relative humidity was lower in December to January during the dry season and the highest relative humidity was in September. The average light intensity was high in March to May during the hot season (Figure 2). Seasonal variations in the abundance of *Stomoxys* species are presented at the species and site levels in Figures 3 and 4. The highest numbers of each species were recorded in September and gradually decreased in number toward February. The second peak of most species occurred between March and April. The most abundant species was *S. calcitrans* (89–96%) which was found on both farms. *S. calcitrans* was present throughout the year. Collections of other species were too low to be meaningful for the analysis (Table 1 and Figure 7).

On both farms, the numbers of males of *S. calcitrans* were significantly ($p < 0.05$) greater than the females in each season (Figure 5). On farm 1, seasonal variations in the numbers of females were significant ($p < 0.05$), except between the hot and rainy seasons, whereas for males, seasonal variations were significant ($p < 0.05$), except between the hot and dry seasons and the hot and rainy seasons. On farm 2, seasonal variations in the numbers of both sexes were significant ($p < 0.05$). The abundance of *S. calcitrans* peaked in the rainy season and declined in the dry season.

On farm 1, the total number of females of *S. calcitrans* collected did not differ significantly among time intervals in the dry, hot and rainy seasons. The numbers of males collected showed a significant ($p = 0.005$) difference among time intervals in the dry season, but there was no significant difference among time intervals in the hot and rainy seasons (Figure 6). On farm 2, the total number of females collected showed a significant ($p = 0.009$) difference among time intervals in the rainy season, but there was no significant difference among time intervals in the dry and hot seasons. Males showed significant differences in time intervals between the hot and rainy seasons ($p = 0.007$, $p = 0.028$, respectively), but not in the dry season (Figure 6).

During the whole collection period, *S. calcitrans* abundance was not related to air temperature but showed a positive correlation with relative humidity and light intensity (Table 2).

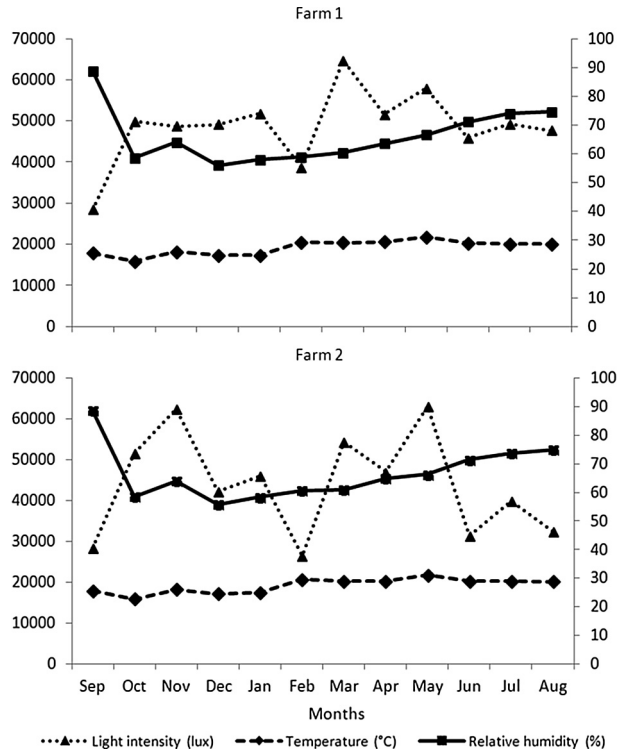


Figure 2. Mean monthly temperature and relative humidity (right axis scale) and light intensity (left axis scale) on the experimental sites (two dairy farms) from September 2010 to August 2011.

Discussion

The present study extends our understanding of the seasonal abundance and daily activity of stable flies in Thailand. In Saraburi Province, there were four species in the genera *Stomoxys* collected from both farms and *S. calcitrans* was active throughout the year. Muenworn *et al.* [13] found *S. calcitrans* to be the most abundant species followed by *S. indicus*. *Stomoxys* populations peaked in September, corresponding to the rainy season. This finding agreed with earlier work by Masmeatathip *et al.* [11] and Muenworn *et al.* [13]. This may have been due to an increase in the rainfall causing a widespread increase in suitable breeding sites which is a

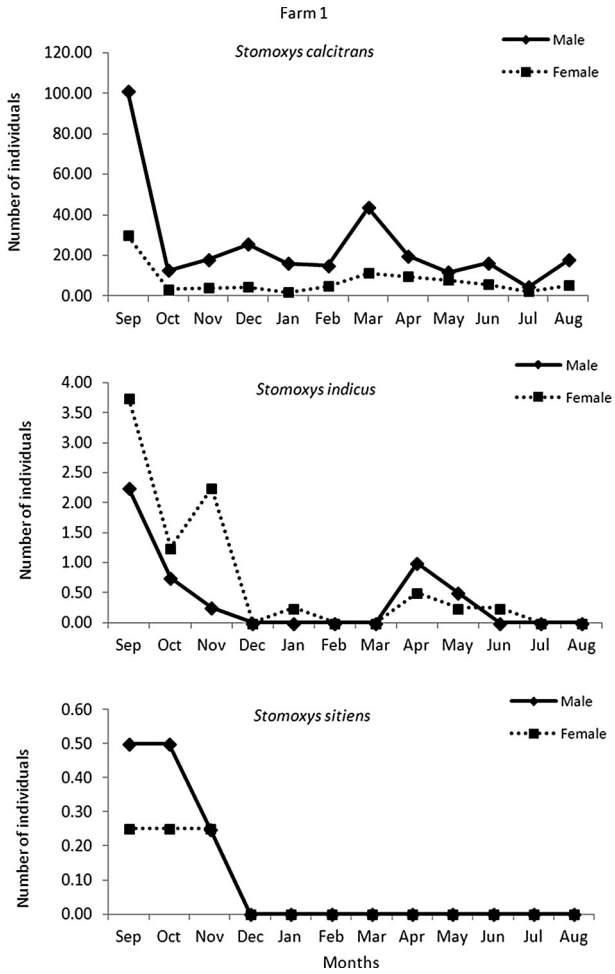


Figure 3. Mean number of *Stomoxys* spp. captured in four traps on dairy farm 1 from September 2010 to August 2011.

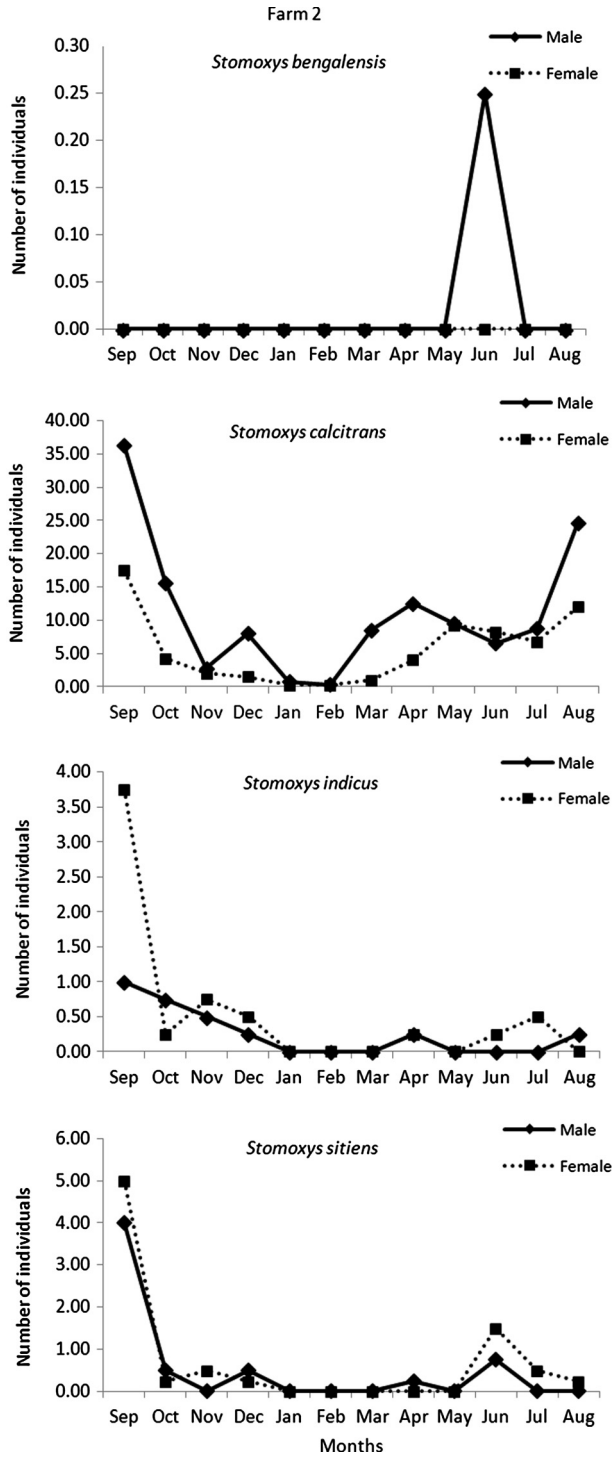


Figure 4. Mean number of *Stomoxys* spp. captured in four traps on dairy farm 2 from September 2010 to August 2011.

critical factor for eggs to hatch and the larvae to survive and successfully develop to pupae and adults. Earlier work shows that immature stages of *Stomoxys* (eggs and larvae) are highly sensitive to the following environmental conditions to survive and successfully develop to pupae and adults: temperature, humidity and rainfall [6, 18].

The numbers of male *S. calcitrans* were significantly higher than females, in agreement with the reports of Masmeatathip *et al.* [11] and Muenworn *et al.* [13]. More female *S. indicus* were collected than males on both farms. Similar findings were reported by Masmeatathip *et al.* [11].The sex ratios of *S. sitiens* varied between farms. Only one male of *S. bengalensis* was seen and that was on farm 2. The variation in the abundance of *Stomoxys* species that were observed may have depended on the temperature, precipitation, types of trap, trap locations and trap height [1, 7, 10, 16]. Further studies are needed to evaluate whether these differences are associated with the location or other factors that attract *Stomoxys* species.

The season seemed to play a major role in the daily activity patterns of *S. calcitrans*. In the dry and rainy seasons, the number of *S. calcitrans* increased throughout the day until 1500 h and gradually decreased in number toward 1800 h. In the hot season, this species was present throughout the day and peaked in abundance in the evening and a secondary peak occurred in the late morning although the numbers of females were not statistically significant among the time intervals. In previous studies, *S. calcitrans* was reported to have a bimodal pattern of feeding with a major peak during 0800–1000 h and a minor peak occurred during the afternoon [11]. Muenworn *et al.* [13] showed a bimodal activity of male *S. calcitrans* with peaks

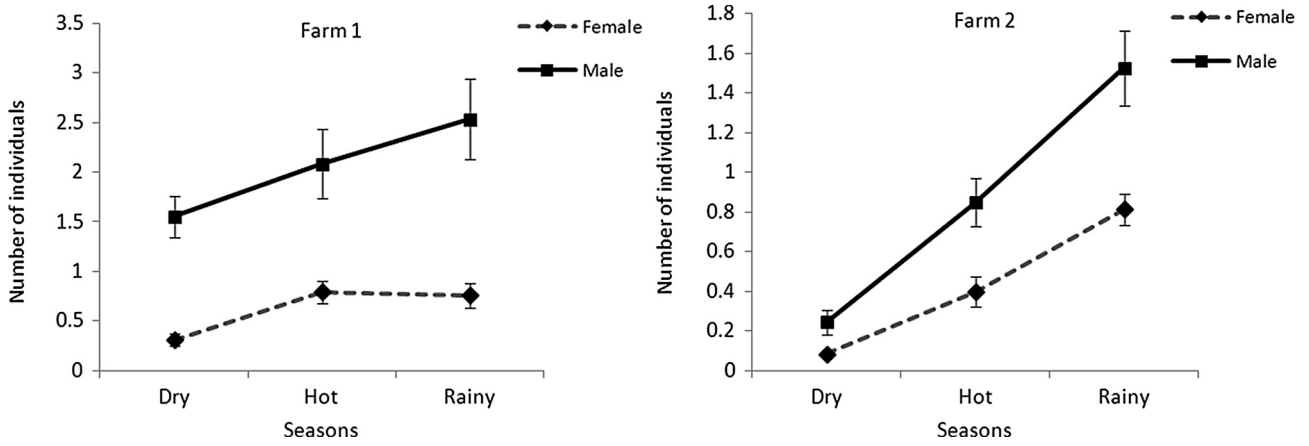


Figure 5. Mean numbers ± standard error bars of *Stomoxys calcitrans* males and females collected during three seasons on two dairy farms.

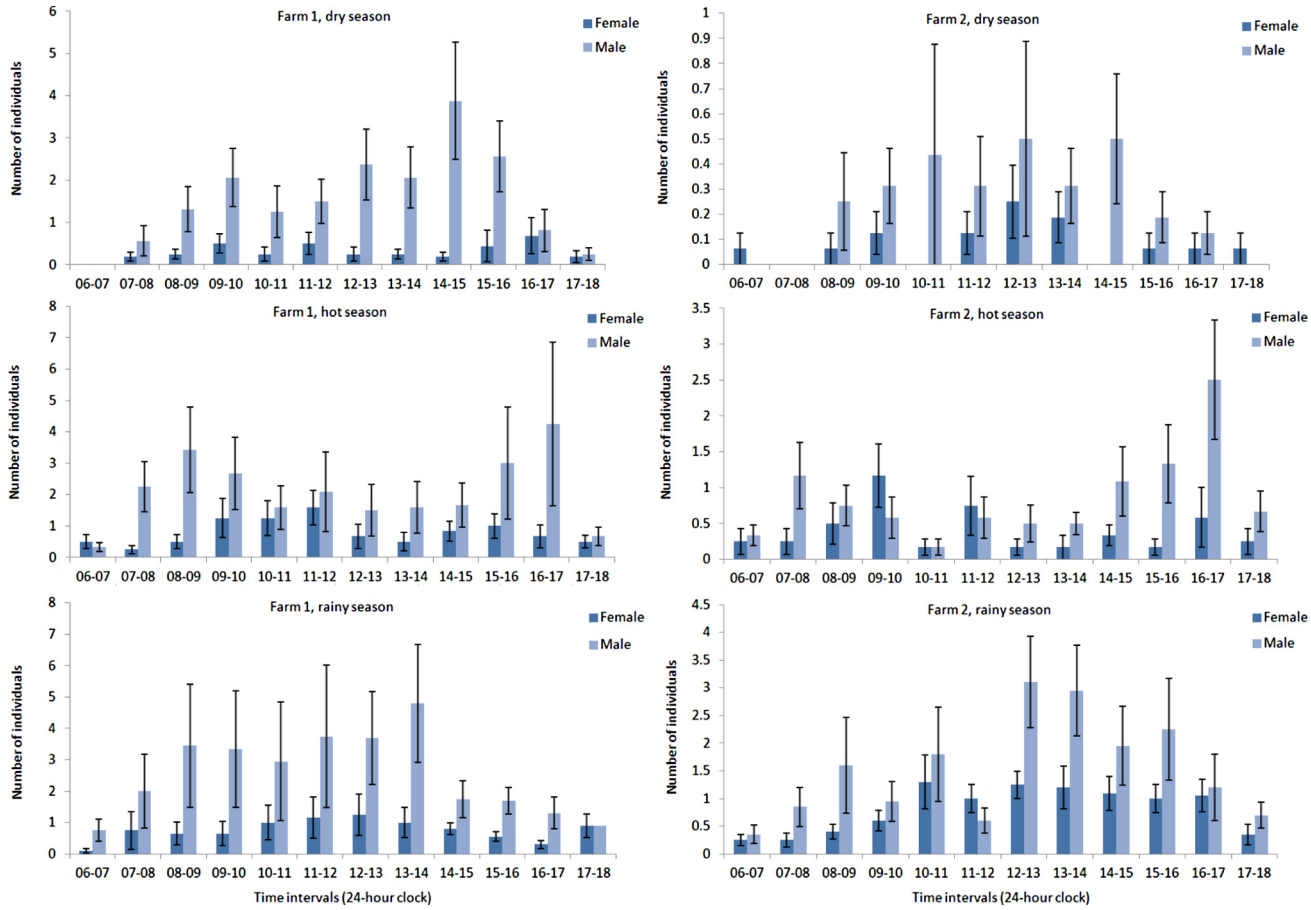


Figure 6. Mean numbers ± standard error bars of *Stomoxys calcitrans* males and females collected during one-hour intervals in three seasons on two dairy farms.

at 0800–1000 and 1400–1600 h while female activity increased throughout the day until 1600 h, when the activity of both males and females declined steadily until it was dark. Masmeatathip *et al.* [11] reported bimodal activity of *S. indicus* and *S. sitiens* with population peaks in the early morning and in the late afternoon. This finding was similar to that observed in this study. Stable flies require blood for successful mating and ovarian development, but also require nectar as a supplemental energy source for flight activity and successful blood-feeding [20]. Müller *et al.* [14] conducted studies on the diurnal feeding behavior of three *Stomoxys* species in Mali and found bimodal blood-feeding and unimodal sugar-feeding activity periods. The differences in feeding activity patterns were dependent upon the protein and nectar sources, local conditions, sampling methods

Table 2. Stepwise multiple regression analysis with correlation between total number of *Stomoxys calcitrans* and weather parameters in Muak Lek district, Saraburi Province from September 2010 to August 2011.

Effect	*b*	*SE* of *b*	Beta	*t*	*p*	
Intercept	−17.266	4.57		−3.778	0.000	
Relative humidity (%)	0.337	0.062	0.336	5.481	0.000	
Light intensity (lux)	0.00006	0.000	0.208	3.39	0.001	
	R^2	*SS*	*df*	*MS*	*F*	*p*
	0.1	3,945.72	2	1,972.86	15.89	0.000

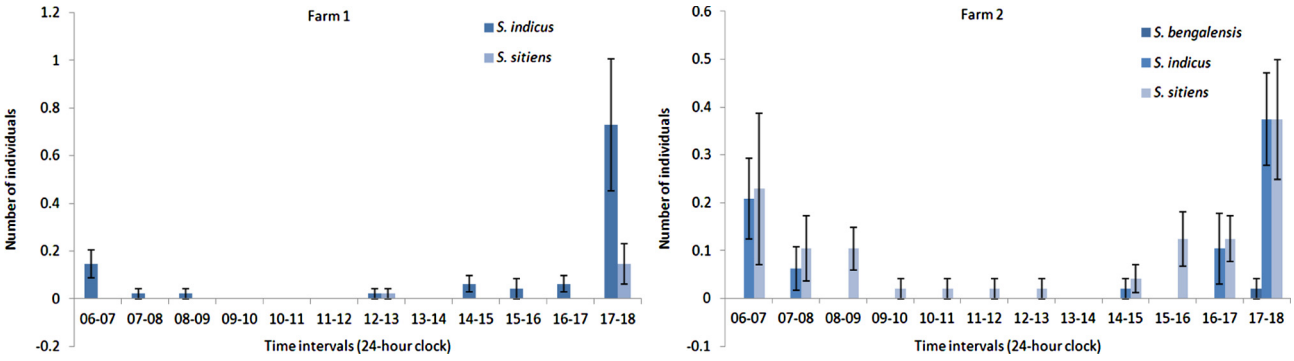


Figure 7. Mean numbers ± standard error bars of *Stomoxys* spp. males and females collected during one-hour intervals on two dairy farms from September 2010 to August 2011.

and seasons. Analysis of the data showed the abundance of *S. calcitrans* was significantly and positively correlated with relative humidity and light intensity although the relationship was weak (R = 0.317). There were probably other factors that affected the flight activity patterns of this species. The current study has provided a step in the evolution of planning and developing control systems for insects.

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Comparison of *Aedes aegypti* (Diptera: Culicidae) Resting Behavior on Two Fabric Types Under Consideration for Insecticide Treatment in a Push-Pull Strategy

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ABSTRACT *Aedes aegypti* (L.), the primary vector of dengue and dengue hemorrhagic fever, breeds and rests predominately inside human dwellings. With no current vaccine available, vector control remains the mainstay for dengue management and novel approaches continue to be needed to reduce virus transmission. This requires a full understanding of *Ae. aegypti* ecology to design effective strategies. One novel approach is the use of contact irritants at target resting sites inside homes to make the surface unacceptable and cause vectors to escape before biting. The objective of the current study was to observe indoor resting behavior patterns of female *Ae. aegypti* within experimental huts in response to two fabrics under consideration for insecticide treatment: cotton and polyester. Results indicate that fabric type, coverage ratio of dark to light fabric and placement configuration (vertical vs. horizontal) all influenced the resting pattern of mosquito cohorts. Findings from this study will guide evaluations of a push-pull strategy designed to exploit contact irritant behaviors and drive *Ae. aegypti* out of homes prefeeding.

KEY WORDS *Aedes aegypti*, contact irritancy, resting behavior, experimental hut, Thailand

Dengue occurs in developing countries of the tropics with an estimated 2.5 billion people residing in endemic areas (World Health Organization [WHO] 2007). Only two species of *Aedes* mosquitoes, *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), are considered as dengue vectors. *Aedes aegypti*, a day biting mosquito, is more prevalent around human dwellings and is a principal vector in urban zones (Gould et al. 1968) while *Ae. albopictus*, the Asian tiger mosquito, is considered a vector in more rural and suburban areas (Chan et al. 1971, Chareonviriyaphap et al. 1999). Controlling these two species is extremely difficult and presents a long-term problem for disease endemic areas. Control strategies aimed at the elimination of immature breeding sites through source reduction, requires full community participation, and often results in failure (Gubler et al. 1998, Kongmee et al. 2004). Arguably, the most effective method for arthropod-borne disease prevention has been through adult vector reduction using various chemical means (Roberts et al. 1997, Grieco et al. 2007, Jirakanjanakit et al. 2007, Thanispong et al. 2008). However, with the rates of dengue and dengue hemorrhagic fever increasing in many endemic areas it is evident that novel control strategies are needed (WHO 2010).

The direct toxic effect of chemicals on mosquitoes has been the focus of control efforts dating back to the WHO malaria eradication efforts of the 1950s (WHO 2009) while limited attention has been given to the benefits of sub-lethal chemical actions (Roberts et al. 1997, 2000; Achee et al. 2012). One of the best known documentations of sub-lethal actions was the behavioral studies of *Anopheles* mosquitoes to DDT (Kennedy 1947). Combined, this work led to the identification of two different types of sub-lethal actions: contact irritancy and noncontact repellency (Kennedy 1947, Muirhead–Thompson 1951, Dethier et al. 1960, Davidson 1953, Lockwood et al. 1984, Roberts and Andre 1994). Contact irritancy elicits an escape response after insects make physical contact with chemical treated surfaces while noncontact or spatial repellency is differentiated from the former because of its ability to prevent vectors from entering chemically treated areas in the vapor phase, that is, without making physical tarsal contact with the treated surface (Lockwood et al. 1984; Roberts et al. 1997, 2000).

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The role of sub-lethal chemical actions in a disease prevention program is dependent on a full understanding of the ecology of the target vector to accurately measure the chemical effects on epidemiologically important mosquito behaviors. Experimental huts have been useful assays for such studies. *Anopheles* mosquitoes have generally been the focus of most behavioral observations using experimental huts (Smith 1965; Roberts et al. 1984; Grieco et al. 2000, 2007; Pates and Curtis 2005; Malaithong et al. 2010) because of their role in malaria transmission. In relation to the global dengue burden, however, relatively few examples exist in the literature for the evaluation of *Ae. aegypti* resting behavior under either laboratory or field conditions (Fay 1968, Pant and Yasuno 1970, Kittayapong et al. 1997, Edman et al. 1997, Perich et al. 2000, Cooperband and Allan 2009).

The current study represents one component in the development of a push-pull strategy for *Ae. aegypti* control. The overall goal of the strategy is to reduce vector densities inside homes using contact irritant and/or spatial repellent chemical actions (i.e., 'push' components) thereby reducing the probability of a vector feeding on a human host and subsequently reducing the probability of virus transmission. Two methods are being evaluated to quantify the effectiveness of the push component: 1) placing repellent-treated fabric around the interior perimeter of house openings (windows and doors) called 'target repellent sites' to prevent entry and 2) placing irritant-treated fabric at preferred indoor resting locations referred to as 'target irritant sites,' or a combination of the two. For either method, we aim to make the interior house space unsuitable and are defining minimum effective chemical dose and surface area coverage required for efficacy. Both approaches (repellent or irritant) could reduce human vector contact by disrupting behavior patterns leading up to indoor feeding, especially in regards to an endophagic species like *Ae. aegypti* (Kroeger et al. 2006, Lenhart et al. 2008). However, the success of a contact irritant push component will depend on characterizing vector resting patterns to properly target preferred sites and deter the vector from finding a suitable refuge before feeding is initiated. Indeed, targeting *Ae. aegypti* inside the home with insecticide treated materials (ITMs) has been the focus of several studies aimed at prevention and control of dengue transmission (Nam et al. 1993, Lenhart et al. 2008, Suwannachote et al. 2009).

Our objectives here were to quantify the resting patterns of female *Ae. aegypti* under controlled experimental conditions on two fabrics under consideration for insecticide treatment during push-pull evaluation trials. Our approach was based on the use of known *Ae. aegypti* visual cues (i.e., dark color and contrast) to identify an optimum treatment scheme (position and fabric type) based on observed proportions of mosquitoes resting. Resting patterns were observed in response to cotton and polyester, varying dark: light surface area coverage ratios (25, 50, and 75%) and fabric configuration (vertical vs. horizontal) under chemical-free conditions. Using the optimum resting scheme, we hope to maximize our contact irritant push effects when fabric is treated with chemical.

Materials and Methods

Study Site. Pu Teuy Village is located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17′ N, 99° 11′ E). It is ≈150 km northwest of Bangkok and is located in a mountainous area (420 m above sea level) surrounded by dense primary forest. The village contains ≈1,400 people with the primary occupation coming from agriculture. Pu Teuy was chosen as the field site based on the criteria of high densities of *Ae. aegypti* without evidence of dengue transmission to facilitate the use of mark-release methodologies. Before beginning the study, the village was mapped using a hand-held global positioning system (GPS) (Garmin International Inc., Olathe, KS) to ensure that there were no indigenous homes within an 800 m radius buffer zone around the study area (i.e., beyond average flight range of *Ae. aegypti*) (Reiter et al. 1995, Muir and Kay 1998, Harrington et al. 2005).

Mosquito Test Populations. *Ae. aegypti* test populations originated from F2-F5 colony material collected as immature stages (larvae and pupae) from various areas throughout Pu Teuy Village and maintained in an insectary established at the field site. Temperature in the field insectary ranged between 25 and 28°C and relative humidity (RH) between 80 and 90%. The photoperiod approximated natural conditions of 12:12 (L:D). Adults were provided cotton soaked with 10% sucrose solution from time of eclosion until 24 h before evaluation in experimental huts. Before the day of testing, 3–5 d old nonblood fed females were grouped into three cohorts: one cohort of 50 for behavioral observation, one cohort of 25 to serve as controls to monitor indoor knockdown rates, and one cohort of 10 mosquitoes to serve as replacements because of mortality before release. All cohorts were marked using fluorescent dusting powder (BioQuip Product, Inc., Rancho Dominguez, CA, and Shannon Luminous Materials, Inc., Santa Ana, CA) using the procedure of Achee et al. 2007 to facilitate observations. Mosquitoes were marked with a unique color for each replicate (i.e., each day).

Experimental Hut Design. Previous descriptions of the experimental hut design have been reported (Chareonviriyaphap et al. 2010). One experimental hut was used for all behavioral evaluations, except in one study comparing 50% coverage of dark cotton and polyester in which two experimental huts were used for simultaneous comparisons. The dimensions, basic design and construction material of the experimental huts mimic those of the indigenous homes in Pu Teuy village. The huts are 4 m wide × 5 m long × 2.5 m high with three windows (0.9 m wide × 0.6 m high) and one door (1 m wide × 2.4 m high) onto which can be affixed removable window and door interception traps, respectively, used during experiments evaluating vector movement patterns. Walls are constructed of untreated wood planks with a metal frame lining the interior wall surface. The metal frame is used to po-

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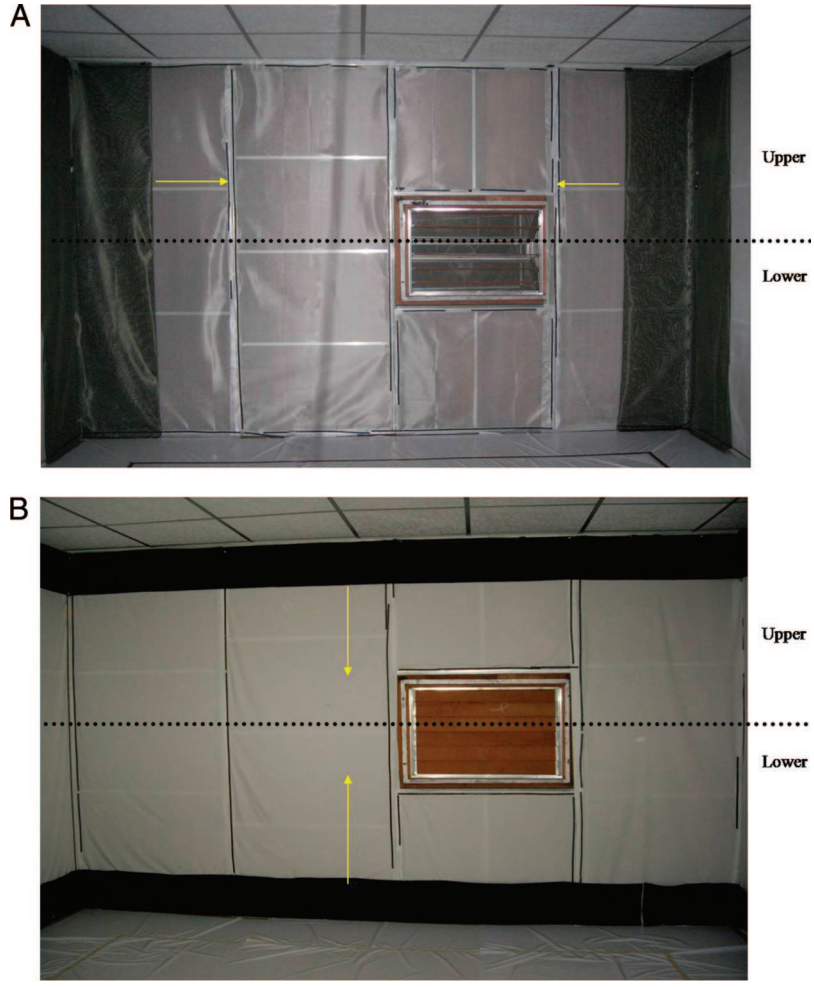


Fig. 1. Resting observations of female *Ae. aegypti* were conducted inside experimental huts using two fabric types and three dark: light coverage ratios (25, 50, and 75%) under (A) vertical configuration (polyester at 25% coverage shown) and (B) horizontal configuration (cotton at 25% coverage shown). L:D coverage ratios of 50 and 75% were achieved by incrementally adding dark fabric along interior wall panels as indicated by the arrows. (Online figure in color.)

sition chemical-free and treated fabrics inside the experimental huts and prevent chemical contamination of the wood for subsequent studies. The windows and the door of each hut were opened during trials to allow natural light to enter and maintain air exchange but were fitted with interception traps (in closed position) to prevent mosquitoes from exiting huts.

Test Fabric and Placement. Two fabric types were used for evaluation: 100% cotton (black and white; Lampang Store, Co. Ltd, Samphanthawong, Bangkok) and 100% polyester (green and white; mesh size 24 by 20/inch, BioQuip Products, Rancho Dominguez, CA). Each fabric type was evaluated at varying surface area coverage of corresponding dark fabric (black for cotton and green for polyester) representing 25, 50, 75% L:D ratios under two placement configurations (vertical and horizontal). The term 'dark' refers to the relative difference in color compared with the corresponding 'light' white color of each fabric. Although the medium olive green color of dark polyester is indeed lighter than the black color of cotton, it is darker than the white polyester of which it was positioned against during experiments.

Fabric was cut into individual panels according to L:D ratio being evaluated and were affixed to the metal frames positioned along the interior hut walls using magnets. All of interior walls (front, back, and two side walls) were covered by fabric. In addition, the floor was covered with white plastic to facilitate observation of knockdown mosquitoes. Each fabric panel was numerically labeled to facilitate data recording during observations. Under vertical configuration, each panel was divided horizontally at the halfway point and the numbers of resting mosquitoes were recorded for each the upper and lower half of each panel (Fig. 1). Dark fabric was positioned starting from the corners of each wall (expected target resting sites) then incrementally added toward the

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Table 1. Summary of female *Aedes aegypti* resting observations (06:00–18:00 hours; $n = 48$ trials) against varying treatment conditions using experimental huts in Kanchanaburi, Thailand

Treatment scheme	Mean no. resting (mosquito/hour) (SE ± 0.20)	Mann–Whitney/Kruskal–Wallis	
Fabric			
Cotton	12.8	$Z = -12.14$	$P < 0.05$
Polyester	3.3		
Color			
Dark[a]	12.9	$Z = -0.61$	$P < 0.05$
Light	3.2		
Configuration			
Vertical	8.5	$Z = -18.76$	$P = 0.54$
Horizontal	7.7		
L:D coverage			
25%	6.1	$\chi^2 = 64.34$, df = 2	$P < 0.05$
50%	7.9		
75%	10.3		

[a] Dark cotton, black; dark polyester, green. Light for both materials, white.

center of the walls with each increasing L:D coverage level (Fig. 1A). For horizontal configuration, dark fabric was positioned along the upper and lower intersections of ceiling to wall and wall to floor, respectively (Fig. 1B). Dark fabric was then added in equal portions incrementally toward the center of each wall as the coverage level increased such that for 25% coverage, 12.5% of the total dark fabric was positioned at the uppermost part of the wall and 12.5% at the lowermost part.

Observation Techniques. Marked female *Ae. aegypti* (50) were released inside the experimental hut, ≈30 min before sunrise (i.e., at 05:30 hours). The number of knocked down mosquitoes from the release population (defined as unable to right themselves when puffed with air; Chadwick 1975) were recorded before each experiment and replaced with live specimens. The control cup containing 25 females (from the same population used for release) was placed at the inside center of the hut at the start of each experiment and was provided a water-soaked cotton pad during the course of the trial. Mortality from the control cup was recorded every hour to correct for the total number of the release test population available to rest during each observation period. Two observers entered the hut at the top of each hour and systematically recorded the number of mosquitoes resting on each wall panel from a "line of observation" (≈50 cm from each wall) during a standard 3 min observation period. Flashlights were used to facilitate observations of marked specimens. Sampling initiated at 06:00 hours and continued until 18:00 hours. Temperature (°C) and humidity (RH%) were manually recorded each hour when observers entered the hut. A backpack aspirator was used for 15 min at the conclusion of each experiment (i.e., 18:00 hours) to remove marked mosquitoes inside the hut in preparation for the next replicate. Each treatment scheme (i.e., fabric type, L:D, and configuration) was conducted in four replicates (i.e., 4 d) making up a single experimental trial. Trials for cotton were performed from December 2008 through February 2009 and for polyester from February through May 2009. An additional trial was performed in December 2009 to simultaneously compare cotton and polyester at 50% L:D in vertical configuration to exclude environmental confounders.

Data Analysis. Numbers of resting mosquitoes observed by hour were calculated for each fabric panel to include upper and lower portions. Resting patterns were evaluated by fabric type (cotton and polyester), color (dark and light), configuration (vertical and horizontal), percent dark coverage (25, 50, and 75%), and observation time (06:00–18:00 hours) by full factorial designs and each pairs between or among factors were compared. Log transformation was applied to all data and tested for normality. Nonparametric Mann–Whitney and Kruskal–Wallis tests were used for analyses with one independent variable (no of resting mosquito) and four dependent variables (fabric, color, percent coverage, and configuration). Pearson correlation was used to examine the relationship and estimated differences between number of resting mosquitoes and the independent environmental variables of ambient temperature and relative humidity by hour of observation. Correlation coefficients (r) between number of mosquitoes resting on either cotton or polyester and hourly mean indoor temperature, hourly mean outdoor temperature, and hourly mean indoor humidity were based on the H_0: $r = 0$; H_1: $r \neq 0$, $r > 0$, $r < 0$. The discriminating level for all significance tests was set at 0.05%. All analyses were performed using the SPSS program (SPSS Inc., version 15.0, Chicago, IL).

Results

General Resting Patterns. Total mean *Ae. aegypti* resting observations from all trials is summarized in Table 1. In general, a significantly higher mean number of *Ae. aegypti* rested on cotton material (12.8 ± 0.20) as compared with polyester (3.3 ± 0.2) ($Z = -12.14$; $P < 0.05$). Most *Ae. aegypti* preferred to rest on dark material (12.9 ± 0.2) rather than light (3.2 ± 0.2), regardless of fabric type ($Z = -0.61$; $P < 0.05$). Overall, resting patterns observed between vertical (8.5 ± 0.2) and horizontal (7.7 ± 0.2) configurations

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Table 2. *Aedes aegypti* resting observations (06:00–18:00 hours; $n = 48$ trials) on dark material and other locations inside experimental huts under different fabric configuration and L:D coverage ratio schemes

Treatment scheme			Mean no. (±SE) of resting *Aedes aegypti*		
Fabric	Coverage	Coverage (%)	Upper portion	Lower portion	Ceiling
Cotton	Vertical	25	5.1 (1.1)	6.8 (0.7)	0.09 (0.60)
		50	10.0 (0.6)	15.4 (1.0)	0.75 (0.52)
		75	8.6 (0.5)	18.7 (0.9)	0.13 (0.12)
	Horizontal	25	16.9 (1.1)	5.1 (0.4)	3.25 (1.60)
		50	12.6 (0.8)	9.4 (0.5)	0.63 (0.26)
		75	7.9 (0.6)	12.3 (0.6)	0.25 (0.16)
Polyester	Vertical	25	3.8 (0.4)	1.5 (0.3)	2.38 (0.37)
		50	5.3 (0.7)	1.6 (0.2)	1.38 (0.53)
		75	3.9 (0.6)	2.2 (0.4)	4.75 (1.08)
	Horizontal	25	2.8 (0.5)	0.7 (0.2)	8.75 (2.39)
		50	2.4 (0.4)	0.2 (0.1)	3.00 (1.5)
		75	2.7 (0.3)	0.5 (0.1)	6.25 (0.75)

were similar ($Z = -18.76$; $P = 0.54$). Significant differences existed in median numbers of *Ae. aegypti* observed resting among the three test coverage ratios ($\chi^2 = 64.34$; df = 2; $P < 0.05$) with greatest resting elicited using 75% (10.3 ± 0.2) dark material and lowest using 25% (6.1 ± 0.2) (Table 1).

Influence of Configuration and Upper versus Lower Preferences. *Cotton.* Significantly greater mean resting observations were made during cotton trials from the lower portion of wall panels (6.8–18.7) as compared with upper wall portions (5.2–10.0) when fabric was configured in vertical configuration (Table 2). This was true for all test coverage ratios evaluated. In contrast, horizontal configuration trials elicited greater mean number resting on upper portions of wall panels ranging from 7.9 to 16.9 as compared with lower portions 5.1–12.3 (Table 2).

Polyester. On the whole, significantly greater resting observations were made on the upper portion of wall panels when using polyester material in vertical configuration (3.8–5.3), regardless of percent coverage, as compared with observations made from the lower wall portions (1.5–2.2; $P < 0.05$; Table 2). Likewise, horizontal configuration of polyester resulted in significantly greater mean resting counts on upper wall panels (2.4–2.8) versus the lower portions (0.2–0.7) at all test coverages (Table 2).

Cotton versus Polyester Texture. Simultaneous testing of black cotton and green polyester (50% L:D) (i.e., to control for dark color variation and environmental condition bias) using two experimental huts during the same trial indicated the mean number of *Ae. aegypti* females observed resting on black cotton (22.8 ± 0.8) was approximately twice that of green polyester (12.3 ± 0.8) ($F = 85.8$; df = 1; $P < 0.05$) and the mean number of *Ae. aegypti* females observed resting on white polyester (4.0 ± 0.3) was approximately quadruple that of white cotton (1.1 ± 0.1) ($F = 95.3$; df = 1; $P < 0.05$).

Influence of Time. A greater number of mosquitoes rested on wall surfaces during the early morning hour observation period (06:00–09:00 hours) 35.1% and 52% compared with late morning (09:00–12:00 hours) 25.9% and 26.0%, early afternoon (12:00–15:00 hours) 21.0% and 11.4% and late afternoon (15:00–18:00 hours) 18.0% and 10.7% observation times for cotton and polyester trials, respectively (Fig. 2). No significant differences in resting observations were found among sampling hours in cotton trials ($F = 1.48$; df = 12; $P = 0.13$), but significant differences were indicated for polyester trials ($F = 16.38$; df = 12; $P < 0.05$).

Influence of Environmental Parameters. Mean temperature (indoor and outdoor), and indoor humidity during cotton trials were 22.3°C, 23.4°C, and 65.9% RH, respectively, and during polyester trials were 27.8°C, 29.4°C, and 65.6% RH, respectively. Overall, no significant correlations were found between mean hourly *Ae. aegypti* resting observations on cotton fabric and indoor humidity or mean difference between outdoor and indoor temperature except at time point 17 (i.e., 17:00 hours), $r = 0.1$; $P = 0.013$ (Fig. 2). For polyester fabric, significant correlations were found between resting and temperature at four observation time points (9, 10, 13, and 14) ($r = 0.2$; $P = 0.019$, $r = 0.2$; $P = 0.017$, $r = -0.2$; $P < 0.01$ and $r = 0.2$; $P < 0.01$, respectively) (Fig. 2). In addition, in more than half of all the observation time points for polyester trials (7/13), a significant positive correlation was noted between hourly mean indoor humidity and mean number of resting *Ae. aegypti.* However, trials using polyester fabric exhibited more negative correlations between indoor humidity and resting observations than results using cotton fabric. These negative associations with polyester generally occurred in the afternoon hours (time points 11–18) (Fig. 2).

Discussion

The significance of *Ae. aegypti* as an important vector of dengue and yellow fever has led many researchers to search for novel and effective techniques to minimize the risk of virus transmission (Service 1993, Kittayapong et al. 1997). As *Ae. aegypti* is highly anthropophilic and anthropophagic, with reports documenting ≈95% of *Ae. aegypti* mosquitoes preferentially resting on surfaces inside homes (Pant and Yasuno 1970, Thavara et al. 2001), exploiting the rest-

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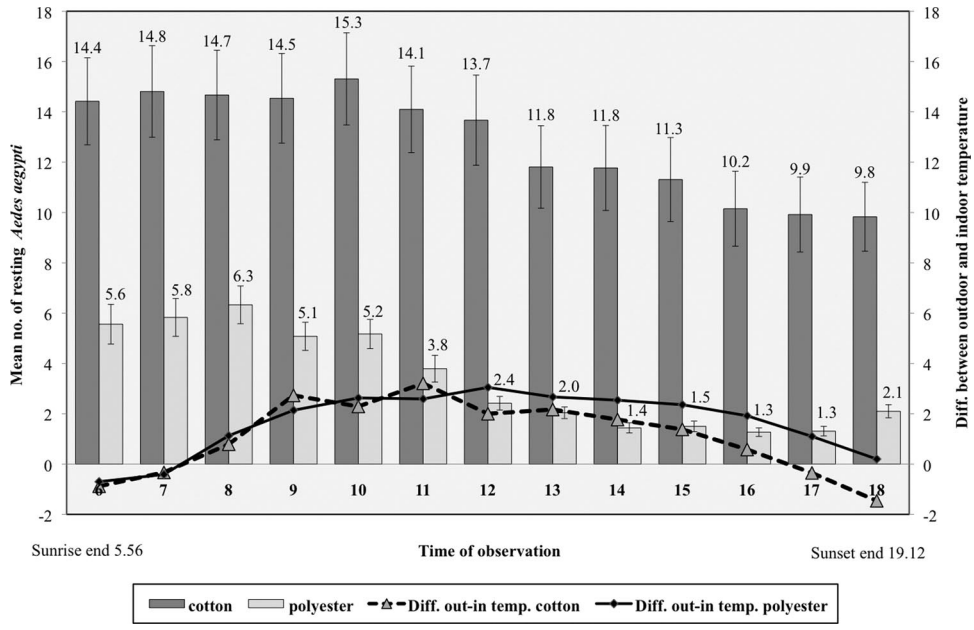


Fig. 2. Relationship between hourly mean resting counts of female *Ae. aegypti* ($n = 48$ replicates) on different fabric types (cotton and polyester) inside experimental huts and the difference between hourly mean outdoor and indoor temperature from all trials July 2008 through May 2009.

ing behaviors of these mosquitoes is one approach that could potentially have a dramatic impact on disease.

Previous work has demonstrated that the use of color and contrasts can be used to develop effective *Ae. aegypti* adult traps such as the Fay–Prince (Fay and Prince 1970) and BG-Sentinel (Maciel de Feitas et al. 2006) and that *Ae. aegypti* prefers dark, humid locations inside homes (Reiter 1991, Thavara et al. 2001, Scoof 1967, Nelson 1986). Targeting indoor resting sites with chemical irritant-treated materials through a mechanism that exploits a color-concept contrast could therefore hold the potential for disrupting dengue virus transmission (Lenhart et al. 2008). Methods using ITMs appear to potentially control diurnally-active *Ae. aegypti* mosquitoes (McCall and Kittayapong 2006) but limited research has been conducted to quantify those variables that influence the resting behavior of adult *Ae. aegypti* inside homes on these materials (Schoof 1967). Such studies are vital in further development of strategies that require mosquitoes to contact chemical-treated surfaces for success.

In this study, we observed the resting behavior of host-seeking (i.e., nonblood fed and sugar-starved) female *Ae. aegypti* using a controlled experimental hut design against two fabrics under consideration for insecticide treatment. The objective was to determine optimum treatment scheme to use during push-pull trials as part of the larger research program. The goal of the overall push-pull strategy is to either prevent vectors from entering the home to begin with (i.e., repellency) or to remove them from the interior space if already indoors (i.e., irritancy). The key to preventing vector–human contact indoors is to deter the vector from finding a suitable refuge for resting before feeding is initiated. This irritation may cause disruption in the host-seeking response (i.e., escape from indoors) and thereby reduce probability of biting.

Not surprisingly, results indicate that all test variables evaluated influenced the resting patterns of *Ae. aegypti* test populations. As expected, dark surfaces of both test fabrics (black cotton and green polyester) elicited greater resting counts compared with white counterpart surfaces. This behavior pattern was exhibited for both vertical and horizontal configurations even at the 25% L:D coverage ratio for each fabric type, indicating the intensity of this behavioral response. However, the number of *Ae. aegypti* resting on dark cotton (black) was significantly greater than on dark polyester (green).

The reason for variations in resting response between the test fabrics was not unequivocally explained using the current experimental design but could be associated with the differences in fabric color and/or characteristics of the textile fabric itself. It should be noted that preliminary tests were conducted in the laboratory to determine potential pretreatment effects from the manufacturer. Results from standard WHO tube tests indicated no significant difference in mortality rates in mosquito cohorts between control (no fabric) and treatment (cotton or polyester fabric) conditions (data not shown). These two fabrics are rather different in fiber properties, especially moisture absorption and density (specific weight) with cotton exhibiting greater moisture absorption than that of polyester (Ching–Luan et al. 2007). For example, the moisture regain (a percentage of moisture present in textile material brought in to equilibrium with a standard atmosphere after partial drying, as a percentage

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of the moisture-free weight; Das et al. 2009), of cotton is ≈7.1–8.5% while polyester fiber regain ranges from 0–0.4% (Brandrup and Immergut 1989). This difference may positively influence resting behavior of female *Ae. aegypti* as they are commonly found in humid environments inside homes (Perich et al. 2000).

In the current study, when black cotton and green polyester were evaluated simultaneously using the same treatment scheme (50% L:D in vertical configuration) greater overall resting occurred in the cotton treated hut despite matched environmental conditions (i.e., trials conducted on the same days). Laboratory studies using *Ae. aegypti* populations originating from Kanchanaburi, Thailand, have also shown similar results when green cotton and green polyester fabrics have been used in choice tests (Manda et al. 2011). Combined, these results indicate that color is not the only variable driving resting behavior. Indeed, there are many other factors of fabrics that will influence tactile response and thereby resting to include whether the fabric is woven or knit, thread count, thread size (relating to thickness of cloth), and optical characteristics (cotton fibers show double refraction when observed in polarized light, while polyester shares similarities of many thermoplastics, providing bright, shiny effects) (Mototada 1993). Exploration of these variables were outside the scope of the current study but could be considered in future behavior evaluations as warranted.

Based on observations from upper and lower wall heights, more female *Ae. aegypti* populations were observed resting on lower portions of the wall as compared with upper portions when exposed to cotton fabric whereas against polyester, upper wall portions were preferred. These differences could be attributed to differences in overall indoor temperature and indoor humidity between experimental trials as studies were conducted during different months of the year. However, mean indoor humidity values were similar among all trials (cotton = 65.9% RH; polyester = 65.6% RH) and in fact, mean indoor temperature for cotton trials (cotton = 22.3°C) was lower than that recorded during polyester experiments (polyester = 27.8°C) that intuitively, might indicate that greater resting would be expected at lower portions of the wall when using polyester and the opposite when using cotton. In other words, the higher the indoor temperature, the more likely it might be to expect more resting at lower regions within the experimental hut. This assumption is based on previous observations that describe *Ae. aegypti* resting sites indoors (Schoof 1967) and those reported from laboratory studies using both *Culex* and *Anopheles* mosquitoes in which resting on lower portions of a test box that was cooler was preferred to that of the upper portion (Gjullin et al. 1963).

It is possible that in the current study the potential for increased humidity levels along the microenvironment of the intersection between the wall and ceiling versus the wall and floor during polyester trials could have influenced results. Indeed, the relatively high mean resting counts (3.0–8.75) made from the ceiling during polyester trials supports such a theory. Unfortunately, environmental parameters were only measured from a central location inside each hut rather than along the wall surfaces so it is not possible to validate this theory using our datasets. Future experiments should integrate microclimate data collected from various positions within the evaluation area to better understand external behavioral drivers.

In this study, the mean hourly difference between outdoor and indoor temperature and indoor relative humidity were found to have no statistically significant influence on the mean number of *Ae. aegypti* resting for the majority of observation time points. However, for both cotton and polyester fabrics, less resting was observed overall during observation periods late in the day when mean temperature was highest and humidity lowest. It has been previously documented that environmental parameters such as temperature and humidity can influence movement patterns of *Ae. aegypti* mosquitoes (Schoof 1967, Clements 1999, Suwannachote et al. 2009). This information has operational significance to guide implementation of *Ae. aegypti* interventions targeting resting behavior. Effectiveness of such treatments may be greater during the early morning hours when resting is highest compared with late in the day when resting is lowest. This would require the surface area with chemical treatment to be assessable to vectors during peak resting time periods and may define patterns of use. We were unable to record light intensity levels within the huts during experiments but these measures should be integrated into future studies to determine correlations between time and ambient light with *Ae. aegypti* resting.

Perhaps most pertinent to a push-pull strategy for *Ae. aegypti* control, a high number of resting observations were recorded against 25 and 50% dark coverages. As the strategy is focused on using minimal treatment coverage at targeted resting sites for a more cost-effective approach these findings are encouraging to support the ability to exploit mosquito behavior at reduced chemical levels inside homes. We recognize the conditions under which these studies were performed (experimental huts) may not match a real-world setting inside a local home in which competitive resting sites would be available (i.e., table, chair, bed frame, and so forth) thereby potentially resulting in artificially high resting on wall surfaces. However, resting was also observed on the ceiling and floor (albeit at very low levels; data not shown) indicating that other resting sites were available and being used by test mosquitoes. In addition, there were sites such as upper eaves, within window and door frames, and so forth, that were more difficult to observe and therefore make measures. The study design selected was based on the experimental condition for which the evaluation for contact irritancy will be conducted for push-pull trials.

Lastly, observing resting behavior during the diurnal phase of activity when *Ae. aegypti* are presumably most active and more disrupted by the human observers could have attenuated results. The sampling

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scheme selected (06:00–18:00 hours) represents a range of time for which multiple mosquito behaviors occur to include resting, flying, and feeding. Despite humans being inside experimental huts in the current study, resting behavior was still observed. Most relevant to objectives of the research, we wanted to observe resting responses during a matched time period for which push-pull trials are planned to occur (i.e., 06:00–18:80 hours). This represents diurnal periods when human activity might also be highest in and around local home environments and therefore highest probability of human–vector contact. A key component of a contact irritant approach is to force mosquitoes that have entered a treated space to escape before biting. This chemical effect may occur at any stage of the host-seeking process encompassing both diurnal and nocturnal time periods. The inclusion of evaluating the nocturnal resting behavior of *Ae. aegypti* could be integrated in future studies to enhance ecological datasets and determine changes in resting patterns.

Despite study weaknesses, these experiments have characterized *Ae. aegypti* resting patterns in response to untreated surfaces of two different fabrics (cotton and polyester), L:D surface area coverage ratios (25, 50, and 75%) and placement configurations (vertical and horizontal). Ongoing studies will evaluate if these resting patterns are modified when dark fabric surfaces are treated with irritant chemicals. It will be vital to determine if a shift in resting occurs to untreated 'safe-site' surfaces (i.e., white fabric) or if flying and escape behaviors are increased. The outcome of each of these scenarios will have different results in regards to reduction in virus transmission; the former a potential negative impact and the latter a positive impact. To date, our laboratory studies indicate that significant escape responses from a treated space occur after exposure to contact irritant-treated material at 25% coverage levels such that untreated sites are not significantly attenuating exit behavior (Manda et al. 2011).

In conclusion, *Ae. aegypti* resting behavior patterns remain poorly underexplored despite the role of vector ecology in developing novel disease control strategies. Knowledge of mosquito behavior is of prime epidemiological importance to understanding how vector control tools function and therefore making evidence-based decisions for disease management. Results from the current study will guide the fabric-treatment scheme in push–pull experimental trials to elicit desired escape effects of a contact irritant element.

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Contact Irritant Responses of *Aedes aegypti* Using Sublethal Concentration and Focal Application of Pyrethroid Chemicals

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Abstract

Background: Previous studies have demonstrated contact irritant and spatial repellent behaviors in *Aedes aegypti* following exposure to sublethal concentrations of chemicals. These sublethal actions are currently being evaluated in the development of a push-pull strategy for *Ae. aegypti* control. This study reports on mosquito escape responses after exposure to candidate chemicals for a contact irritant focused push-pull strategy using varying concentrations and focal application.

Methods: Contact irritancy (escape) behavior, knockdown and 24 hour mortality rates were quantified in populations of female *Ae. aegypti* under laboratory conditions and validated in the field (Thailand and Peru) using experimental huts. Evaluations were conducted using varying concentrations and treatment surface area coverage (SAC) of three pyrethroid insecticides: alphacypermethrin, lambacyhalothrin and deltamethrin.

Results: Under laboratory conditions, exposure of *Ae. aegypti* to alphacypermethrin using the standard field application rate (FAR) resulted in escape responses at 25% and 50% SAC that were comparable with escape responses at 100% SAC. Significant escape responses were also observed at <100% SAC using ½FAR of all test compounds. In most trials, KD and 24 hour mortality rates were higher in mosquitoes that did not escape than in those that escaped. In Thailand, field validation studies indicated an early time of exit (by four hours) and 40% increase in escape using ½FAR of alphacypermethrin at 75% SAC compared to a matched chemical-free control. In Peru, however, the maximum increase in *Ae. aegypti* escape from alphacypermethrin-treated huts was 11%.

Conclusions/Significance: Results presented here suggest a potential role for sublethal and focal application of contact irritant chemicals in an *Ae. aegypti* push-pull strategy to reduce human–vector contact inside treated homes. However, the impact of an increase in escape response on dengue virus transmission is currently unknown and will depend on rate of biting on human hosts prior to house exiting.

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Introduction

Dengue, transmitted primarily by *Aedes aegypti* mosquitoes, is the most important mosquito-borne viral disease affecting humans worldwide [1]. It is caused by four serotypes that produce a spectrum of clinical illness ranging from unapparent or mild disease, to an influenza-like illness, to a fatal shock syndrome.

Due to the current lack of a licensed vaccine, dengue prevention is limited to vector control. *Aedes aegypti* control programs are based on two main targets: (1) the immature stages (egg, larvae, and pupae) through environmental management (source reduction), larvicides and/or biological control; and (2) the adult stage using space or residual sprays of chemical insecticides and more recently insecticide treated materials [2]. *Aedes aegypti* has strong associations with human habitations, living and breeding very near or inside human dwellings [3–5]. This extensive use of the human indoor environment poses challenges to traditional adult control methods and as well as in devising new or improved methods to sufficiently reduce disease transmission risk [6]. Pyrethroids have commonly been employed in dengue endemic countries such as Thailand and Peru for peridomestic and/or indoor residual/space-spraying to reduce adult mosquito populations [7,8]. However, despite the fact that selective use of pyrethroids and other residual insecticides applied indoors have successfully controlled *Ae. aegypti* and dengue [9–13], outdoor and peridomestic space-spraying alone has often failed to achieve any meaningful

Author Summary

Dengue virus is spread by *Aedes aegypti*, a mosquito that prefers to feed on humans. Chemicals used at toxic levels is currently the only confirmed effective strategy for dengue vector control, but insecticide resistance issues are threatening this approach. Other chemical actions that break vector-human contact exist: contact irritant effects, causing escape from homes and spatial repellent effects, preventing house entry. These actions are being evaluated in the development of a push-pull strategy to target indoor resting sites (using contact irritants) and/or portals of house entry (using spatial repellents) at sublethal concentrations to push the vector away from the treated structure and associated human hosts. When combined with an attractant trap placed outdoors to pull the irritated/repelled vectors from the peridomestic environment, the integrated stimuli form a push-pull system that can be used to further enhance disruption of human-vector contact. Here we report on the quantification of contact irritancy (escape) behavior, knockdown and 24 hour mortality rates in populations of *Ae. aegypti* under both laboratory and field conditions. Evaluations were carried out against three pyrethroid insecticides. Findings indicate an increase in escape response using sublethal concentrations and focal application suggesting a potential role for contact irritants in a push-pull strategy.

control of indoor adult *Ae. aegypti* populations. This is because the chemical fails to reach the preferred resting sites inside homes [14,15] and indoor residual or space-spraying can be resource limited and hampered by poor public perception that limits indoor access [16] therefore reducing treatment coverage. However, the high affinity of *Ae. aegypti* for the human indoor environment also provides unique opportunities for innovative approaches to control the adult vector [16].

During dengue outbreaks, the methods of choice for emergency interventions remain outdoor ultra-low-volume (ULV) application of insecticides and/or indoor thermal fogging [17,18]. While effective, these interventions usually follow onset of epidemics, are difficult to implement in urban environments where *Ae. aegypti* is most common, and more importantly very difficult to sustain [16]. For these and other reasons such as insecticide resistance [7,8], there is a need for proactive measures that have potential to prevent virus transmission and avert dengue epidemics as well as reduce selection pressure for insecticide resistance.

Research has shown that the impact of public health insecticides on vector populations is much more complex than simple toxic actions (i.e., direct mortality following exposure). There are other chemical actions that exist to break human-vector contact [19–28]. Such actions include among others, contact irritant effects, causing an escape response from homes, and initiating a spatial repellent or deterrent effect, thereby preventing house entry [26,28,29]. Both contact irritant and spatial repellent behaviors have been demonstrated in vector populations using sublethal chemical concentrations under both laboratory and field environments [26,28]. The importance of these findings is accentuated as both organochlorine resistant and pyrethroid tolerant *Ae. aegypti* were included in the vector test populations, indicating that behavior-modifying actions exist even when mosquitoes are not susceptible to their toxic actions and as such, may play a role in insecticide resistance management. Current issues of insecticide resistance are creating a necessity for development of new strategies [30] and/or chemical products that act to prevent vector biting. Such approaches will require an understanding of the range of biological actions (independent from toxicity) elicited by vectors exposed to chemical tools to help drive such methods for disease control.

Both contact irritancy and spatial repellency are currently being evaluated in the development of a push-pull strategy for *Ae. aegypti* control. The goal of the push-pull strategy is to reduce the probability of human-vector contact and therefore dengue virus transmission. The approach is to target preferred indoor resting sites (using contact irritants) and house portals of entry (using spatial repellents) using minimum effective chemical concentration and treatment surface area coverage (SAC) to make these sites unsuitable, and thereby drive (push) the vector away from the treated structure and human hosts in a cost-effective manner. To further enhance the effect of a push response, an attractant trap is placed outdoors to pull the irritated/repelled vectors from the peridomestic environment thereby disrupting human-vector contact in the peridomestic environment. However, a component to system success based on a contact irritant push element is the quantification of a contact irritant response - movement of *Ae. aegypti* test populations away from a chemical source following tarsal contact–when less than 100% of a surface is treated (i.e. focal application). Target application could prove cost-effective; however, untreated sites inside homes may serve as refuge locations for exposed vectors thereby diminishing efficacy of the approach.

Our previous study quantified *Ae. aegypti* resting behavior under laboratory conditions following exposure to chemical-treated surfaces at various treatment coverage area (100%, 75%, 50% and 25%) to define the effects of "safe sites" (untreated surfaces) on irritancy behavior [31]. Results indicated that when preferred *Ae. aegypti* resting sites were treated with irritant chemicals, even at a treatment coverage of 25%, test populations did not simply move to untreated areas but became agitated, using increased flight as a proxy indicator. It is this contact irritant response that may result in an escape behavior and could be exploited as one type of push component in a push-pull strategy.

The objectives of the current study were to quantify contact irritancy (escape) response, knockdown, and 24 hour mortality rates in populations of female *Ae. aegypti*. Evaluations were carried out at varying chemical concentrations and treatment surface area coverage using three standard pyrethroid insecticides: alphacypermethrin, lambdacyhalothrin and deltamethrin. We present findings from the laboratory and two field sites (Thailand and Peru) where validation studies using experimental huts were conducted.

Methods

Mosquitoes

Laboratory. *Aedes aegypti* test populations (F_2-F_5 generations) from Iquitos, Peru (PERU strain) were used for all laboratory experiments. Larvae were reared from eggs shipped to the Uniformed Services University of the Health Sciences (USUHS), Bethesda, USA from the Naval Medical Research Unit No. 6 (NAMRU-6) Iquitos Entomology Laboratory, Iquitos, Peru (INRENA authorization number 128-2007-INRENA-IFFS-DCB, permit number 000703-AG-DGFFS), following previously described protocols [32]. Females (4–7 days old) were provided with sugar pads saturated in a 10% sucrose solution until 24 hours prior to the day of testing. The USUHS colonies were maintained using a membrane blood-feeding system until the F_5 generation at which time the colony was refreshed with F_1 field material to control for behavioral comparability between laboratory and field populations.

Field. *Aedes aegypti* were collected as immatures from experimental hut locations: Pu Teuy Village, Kanchanaburi Province, Thailand (THAI strain); and Iquitos, Peru (PERU strain). Larval populations were reared to adults at respective field insectaries using site-specific established protocols and colonized until the F5 generation. *Aedes aegypti* from Pu Teuy Thailand has been described as pyrethroid tolerant [27,33]; while a recent survey from neighborhood sites in Iquitos, Peru have reported *Ae. aegypti* populations susceptible to pyrethroids (Vasquez La Torre, personal communication). As with laboratory studies, mosquito test populations were maintained with sugar pads saturated in a 10% sucrose solution until 24 hours prior to day of testing. Test cohorts (100 per hut) were marked with a unique color fluorescent dust (BioQuip Products, Inc., Gardena CA) to facilitate visual observation of mosquitoes during experimental hut trials. The use of a unique marking color per hut also allowed to determine the total number of mosquitoes that were present inside each hut during each experimental day, and thus defining proportion escape using a known denominator.

Test compounds

Chemicals evaluated were chosen based on current World Health Organization Pesticide Evaluation Scheme (WHOPES) recommendations and/or historical use in vector control programs [34]. Compounds were acquired as neat grade material purchased from Sigma-Aldrich (St. Louis, MO): alphacypermethrin (CAS 67375-30-8), lambdacyhalothrin (CAS 91465-08-6), and deltamethrin (CAS 52918-63-5). Test concentrations evaluated included WHO recommended field application rates (FAR) and ½FAR; where FAR = 7.2 nmol/cm^2 (0.03 g/m^2) for alphacypermethrin and lambdacyhalothrin, and 4.9 nmol/cm^2 (0.025 g/m^2) for deltamethrin.

Laboratory evaluations

Assay device. The previously described box assay was used for all laboratory evaluations [31]. Briefly, the assay is composed of metal and Plexiglas chambers (30 cm^3) that are joined together using draw latches. The metal test chamber is fitted with textile panels (either chemical-free or treated) and has a funneled exit portal through which mosquitoes can escape into the Plexiglas receiving chamber. The metal test chamber is covered with a Plexiglas lid to facilitate viewing of mosquito behavior during testing, as well as, a removable tinted lid that can be added to maintain darkness throughout the test procedure as desired. A full description of the assay along with accompanying standard operating procedures can be found at www.usuhs.mil/pmb/gsvc.

Contact irritant assay. Laboratory studies were conducted using both black and white cotton fabric (Natural Charm 43/44″ wide 100% cotton 68×68 D/R-black and white, Bruce Variety, Bethesda, MD, USA) based on results from previous *Ae. aegypti* resting preference evaluations [31]. All fabric pieces were treated approximately 30 min prior to initiating the first assay replicate and allowed to air-dry for at least 15 min prior to inserting it into the test chambers. New fabric panels were prepared daily. Treated and chemical-free cotton were fitted into the metal chamber at 100% dark (D), 100% light (L), and 75%:25%, 50%:50%, and 25%:75% D:L surface area coverage (SAC) ratios [31]. Only dark material strips were treated with chemical solutions except during 100%L evaluations. Fabric panels were attached to assay walls using magnets. Groups of 20 female mosquitoes were introduced into the metal chamber and allowed to rest for 30 sec after which time the funnel gate linking the metal and Plexiglas chambers was opened to allow mosquito escape during a 10 min sampling period. The following data was then collected: 1) the number of *Ae. aegypti* in the Plexiglas chamber (i.e., escaping); 2) the number of mosquitoes within the metal chamber (mosquitoes that did not escape) and; 3) knockdown (KD) (defined as mosquitoes lying on their side or back that are unable to right themselves) in both clear and metal chambers. Mosquitoes were immediately removed from the assay using vacuum power hand aspirators and placed into containers labeled according to the respective chamber and maintained at 28°C and 80% RH to monitor 24 hour mortality. A total of 6 replicates were performed for each chemical concentration and D:L coverage. A matched control assay fitted with acetone-only treated fabric at the same D:L coverage was conducted simultaneously for each replicate. All testing was performed under controlled temperature (28–30°C) and relative humidity (50–60%) conditions. Box assays were cleaned with a 10% bleach solution at the end of each testing day to control for residual chemical and allowed to air-dry overnight before reuse.

Field evaluations

Experimental huts. Trials were conducted in both Thailand and Peru using experimental huts fitted with window and door interception traps. Three (Thailand) and five (Peru) portable huts were constructed using dimensions and materials that mimic indigenous homes at each site (Figure 1). Details of the experimental hut and intercept trap design in Thailand have been previously described [35]. In Peru, the huts measured 4 m wide×6 m long×6 m height. Each hut had two windows (96 cm wide×96 cm height) and two doors (1 m wide×2 m height); one window and one door each at the front and the back, which were fitted with interception traps. At both field sites, the huts were positioned on raised platforms 30 cm above ground. The support columns for the platform sit atop cement ant traps to prevent predation on indoor KD mosquitoes. Floors were covered with white plastic sheeting to facilitate the detection of KD responses. Huts at both locations contained metal frame panels with wire-mesh backing positioned along the interior walls. Treated fabric panels were fixed to these metal panels using magnets, thereby avoiding chemical contact with the wall surface and potential contamination during subsequent chemical testing or treatment concentration. Environmental parameters of indoor/outdoor temperature and relative humidity were recorded using HOBO data loggers (series H08-004-02 and H21-001, respectively). HOBO U30/NRC Data Logging Weather Stations (Onset Computer Corporation Cape Cod, MA, USA) were used at both sites and were positioned centrally to the experimental huts.

Escape trials. Black and white cotton (Consorcio la Parcela Products, Ate, Lima, Peru; size 39.4×0.8/inch); and green and white polyester (BioQuip Products, Rancho Dominguez, CA, USA; mesh size 24×20/inch) fabric panels were used in Peru and Thailand, respectively. Experimental design for measuring mosquito escape behavior followed previously established protocols [26]. Briefly, fabric panel strips were measured to cover a specific surface area coverage (SAC) of interior hut walls. Chemical solutions were prepared using either acetone (in Thailand) or absolute ethanol (in Peru) to test concentration and applied to fabric panels using a pipette. Additional fabric panels were treated with solvent only, to serve as matched chemical-free controls. Panels were treated no more than 48 hours prior to initiating the first replicate of a trial. Following treatment, the fabric was wrapped in aluminum foil and placed under cold storage (4°C) for transport to the field. The day before testing, chemical-treated and chemical-free fabric panels were affixed onto the metal frames inside each hut at 100% D, 75%:25%, 50%:50%, and 25%:75% D:L ratios where only dark fabric was chemical-treated. A



Figure 1. Experimental huts fitted with window and door interception traps. (A) Thailand, (B) Peru.
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matched control hut with similar SAC was performed simultaneously for each D:L trial using solvent-only treated fabric.

Interception traps were affixed on the outside of doors and windows to capture exiting mosquitoes. Three to seven day-old marked female *Ae. aegypti* (100 per hut) were released inside each hut at 05.30 Hrs each experimental day. An additional container holding 25 marked mosquitoes was positioned in the center of each hut to serve as an internal control for monitoring KD due to indoor environmental conditions. During testing, one person was positioned indoors underneath an untreated bed net to generate host cues and monitor hourly KD. From 06.00 Hrs–18.00 Hrs at 20 min (Thailand) and 30 min (Peru) intervals, collector pairs located outside each hut removed mosquitoes within interception traps. Captured mosquitoes were placed into individual holding cups labeled by hut, trap number and time. At the top of each hour, all KD mosquitoes on the floor were collected and placed into holding cups labeled by hut and time. Both escape and KD mosquito samples were provided with cotton pads soaked in 10% sugar solution and held overnight in field insectaries to monitor 24 hour mortality. Four (Thailand) or five (Peru) replicates (i.e. experimental days) were performed for each experimental trial. Outdoor collector pairs rotated between huts every sampling period and indoor hosts at mid-day during an experimental day to control for individual bias. At the end of each experimental day, all mosquitoes that remained inside the huts were collected using backpack (Thailand) and Prokopack (Peru) [36] aspiration. The following treatments were tested in Thailand: alphacypermethrin at FAR and ½FAR at 75% D:L SAC; and in Peru: alphacypermethrin at FAR and ½FAR at 100%, 75%, 50%, and 25% D:L SAC.

Data analysis

Laboratory evaluations. All analyses were performed using SAS v. 9.2 software [37]. Proportional data were subjected to arcsine square root transformation before statistical analysis. Output tables represent back-transformed values. Mean percentage escaping (or corrected percentage escaping) was obtained from the number escaping in the treatment over the number released after correcting for the number escaping in the control and KD in the metal test chambers). Knockdown and 24 hour mortality rates of populations that escaped and those that did not escape were corrected based on measurements from populations in control assays using Abbott's formula [38]. For each chemical dose and SAC ratio trial, the difference between the number escaping from treated and control test chambers; and the difference between KD or dead in mosquitoes that escaped and those that did not escape were analyzed using the Wilcoxon 2-sample test as previously described [26,28,39]. Variations in the percentage of mosquitoes escaping, KD and dead after 24 hours per trial were analyzed for each chemical by 2-way analysis of variance (ANOVA) with 2 main factors (treatment concentration and surface area coverage). For those chemicals that showed significant variations, an additional one-way ANOVA was conducted to examine the main factors (treatment concentration or SAC ratio), independently, on percentage escaping, KD and mortality. Means were separated using t-test and Sheffe's test ($\alpha = 0.05$).

Field evaluations. The mean daily percentages of mosquitoes that escaped, indoor KD and 24 hour mortality rates from those collected inside the huts (mosquitoes that did not escape) and those that escaped were compared between treatment and control huts using one-way ANOVA with means separated using Student Newman Keuls (SNK) tests ($\alpha = 0.05$). The percentage of mosquitoes that escaped was compared between huts at three time periods: 06.00–10.00 Hrs, 11.00–14.00 Hrs and 15.00–18.00 Hrs corresponding to expected morning, mid-day and afternoon *Ae. aegypti* resting behavior patterns (Castro et al. unpublished data). Percentage escape was calculated using the number of mosquitoes that escaped over the number of mosquitoes that were available to escape (total number of mosquitoes recaptured from the hut each day minus number knockdown within the hut).

Results

Laboratory evaluations

Escape responses. The mean number of female *Ae. aegypti* escaping in the treatment and matched control assays, with corrected percentage escaping for all trials is presented in Tables 1–3. In general, escape was higher in treatment assays as compared to matched controls. Most importantly, significant escape response was observed at ½FAR and/or at SAC less than 100% for each pyrethroid evaluated (Tables 1–3). Against alphacypermethrin treatment, corrected percent escape ranged from 8–49% with highest responses observed at FAR for all SAC evaluated (Table 1). Percent escape was comparable among all treatment coverages at the ½FAR dose (Table 1). With lambdacyhalothrin and deltamethrin treatments, corrected percent escape ranged from 1–59% (Table 2) and from −1–40% (Table 3), respectively. At

Table 1. Escape responses of *Aedes aegypti*[1] to different concentrations and coverage of alphacypermethrin under laboratory conditions.

				No. escaping (mean[2] ± SE)				
Alphacypermethrin concentration (nmoles/cm^2)	**SAC[3] (%)**	**No. of mosquitoes Treatment**	**No. of mosquitoes Control**	**Treated**	**Control**	**% escaping[4] (mean ± SE)**	***P*[5]**	***P*[6]**
½FAR[7] (3.6)	25	119	119	0.0±0.0	0.2±0.2	8±4[a]	0.06	<0.01
	50	118	119	0.2±0.2	0.5±0.3	8±4[a]	0.02	<0.01
	75	118	118	0.2±0.2	0.0±0.0	8±5[a]	0.06	0.30
	100D	118	116	0.2±0.2	0.0±0.0	20±4[a]	<0.01	<0.01
	100L	117	121	1.8±0.6	0.5±0.5	24±6[a]	<0.01	0.36
	P[8]			-	-	0.05		
FAR (7.2)	25	123	120	0.3±0.2	0.0±0.0	35±6[ab]	<0.01	
	50	118	120	0.5±0.2	0.3±0.2	35±5[ab]	<0.01	
	75	118	125	0.7±0.3	0.2±0.2	14±4[b]	<0.01	
	100D	119	118	0.8±0.7	0.2±0.2	49±6[a]	<0.01	
	100L	108	116	6.5±0.6	5.2±1.0	36±11[ab]	0.01	
	P			-	-	0.01		

[1]Four to seven day-old females, non-blood-fed, 24 hour sugar starved (PERU).
[2]For each trial (n = 6 replicates).
[3]Surface area coverage (SAC) of treated material.
[4]For each trial percentage escaping after correction based on escape in the control and knockdown in the metal test chambers using Abbott's formula. Means in the same column followed by the same letter were not significantly different. Multiple comparisons of means were done using Scheffe's test ($\alpha = 0.05$).
[5]P values are from Wilcoxon 2-sample test for difference between the number escaping in a chemical treatment chamber and an acetone treatment (control) chamber.
[6]P values are from t-test examining the effect of treatment concentrations on corrected percentage escaping at each treatment coverage.
[7]WHO recommended field application rate (FAR) = 7.2 nm/cm^2 or 0.03 g/m^2.
[8]P values are from one-way ANOVA examining the effect of treatment coverage on corrected percentage escaping at each treatment concentration.
doi:10.1371/journal.pntd.0002074.t001

Table 2. Escape responses of *Aedes aegypti*[1] to different concentrations and coverage of lambdacyhalothrin under laboratory conditions.

				No. escaping (mean[2] ± SE)				
Lambdacyhalothrin concentration (nmoles/cm^2)	**SAC[3] (%)**	**No. of mosquitoes) Treatment**	**No. of mosquitoes) Control**	**Treated**	**Control**	**% escaping[4] (mean ± SE)**	***P*[5]**	***P*[6]**
½FAR[7] (3.6)	25	119	122	2.7±0.6	0.8±0.5	12±3[a]	0.03	0.99
	50	120	122	4.0±0.7	2.3±0.7	9±9[a]	0.16	0.23
	75	116	114	6.2±1.0	3.3±0.4	15±11[a]	0.08	0.50
	100D	115	114	0.5±0.3	0.0±0.0	1±4[a]	0.45	0.22
	100L	118	122	6.2±1.3	0.2±0.2	38±5[a]	<0.01	0.03
	P[8]			-	-	0.06		
FAR (7.2)	25	122	122	3.0±0.8	1.0±0.5	13±5[b]	0.08	
	50	116	122	3.2±0.7	1.0±0.4	21±2[b]	0.01	
	75	114	117	4.3±1.0	0.8±0.5	23±7[b]	<0.01	
	100D	116	115	3.0±0.9	0.3±0.2	13±8[b]	0.04	
	100L	119	116	11.2±1.8	1.0±0.4	59±9[a]	<0.01	
	P			-	-	<0.01		

[1]Four to seven day-old females, non-blood-fed, 24 hour sugar starved (PERU).
[2]For each trial (n = 6 replicates).
[3]Surface area coverage (SAC) of treated material.
[4]For each trial percentage escaping after correction based on escape in the control and knockdown in the metal test chambers using Abbott's formula. Means in the same column followed by the same letter were not significantly different. Multiple comparisons of means were done using Scheffe's test ($\alpha = 0.05$).
[5]P values are from Wilcoxon 2-sample test for difference between the number escaping in a chemical treatment chamber and an acetone treatment (control) chamber.
[6]P values are from t-test examining the effect of treatment concentrations on corrected percentage escaping at each treatment coverage.
[7]WHO recommended field application rate (FAR) = 7.2 nm/cm^2 or 0.03 g/m^2.
[8]P values are from one-way ANOVA examining the effect of treatment coverage on corrected percentage escaping at each treatment concentration.
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Table 3. Escape responses of *Aedes aegypti*[1] to different concentrations and coverage of deltamethrin under laboratory conditions.

			No. escaping (mean[2] ± SE)				
Deltamethrin concentration (nmoles/cm^2)	**SAC[3] (%)**	**No. of mosquitoes) Treatment**	**No. of mosquitoes) Control**	**Treated**	**Control**	**% escaping[4] (mean[2] ± SE)**	***P*[5]**	***P*[6]**
½FAR[7] (2.47)	25	118	115	0.3±0.2	0.0±0.0	−1±3[b]	0.45	0.30
	50	121	119	1.2±0.5	0.3±0.2	2±3[ab]	0.27	0.89
	75	114	127	4.5±0.7	1.7±0.8	24±9[a]	0.03	0.11
	100D	121	120	0.7±0.3	0.2±0.2	2±5[ab]	0.30	0.16
	100L	115	123	4.0±1.0	0.2±0.2	26±4[a]	<0.01	<0.01
	P[8]			-	-	<0.01		
FAR (4.94)	25	121	119	1.2±0.5	0.0±0.0	6±5[b]	0.06	
	50	118	120	0.8±0.5	0.0±0.0	5±6[b]	0.45	
	75	121	114	2.0±0.5	0.0±0.0	7±6[b]	0.01	
	100D	122	118	2.7±0.5	0.0±0.0	11±5[b]	<0.01	
	100L	119	118	7.8±1.2	0.2±0.2	40±6[a]	<0.01	
	P			-	-	<0.01		

[1]Four to seven day-old females, non-blood-fed, 24 hour sugar starved (PERU).
[2]For each trial (n = 6 replicates).
[3]Surface area coverage (SAC) of treated material.
[4]For each trial percentage escaping after correction based on escape in the control and knockdown in the metal test chambers using Abbott's formula. Means in the same column followed by the same letter were not significantly different. Multiple comparisons of means were done using Scheffe's test ($\alpha = 0.05$).
[5]P values are from Wilcoxon 2-sample test for difference between the number escaping in a chemical treatment chamber and an acetone treatment (control) chamber.
[6]P values are from t-test examining the effect of treatment concentrations on corrected percentage escaping at each treatment coverage.
[7]WHO recommended field application rate (FAR) = 4.9 nm/cm^2 or 0.025 g/m^2.
[8]P values are from one-way ANOVA examining the effect of treatment coverage on corrected percentage escaping at each treatment concentration.
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Table 4. Percentage mortality *Ae. aegypti*[1] that escaped and those that did not escape under laboratory conditions.

Chemicals	SAC[3] (%)	Mean[2] ± SE Percentage mortality					
		Escaped			Did not escape		
		½FAR[4]	FAR	*P*[5]	½FAR	FAR	*P*
Alphacypermethrin	25	NA[7]	0±0[c]	-	49±15[a]	84±7[a]	0.04
	50	0±0[a]	0±0[c]	-	48±14[a]	73±8[a]	0.14
	75	0[a]	0±0[c]	-	45±11[a]	60±10[a]	0.32
	100D	100[a]	100±0[a]	-	47±10[a]	69±10[a]	0.14
	100L	18±13[a]	45±9[b]	0.06	54±7[a]	52±8[a]	0.84
	P[6]	0.07	<0.01		0.98	0.11	
Lambdacyhalothrin	25	0±0[a]	10±10[a]	0.29	0.5±0.3[b]	20±7[a]	<0.01
	50	0±0[a]	5±5[a]	0.34	14±3[a]	28±7[a]	0.12
	75	8±8[a]	17±9[a]	0.37	8±4[ab]	17±4[a]	0.09
	100D	25±25[a]	0±0[a]	0.42	13±2[a]	17±7[a]	0.75
	100L	6±4[a]	10±6[a]	0.55	13±4[a]	25±5[a]	0.07
	P	0.27	0.67		<0.01	0.58	
Deltamethrin	25	0±0[a]	0±0[a]	-	8±4[ab]	5±3[a]	0.49
	50	0±0[a]	0±0[a]	-	1±1[b]	10±4[a]	0.02
	75	0±0[a]	7±7[a]	0.29	5±2[ab]	4±2[a]	0.88
	100D	0±0[a]	0±0[a]	-	7±3[ab]	10±4[a]	0.46
	100L	6±6[a]	2±2[a]	0.66	16±4[a]	7±3[a]	0.09
	P	0.68	0.64		0.02	0.36	

[1]Four to seven day old females, non-blood-fed, 24 hour sugar starved (PERU).
[2]For each trial (n = 6 replicates), percentage 24 h mortality is corrected for control using Abbot's formula. Means in the same column followed by the same letter were not significantly different. Multiple comparisons of means were done using Scheffe's test ($\alpha = 0.05$).
[3]Surface area coverage (SAC) of treated material.
[4]WHO recommended field application rate (FAR) = 7.2 nm/cm^2 or 0.03 g/m^2 for alphacypermethrin and lambdacyhalothrin; and 4.9 nm/cm^2 or 0.025 g/m^2 for deltamethrin.
[5]P values are from t-test examining the effect of treatment concentrations on corrected percentage 24 h mortality at each treatment coverage.
[6]P values are from one-way ANOVA for difference in corrected percentage 24 h mortality between treatment coverage at each treatment concentration.
[7]NA = Not available (no escapee).
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each treatment concentration, escape response was highest using 100%L SAC, while for each treatment coverage (except 100%L), escape response was comparable among doses (Tables 2 and 3).

Knockdown and 24 hour mortality. In most trials, KD and 24 hour mortality rates were higher in *Ae. aegypti* that did not escape than in those that escaped (Table S1 and Table 4, respectively); however, there was minimal KD observed overall in test populations that escaped with rates ranging from 0–25% in all test chemicals. Highest KD was observed using alphacypermethrin at 100%D SAC and FAR (Table S1). No KD was observed in mosquitoes that escaped against lambdacyhalothrin or deltamethrin (Table S1). For mosquitoes that did not escape, percentage KD ranged from 0–48%, 0–15% and 0–1% using alphacypermethrin, lambdacyhalothrin and deltamethrin, respectively, with highest rates occurring in alphacypermethrin trials using FAR (Table S1). Most importantly, KD observed in both mosquito cohorts that escaped and those that did not were comparable between 100% and 25% SAC for each test concentration and chemical evaluated.

In general, 24 hour mortality rates were highest in mosquitoes that did not escape compared to *Ae. aegypti* test populations that escaped the laboratory assay (Table 4). Mortality ranged from 0–100%, 0–25%, and 0–7% in mosquitoes that escaped and from 45–84%, 0.5–28%, and 1–16% in those that did not escape for alphacypermethrin, lambdacyhalothrin and deltamethrin, respectively. For alphacypermethrin, highest mortality was observed at FAR 100%D in mosquitoes that escaped and 25%D in those that did not escape, although the latter was statistically comparable among all SAC using both test concentrations (Table 4). Percentage mortality in mosquitoes that escaped was comparable among all SAC using each test concentration of lambdacyhalothrin and deltamethrin (Table 4). Trials using ½FAR lambdacyhalothrin and deltamethrin indicated percentage mortality in mosquitoes that did not escape varied significantly among SAC, with mortality significantly low at 25% (lamdacyhalothrin) and 50% (deltamethrin) SAC (Table 4).

Field evaluations

Thailand. In comparison to the matched control hut, there was a 42.3% and 46.4% increase in the percentage of marked *Ae. aegypti* females that exited huts containing fabric panels treated at FAR and ½FAR alphacypermethrin, respectively (Table 5); although these results were statistically comparable among treatment and control conditions (P = 0.4). In both treatment huts, the percentage of KD mosquitoes collected indoors were comparable but both significantly higher compared to the control hut (P<0.01). Similar results were obtained from analyses of mosquitoes collected inside the exit traps that were dead 24 hours post-escape (P = 0.02) (Table 5). In both treatment huts, more mosquitoes exited during the early sampling periods of 06.00–14.00 Hrs (10.0%±3.8 and 9.7%±1.2 for FAR and ½FAR, respectively) as compared to the untreated control hut (1.9%±1.2) (P = 0.05), which had highest escape later in the day (15.00–18.00 Hrs) (Table S2). Mean indoor temperature (P = 0.9) and relative humidity (P = 0.6) readings were comparable among all huts during the trials (Table 5).

Peru. The percentage of *Ae. aegypti* that exited from huts treated with alphacypermethrin at FAR (at all SAC) (P = 0.8) and percentage dead 24 hours post-escape (P = 0.3) was comparable to control, with 0–11% increase in escape compared to the untreated hut (Table 6). However, the percentage of KD mosquitoes inside the huts (P<0.01) and subsequent 24 hour mortality in the same KD populations (P<0.01) were significantly higher in all the huts treated with chemical (even 25% SAC) than in the untreated hut indicating a chemical effect (Table 6). Similar results in escape (P = 0.2) and mortality (P = 0.9) rates 24 hours post-escape were observed using alphacypermethrin at ½FAR treatment (Table 6). Unlike the FAR trials, percentage KD inside the huts (P = 0.2) and 24 hour mortality rates (P = 0.4) in these KD populations were comparable between treated and untreated huts at all SAC (Table 6). The percentage of exiting mosquitoes during each time period was comparable among treated and the untreated hut for each chemical concentration evaluated (Table S3). Mean indoor daily temperature was comparable among huts during both FAR and ½FAR trials (P = 0.6 and P = 0.2, respectively), while RH significantly varied among huts during both experimental trials (P<0.01) (Table 6).

Discussion

The objective of the current study was to evaluate the efficacy of contact irritant chemicals to elicit *Ae. aegypti* escape responses from a treated space under both laboratory and field conditions. The approach included treating at concentrations below standards for

Table 5. Percentage escape, indoor KD, escapees mortality of *Ae. aegypti*[1] using alphacypermethrin treated huts in Thailand.

Concentrations (nmoles/cm^2)	SAC[2] (%)	Total Nb. Released	Mean (SE) daily escape[3]	Difference % escape treatment and control	Mean(SE) KD (Indoor)	Mean (SE) 24 hour Mort. (escapees)	Mean Temp. (SE) (indoor)	Mean RH (SE) (indoor)
Untreated	75	370	9.70 (2.3)^a	-	1.80 (1.1)^b	2.50 (2.5)^b	24.3 (0.6)^a	70.4 (2.4)^a
FAR[4] (7.2)	75	265	16.8 (6.5)^a	42.3	29.2 (6.3)^a	51.4 (11.3)^a	24.4 (0.6)^a	70.7 (2.5)^a
½FAR (3.6)	75	358	18.1 (6.6)^a	46.4	33.0 (5.9)^a	29.3 (11.1)^{ab}	24.3 (0.6)^a	67.7 (2.4)^a

[1]Three-seven-day old females, non-blood-fed, 24 hour sugar starved (THAI).
[2]Surface area coverage of treated material.
[3]For each trial (n = 4 replicates), percent escaping after correcting for knockdown inside the same hut. Means with same letter in superscript within the same column are not significantly different based on one-way ANOVA and Student Newman Keuls (SNK) tests.
[4]WHO recommended field application rate (FAR) = 7.2 nm/cm^2 or 0.03 g/m^2.
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insect toxicity and at surface area coverage below 100%. The overall goal was to describe the potential of focal application of irritant compounds to drive sublethal vector behaviour (such as escape) that could disrupt human-vector contact. If evident, the role for these approaches in novel control strategies would be strengthened.

In the laboratory, significant escape responses were observed at treatment concentrations equal to but also below corresponding WHO recommended field application rates for the test compounds based on chemical levels required for vector mortality [34]. Our field studies in Thailand corroborate this finding with a 46% increase in the percentage of *Ae. aegypti* exiting a hut treated with alphacypermethrin at ½FAR and significantly more mosquitoes exiting the treated hut prematurely, by four hours, compared to the control hut. The time of vector escape from inside a treated space is as important as the total density of escape since the faster irritation and escape occurs, the greater the probability that contact (i.e., biting) of the human host has or can been prevented. Combined, these results are similar with previous studies conducted in Thailand that found significant escape response in an *Ae. aegypti* local strain to alphacypermethrin tested at concentrations below the WHO FAR and continue to support the fact that insecticides can be used to elicit irritant actions even if applied at concentrations below toxic levels [26,28,39].

The current study also evaluated the escape behavior of *Ae. aegypti* in response to different treatment surface area coverage (SAC) of a space using dark:light fabric panels, where chemical was applied on dark surfaces only representing focal application. The approach of using contrasting colors and treating black fabric alone was based on exploiting known behavior that *Ae. aegypti* adults prefer to rest in dark, damp locations in households, and are generally attracted to black colors [40–42]. The evaluation of varying SAC was founded on the theory that taking advantage of resting characteristics could result in desired outcomes achieved with less than 100% treatment of indoor resting sites. A previous laboratory study [31] reported on the resting patterns of *Ae. aegypti* using different dark: light SAC ratios of sublethal concentrations of various pyrethroid chemicals. Results from these experiments indicated that *Ae. aegypti* preferred to rest on dark versus light-colored surfaces, even at the lowest 25% dark coverage and that when preferred *Ae. aegypti* resting sites (i.e., dark surfaces) were chemical-treated, mosquitoes did not significantly alter their resting behavior to safe-sites (untreated areas). Instead, they became agitated, and took to flight rather than seeking out alternate resting sites. However, in that study, the test populations were not provided the opportunity to escape from the test arena so measurements of exiting were not captured [31].

We report here that exposure of *Ae. aegypti* females to alphacypermethrin using FAR at 25% and 50% SAC resulted in escape responses that were comparable with those reported at 100% SAC. In addition, escape responses greater than controls were also observed using treatment coverage at <100% at the ½FAR sublethal concentration of all test compounds. It is noted that in most trials, escape responses were highest when using treated 100%L SAC. This agrees with previous data [31] that showed less overall *Ae. aegypti* resting on light versus dark fabric material. Higher escape responses on 100%L SAC may be due to the combination effect of light color (reduction of preferred resting sites) and the irritating chemical. Field studies from Thailand also indicated a substantial premature escape and an average of 40% increase in exiting an alphacypermethrin-treated hut using 75% SAC. Combined, these results demonstrate that, irrespective of treatment concentrations, substantial escape responses can be elicited in *Ae. aegypti* test populations at treatment coverage below

Table 6. Percentage escape, indoor KD, and mortality of *Ae. aegypti*[1] using alphacypermethrin treated huts in Peru.

Concentrations (nmoles/cm^2)	SAC[2] (%)	Total Nb. Released	Mean (SE) daily escape[3]	Difference % escape treatment and control	Mean (SE) KD (Indoor)	Mean (SE) 24 hour Mort. (indoor)	Mean (SE) 24 hour Mort. (Responders)	Mean Temp. (SE) (indoor)	Mean RH (SE) (indoor)
FAR[4] (7.2)	100 D untreated	472	80.6 (8.8)^a	-	1.5 (0.5)^b	1.5 (0.5)^b	5.5 (1.4)^a	24.7 (0.5)^a	81.0 (0.9)^b
	100	414	80.6 (8.5)^a	0	37.1 (7.4)^a	20.6 (4.2)^a	4.2 (1.8)^a	24.1 (0.4)^a	85.3 (0.8)^a
	75	452	87.6 (5.3)^a	8.7	28.7 (12.0)^a	14.6 (3.4)^a	7.8 (1.4)^a	24. 8 (0.5)^a	80.1 (1.0)^b
	50	471	82.5 (15.8)^a	2.4	24.1 (5.5)^a	12.9 (3.0)^a	4.5 (1.3)^a	24.1 (0.4)^a	84.7 (0.7)^a
	25	473	89.8 (9.6)^a	11.4	16.6 (6.1)^a	9.8 (4.5)^{ab}	2.9 (1.4)^a	23.9 (0.4)^a	86.1 0.8)^a
½FAR (3.6)	100 D untreated	456	90.9 (2.8)^a	-	2.7 (0.6)^a	2.7 (0.6)^a	13.2 (4.2)^a	26.5 (0.3)^a	80.2 (1.0)^a
	100	464	81.3 (4.7)^a	−11.8	6.4 (2.0)^a	5.8 (2.0)^a	11.2 (4.1)^a	27.2 (0.4)^a	74.5 (1.2)^b
	75	478	88.9 (2.3)^a	−2.24	3.4 (1.4)^a	3.4 (1.4)^a	11.3 (3.5)^a	26.0 (0.3)^a	80.0 (0.9)^a
	50	487	83.0 (5.6)^a	−9.5	4.6 (2.1)^a	4.6 (2.1)^a	14.4 (5.3)^a	27.2 (0.4)^a	74.5 (1.2)^b
	25	490	91.3 (2.8)^a	0.4	1.2 (0.6)^a	1.2 (0.6)^a	9.2 (4.7)^a	26.0 (0.3)^a	81.5 (1.0)^a

[1]Three-seven-day old females, non-blood-fed, 24 hour sugar starved (PERU).
[2]Surface area coverage of treated material.
[3]For each trial (n = 5 replicates), percent escaping after correcting for knockdown inside the same hut. Means with same letter in superscript within the same column are not significantly different based on one-way ANOVA and Student Newman Keuls (SNK) tests for each treatment concentration.
[4]*WHO recommended field application rate (FAR) = 7.2 nm/cm^2 or 0.03 g/m^2.*
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the traditional 100% SAC used in indoor residual spray campaigns. These findings agree with studies conducted in southern Mexico using selective spraying to target the preferred resting sites of *An. albimanus* which showed that reduced coverage application was as effective as full spraying in controlling adult populations [43]. In addition, preferred resting sites, as determined by landing frequencies, times of resting, preferred surface types and resting heights were not modified by the insecticide applications. In essence, the preferred resting sites of the mosquitoes did not change, even on insecticide-treated surfaces after four successive spray rounds [43].

The use of this approach - sublethal concentration and <100% treatment area coverage - against *Ae. aegypti*, or other vector species, if effective, holds the potential for reducing programmatic costs due to reduced requirements for active ingredient. Arredondo-Jimenez et al (1995) showed that selective spraying of preferred resting sites required 46% less time and cost 67% less than conventional full-spraying. Cost and manpower constraints could be further reduced by integrating the proper active ingredients into consumer-based products (pre-treated material strips, plastic sheeting, tiles, wallpaper, paint, etc.) thereby sharing vector control costs with community members. Initial focus group studies evaluating acceptability of sublethal approaches to vector control have indicated that treated materials are viable options for chemical application inside homes [44]. While all trials in the current study were performed with fresh chemical applications, the pyrethroids evaluated are known to have residual properties [34,45–47], therefore their effectiveness over a long period of time would be anticipated, further supporting a cost-effective approach. In addition, such control strategies may increase sustainability of an intervention through home ownership, and broaden the delivery platforms available for traditional house treatment. This concept recognizes that householders in many developing countries already buy pesticides to control insects in the homes [44,48], and there are advantages in engaging market forces to promote such products, rather than relying solely on public health appeals.

Experimental hut experiments in Peru showed only an 8.7% increase in escape from huts treated with alphacypermethrin at FAR using 75% SAC as compared to an untreated hut and no increase in escape rate when alphacypermethrin was used at ½FAR, irrespective of the SAC. In addition, the time of exit in Peru trials was comparable between treatments and chemical-free control. These results are in contrast to both laboratory and Thailand field trials that showed substantial increases in escape rates for the same compound and treatment coverage in relation to controls. These differences are most likely due to the naturally high exit rate of *Ae. aegypti* observed during baseline studies in Peru when no chemicals were used (Castro et al. unpublished data), making any impact due to chemical difficult to measure, and the variation in fabric material type used between the two field sites (polyester in Thailand versus cotton in Peru). Differences in material type may lead to differences in chemical uptake, absorption and release. Previous studies [49,50] showed less mosquito KD and mortality when locally sourced mosquito nets (treated with K-O Tab 1-2-3 'dip-it-yourself' long-lasting formulation) were made from cotton as opposed to polyester. Although cotton retained a higher concentration of insecticide, the majority of the chemical is bound within the cotton fibers rather than remaining on the surface where the mosquitoes make contact [49]. However, the chemical was shown to be available for uptake and effect based on indoor KD rates observed within alphacypermethrin-treated huts. The difference between our laboratory findings, where minimal escape occurred from control

chambers, and results from our field studies in Peru, where high escape occurred from chemical-free huts, most likely reflects influences from environmental parameters such as temperature and humidity in the micro-environment of the experimental huts. A previous study in Thailand demonstrated how *Ae. aegypti* exit behavior is affected by ambient environmental factors of temperature and humidity [51]. The possibility that behavioral variation between the Thailand and Peru study sites in the current study is the result of the geographically different strains is unknown. These results illustrate that despite considerable progress in the field of vector behavior as a whole, there remains much to understand on how external and genetic factors affect the biology and behavior of important disease vectors.

Both laboratory and field results from the current study showed that KD and mortality were considerably low (<90%) in our *Ae. aegypti* test populations despite using FARs that are based on concentrations required to produce lethal outcomes (i.e. LD90) [52]. *Aedes aegypti* THAI strain from Pu Tuey has been characterized as pyrethroid tolerant [27,33] while the PERU strain from Iquitos, Peru is most likely susceptible to the chemicals evaluated in this study (Vasquez La Torre, personal communication). It was therefore expected that KD and mortality would be low in test populations from Thailand. However, the low mortality rates observed in *Ae. aegypti* from Peru that did not escape in our laboratory tests could be explained by the minimum exposure time compared to standard resistance testing [52,53] (i.e. 10 min in a single contact irritancy replicate versus standard one hour used in toxicity assays); also by the potential difference in chemical uptake, absorption and release between the cotton material used in this study and bottle and/or filter test methods used in many resistance testing [52,53].

More importantly, our *Ae. aegypti* assay cohorts from Thailand continued to exhibit a contact irritant response when exposed to test pyrethroids, despite indication of tolerance, thus indicating that a sublethal approach to vector control may be effective in locations where insecticide resistance occurs. It is worth noting that highest mortality was observed at FAR at 25%D in mosquitoes that did not escape. This finding suggests that although they did not escape, the mosquitoes rested on treated material long enough to receive a lethal dose. Combined, results suggests that escape can occur from a treated area at <100% SAC and of those that do not escape, resting on treated surfaces will continue as supported by a previous study [31] and result in mortality. As expected, we also show that KD and mortality were lower in mosquitoes that escaped as compared to those that did not escape. It is logical that killing those vectors that remain in a treated space (i.e. inside a home) and may have contact with humans will impact pathogen transmission. However, the role of escape and survival for disease management seems counterintuitive. We theorize that an escape and survival response could lead to reduced selection pressure for insecticide resistance which would not only potentially interrupt human-vector contact inside the home through contact irritancy but also extend the effective life of the chemical. Those mosquitoes that are irritated and exit have the opportunity to transfer their genetic material to the next generation, thereby potentially selecting for the mechanistic pathway that result in behavior modification – i.e., irritancy. Of course, this is dependent on linking the mode of action for contact irritancy to one that is dependent on up- or down-regulation of particular cascade drivers. Such information is not yet available. The inclusion of an outdoor trap, such as in a push-pull strategy, could serve to remove irritated vectors from the peridomestic environment thereby controlling potential movement to an untreated location and hosts.

Pertinent to the goal of developing a push-pull strategy, results presented here suggest a role for contact irritancy to drive vectors from a space occupied by a human host, and indicate efficacy using minimal chemical concentration (<LD90) and treatment coverage area (<100%). This in spite of the fact that the chemical delivery platform and formulation we evaluated were not formulated (i.e., optimized for chemical residuality). Although encouraging, current results suggest that behaviors observed in one species or strains might not translate into other mosquito species or strains found in different environmental settings. In addition, the inside environment of an experimental hut is much different than that of a local home; therefore future evaluations of contact irritant efficacy must be performed using indigenous houses against natural *Ae. aegypti* populations. Lastly, although contact irritancy may promote escape, it will be critical to measure the level of reduction, if any, in human-vector contact (i.e., biting) to truly understand the impact of sublethal behaviors on pathogen transmission. The irritant response must occur prior to blood meal acquisition. Due to this challenge, we are therefore currently applying a similar concept of sublethal concentrations and focal treatment application to the use of spatial repellent compounds in a push-pull strategy. This is an attempt to exploit the deterrent actions of chemicals to prevent *Ae. aegypti* entry into homes and thereby reduce indoor densities of mosquito vectors available for human-vector contact. The latter, if successful, may provide greater protection from pathogen transmission as compared to contact irritancy as repellency functions to provide a barrier of protection that is not dependent on vector contact with a treated surface [54].

Supporting Information

Table S1 Percentage knockdown *Ae. aegypti*[1] that escaped and those that did not escape under laboratory conditions.
(DOC)

Table S2 Percentage escape of *Ae. aegypti*[1] against alphacypermethrin (75% SAC) at designated time periods in Thailand.
(DOC)

Table S3 Percentage escape of *Ae. aegypti*[1] against alphacypermethrin at designated time periods in Peru.
(DOC)

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Author Contributions

Conceived and designed the experiments: NLA JPG HM TC. Performed the experiments: HM PS FC SP NLA JPG TC. Analyzed the data: HM PS NLA JPG. Wrote the paper: HM NLA JPG TC RGB AM.

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Dual exposure of *Rickettsia typhi* and *Orientia tsutsugamushi* in the field-collected *Rattus* rodents from Thailand

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ABSTRACT: Field-collected rodents and fleas from ten provinces covering four regions of Thailand were investigated for possible rickettsial pathogen infections. The 257 trapped-rodents belonged to 12 species. Five species of Genus *Rattus* accounted for 93% of the total capture, of which *Rattus exulans* and *Rattus norvegicus* were the two major species caught. All flea specimens, removed from trapped rodents, were identified as *Xenopsylla cheopis*. The PCR technique was performed on ectoparasite specimens to detect the presence of murine typhus pathogen (*Rickettsia typhi*) and scrub typhus pathogen (*Orientia tsutsugamushi*). Thirteen flea specimens (2.6 %) were found to be positive for *R. typhi* but none for *O. tsutsugamushi*. An ELISA technique was used to detect the rodent's antibodies against *R. typhi* and *O. tsutsugamushi*. Sixty-one rodent serum samples (23.7%) were positive for *R. typhi* specific IgM, IgG, or both, while 47 of the samples (18.3%) were positive for *O. tsutsugamushi*. Twenty serum samples from *R. norvegicus* (7.8%) had detectable antibodies against both *R. typhi* and *O. tsutsugamushi*. Our findings revealed the existence of the dual infection of rickettsial pathogens in the same natural hosts. ***Journal of Vector Ecology* 39: 182-189. 2014.**

Keyword Index: *R. typhi*, *O. tsutsugamushi*, Dual Exposure, *Rattus* Rodent, Thailand.

INTRODUCTION

Murine typhus, a flea-borne rickettsial disease, is caused by the pathogen *Rickettsia typhi*, an intracellular, gram-negative bacterium transmitted via the bite of the Oriental rat flea, *Xenopsylla cheopis*, with rodents being the natural reservoir (Azad 1990). Endemic typhus is widely distributed in tropical and subtropical areas of the world, particularly in coastal or port cities (Saah 1990). The disease is found to be endemic in many Southeast Asian countries, e.g., Thailand, Cambodia, Malaysia, and Indonesia (Sankasuwan et al. 1969, Taylor et al. 1986, Brown et al. 1988, Silpapojakul et al. 1993, Strickman et al. 1994, Parola et al. 1998). Rats are the natural reservoirs and belong to the Order Rodentia (a group of small mammals which are characterized by two incisors in the upper and lower jaws). Most cases of murine typhus are associated with areas in which rats accumulate in large numbers. Other natural hosts include domestic cats, mice, squirrels, and shrews. The Oriental rat flea, *Xenopsylla cheopis*, which remains infected with *R. typhi*, is the principal vector of murine typhus. *R. typhi* is transmitted via the bite of an infected flea or by scratching the bite area in the presence of infected feces excreted from the flea during feeding (Vaughan and Azad 1990). The incubation period for murine typhus is six to 14 days. Symptoms are very similar to those of epidemic typhus but are less severe (Fraoult and Roux 1997, Raoult et al. 2001). Most cases are mild, but the fatality rate may be close to 4% (Dumler et al. 1991) and without proper treatment, the disease may last for several months. Most laboratory diagnoses of *R. typhi* in human patients are based on the serological analysis of serum samples to detect pathogen specific antibodies. While the indirect immunofluorescent antibody (IFA) test is considered the 'gold standard,' the immunohistological, immunohistochemical techniques (Walker et al. 1989, 1997), enzyme-linked immunosorbent assay (ELISA), and dot-blot ELISA (Silpapojakul et al. 1995) are acceptable options. The Weil Felix test using the commercial Proteus OX-K and OX-19 antigens is not as good as the other assays but is still widely used in resource limited areas where *R. typhi* antigens are not available. The molecular detection for the presence of *R. typhi* in experimentally infected adult fleas using the polymerase chain reaction (PCR) amplification was first reported by Webb et al. in 1990. Several other rickettsial pathogens have also been detected in rodent hosts, including *Orientia tsutsugamushi* (scrub typhus) and *Ehrlichia* (Ehrlichiosis). Among flea-borne rickettsial diseases, scrub typhus is the most recognized in Southeast Asia, especially in Thailand where human infections occur in most parts of the country (Tanskul et al. 1998, Khuntirat et al. 2003, Lerdthusnee et al. 2003). Scrub typhus is an important human febrile disease caused by the infection of an obligate intracellular bacterium *O. tsutsugamushi*. It can be transmitted transovarially (from adult female to eggs) as well as trans-stadially (from egg to larva to adult) and to humans and rodents via the bite of an infected larval trombiculid (chigger) mite (Acari: Trombiculidae).

The aim of the current study was to investigate the distributions of *R. typhi* and other rickettsial pathogens (e.g., *Orientia*) in natural hosts and their ectoparasites in murine typhus endemic areas in Thailand.

MATERIALS AND METHODS

Collection sites

Ten provinces in four regions of Thailand were selected for the rodent trapping: Nonthaburi (Central); Chiangrai, Chiangmai, Tak

(northern); Khon Kaen, Udon Thani, Sa Kaeo, Ubon Ratchathani (northeastern); Petchaburi and Prachuap Khiri Khan (southern).

Fifty small wire live-traps, (14x14x30 cm in wxhxl) specially designed for rodents, were set up in each of two types of habitats, 'residential' (e.g., housing and surrounding areas) and 'others' (e.g., forest edge, rice field, and orchard), for two to three consecutive nights. Collections were conducted between March and May of 2011. Traps were checked daily and rodents were removed and identified to species. Rodent blood and serum samples were collected at the sites of capture. All procedures for handling captured animals were conducted according to the Approved-Animal Care and Use Protocol for Scientific Research of Kasetsart University (No. ACKU-01956). Flea specimens and other ectoparasites (mites and ticks) were removed from trapped rodents and were identified to species using Nadchatram and Dohany (1974).

Molecular detection of *Rickettsia typhi* and *Orientia tsutsugamushi* in flea specimens

Genomic DNA was extracted from an individual field-collected flea using the modified protocol of Invisorb ® Spin Tissue Mini Kit (Startec Molecular, Berlin, Germany). The extracted DNA was stored at 4° C and used later as the template for PCR amplification. The citrate synthase (*gltA*) gene and 17 kDa antigen gene of *R. typhi* (Regnery et al. 1991, Williams et al. 1992) and the 56kDa outer membrane protein gene of *O. tsutsugamushi* (Horinouchi et al. 1996) were amplified by a modified PCR as described previously. Amplification was conducted in a DNA thermocycler (Biometra T1). The DNA of the controls and the samples were handled separately to avoid contamination. The presence of *R. typhi* was confirmed by restriction fragment length polymorphism analysis (RFLP) of the amplified fragment of *gltA gene* or 17-kDa gene digested by restriction enzyme *Alu*I (Regnery et al. 1991, Boostrom et al. 2002). The presence of *O. tsutsugamushi* was confirmed by RFLP analysis of the amplified fragment, 56 kDa gene, digested by the restriction enzyme *Hha* I (Horinouchi et al. 1996).

Enzyme-linked immunosorbent assay (ELISA) for rodent serum samples

The indirect enzyme-linked immunosorbent assay (ELISA) (Dasch et al. 1979, Suwanabun et al. 1997, Ching et al. 1998, Coleman et al. 2002) was performed on rodent's serum samples for detecting the presence of *R. typhi* and *O. tsutsugamushi* antibodies using the recombinant 56-kd proteins (Ching et al. 1998). A screen at titers of 1:50 was first performed; positive sera at that level were then serially diluted for titer determination. The cut-off values for ELISA were established as the means of net OD (Optimal density) + 2 SD (standard deviation) from 50 rodent serum samples. They were 0.598 for IgM and 0.594 for IgG against murine typhus antigens; 0.515 for IgM and 0.454 for IgG for scrub typhus, respectively.

RESULTS

Field-collected rodent and flea identifications

A total of 257 rodents was captured. Udon Thani province had the greatest number of rodents (n=82), followed by Tak (n=42), Chiang Mai (n=34), Khon Kean (n=32), Nonthaburi (n=18), Chiang Rai (n=14), Prachuap Kirikhan (n=11), Sa Kaeo (n=11), Ubon Ratchathani (n=10), and Petchaburi (n=3). The rodents belonged to three Orders and six Families: Order Insectivora: Family Erinaceidae - *Hylomys suillus* (total number of trapped = 1); Family Tupiidae - *Tupaia glis* (2); Family Soricidae - *Crocidura murina* (8); Order Rodentia: Family Sciuridae - *Menetes berdmorei* (1); Family Muridae - *Bandicota indica* (3), *Mus muscurus* (1), *Rattus bukit* (2), *Rattus exulans* (121), *Rattus koratensis* (2), *Rattus norvegicus* (100), and *Rattus rattus* (15); Order Carnivora: Family Viverridae - *Herpestes javanicus* (1). Ten different species of rodents were collected in Chiang Mai province, demonstrating the greatest diversity. The dominant rodent species belonged to the genus *Rattus*, with five representative species, accounting for 93.4% of the total field-collected rodents. Among them, 86% were of *R. exulans* (121) and *R. norvegicus* (100). A total of 504 fleas were removed from all field-collected rodents. All fleas belonged to one species, *Xenopsylla cheopis*. Approximately half of the fleas (213 fleas or 42.3%) analyzed in this study were removed from rodents collected in Udon Thani province. The majority of the fleas (480 fleas or 95.2%) were removed from the genus *Rattus*, with more than half of them (259 fleas or 51.4%) being found on *R. norvegicus*, followed by 183 fleas (36.3%) from *R. exulans* (Table 1, Figure 1). Two other ectoparasites were removed from field-collected rodents: five ticks of the genus *Ixodes* and 30 chigger-mites of the genus *Leptotrombidium*.

Detections of specific antibodies against *R. typhi* and *O. tsutsugamushi* by enzyme-linked immunosorbent assay (ELISA)

Sixty-one (23.7%) field-collected rodent serum samples examined in this study had either IgM (36 samples), or IgG (56 samples), or both (29 samples) for *R. typhi*. Rodent serum samples from Udon Thani province resulted in the highest number (49 (59.8%)) of positive samples (44 *R. norvegicus*; four *R. exulans* and one *M. muscurus*), followed by four (4 *R. norvegicus*) from Prachuap Kirikhan; three (two *C. murina* and one *R. exulans*) from Khon Kean; two (*R. exulans*) from Tak; one (*T. glis*) from Chiang Mai; one (*R. rattus*) from Sa Kaeo and one (*R. exulans*) from Ubon Ratchathani province, respectively (Table 2, Figure 3). Regarding the species which had *R. typhi* specific antibodies, *R. norvegicus* had the highest number of 48 samples (out of the total number of 100 *R. norvegicus* captured) which had either IgM (31 samples), or IgG (46 samples), or both (27 samples) positives, followed by *R. exulans* with eight (two IgM, seven IgG, and one with both IgM and IgG positives); *C. murina* with two (IgM positives) and a *M. muscurus* (with both IgM and IgG positives), *R. rattus* and *T. glis* with one (IgG positive), respectively. Serum samples from Chiang Rai, Nonthaburi, and Petchaburi province tested positive for murine typhus antibodies by ELISA.

Four *X. cheopis* specimens that were removed from two ELISA-positive rodents from the Prachuap Kirikhan province (of *R. norvegicus* (both IgG and IgM) and *R. norvegicus* (IgM)) were positives for *R. typhi*-detection by PCR method. Of the nine flea specimens that were removed from rodents collected from the Udon Thani province and that were found to be positive for *R. typhi* by PCR method, seven were removed from two *R. norvegicus* and one *R. exulans* IgG positive samples and three *R. norvegicus*

Table 1. Summary of total numbers of field-collected rodents, total numbers of positive detections of specific antibodies against *Rickettsia typhi* and *Orientia tsutsugamushi* by ELISA technique, total numbers of field-collected *Xenopsylla cheopis* fleas, and total numbers of *X. cheopis* fleas with positive detections of *R. typhi* by the PCR technique.

Rodent species	No. of field-collected rodents	No. of positive antibodies against *R. typhi* By ELISA			No. of positive antibodies against *O. tsutsugamushi* By ELISA			No. of *X. cheopis* collected-fleas	No. of *X. cheopis with positive- R. typhi* by PCR
		IgM	IgG	IgM & IgG	IgM	IgG	IgM & IgG		
Bandicota indica	3	-	-	-	2	2	1	3	-
Crocidura murina	8	2	-	-	-	-	-	10	-
Herpestes javanicus	1	-	-	-	-	-	-	5	-
Hylomys suillus	1	-	-	-	-	-	-	4	-
Menetes berdmorei	1	-	-	-	-	-	-	1	-
Mus mucurus	1	1	1	1	-	-	-	1	-
Rattus bukit	2	-	-	-	-	-	-	3	-
Rattus exulans	121	2	7	1	-	-	-	183	4*
Rattus koratensis	2	-	-	-	-	-	-	7	-
Rattus norvegicus	100	31	46	27	26	19	11	259	9**
Rattus rattus	15	-	1	-	7	8	5	26	-
Tupaia glis	2	-	1	-	-	-	-	2	-
Total	257	36	56	29	35	29	17	504	13

*Four *X. cheopis*: two fleas removed from two *R. norvegicus* collected from Prachuap Kirikhan Province.
**Nine *X. cheopis*: five fleas removed from five *R. norvegicus*; four fleas removed from four *R. exulans,* all collected from Udon Thani Province.

Table 2. Detection of specific antibodies against both *Rickett*sia *typhi* and *Orientia tsutsugamushi* in field-collected *Rattus norvegicus* by ELISA.

Collection site (Province) –Collection#	Detection of specific antibodies against *R. typhi* by ELISA		Detection of specific antibodies against *O. tsutsugamushi* by ELISA	
	IgM	IgG	IgM	IgG
Udon Thai- UD1	+	+	+	+
Udon Thani-UD3	-	+	+	-
Udon Thai- UD5	-	+	+	-
Udon Thani-UD7	+	+	+	-
Udon Thai- UD8	+	+	+	-
Udon Thani-UD9	+	+	+	+
Udon Thai- UD13	+	+	+	-
Udon Thani-UD15	+	+	+	+
Udon Thai- UD16	-	+	+	+
Udon Thani-UD22	+	+	+	-
Udon Thai- UD23	+	+	+	+
Udon Thani-UD28	+	+	+	-
Udon Thai- UD29	+	+	+	-
Udon Thani-UD30	+	+	+	+
Udon Thai- UD33	-	+	+	-
Udon Thani-UD34	+	+	+	+
Udon Thai- UD68	+	+	+	+
Udon Thai- UD70	+	+	+	+
Prachuab Kirikhan-PK1	+	+	+	+
Prachuab Kirikhan-PK7	+	+	-	+
Total	16	20	19	11

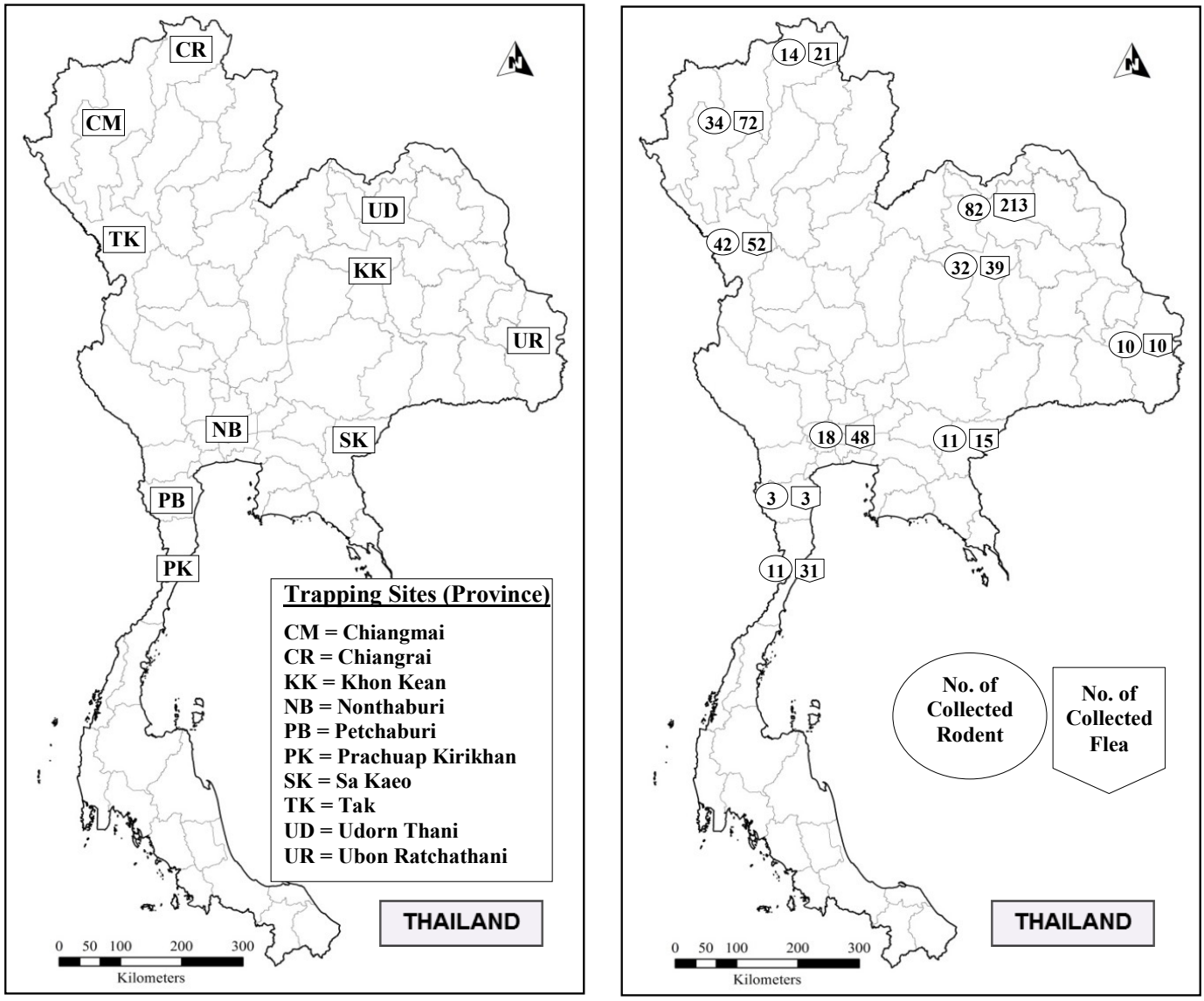


Figure 1. Trap sites (province) in Thailand and numbers of field-collected rodents and fleas by province.

and one *R. exulans* IgM and IgG positive samples. The remaining two fleas which were removed from two *R. exulans* rodents were found negative for antibody by ELISA methods.

In addition, 47 (18.3%) rodent sera had either IgM (35 samples), or IgG (29 samples) or both (17 samples) against scrub typhus pathogen by the ELISA method. All samples testing positive for scrub typhus specific antibodies belonged to three rodent species *B. indica*, *R. norvegicus* and *R. rattus*. Twenty-one of them were from Udon Thani province (all *R. norvegicus*), followed by eight (one *B. indica*; three *R. norvegicus*; four *R. rattus*) from Chinag Mai; four (two *R. norvegicus*; two *R. rattus*) from Chiang Rai; three (one *R. norvegicus*; two *R.rattus*) from Nonthaburi; two (both *R. norvegicus*) from Khon Kean; two (both *R. norvegicus*) from Prachuap Khirikhan; two (*R. norvegicus*) from Sa Kaeo; two (one *B. indica*; one *R. rattus*) from Tak; two (one *R. norvegicus*; one *R. rattus*) from Ubon Ratchathani and one (*B. indica*) from Phetchaburi province, respectively. *R. norvegicus* species had the greatest number of specimens testing antibody positive for *O. tstsugamushi* with 34 (out of the total number of 100 captured), followed by *R. rattus* with ten) and *B. indica* with three (Table 1, Figure 2).

Detection of dual-infection in wild-caught rodents

There were 20 sera samples from *R. norvegicus* that tested positive for antibodies against both murine typhus and scrub typhus pathogen. Among them, 18 were collected from Udon Thani Province and two from Prachuap Kirikhan Province. Eight of the *R. norvegicus* were IgM and IgG positive for *R. typhi* and *O. tsutsugamushi* (seven from Udon Thani and one from Prachuap Kirikhan Province, respectively). The remaining 12 specimens were positive for either IgM, IgG, or both against *R. typhi* or *O. tsutsugamushi*. The distribution is as follows: eight *R. norvegicus* (seven of Udon Thani and one of Prachuap Kirikhan) had both IgM and IgG of *R. typhi* and only IgM of *O. tsutsugamushi*; two *R. norvegicus* from Udon Thai with only IgG of *R. typhi* but both IgM and IgG of *O. tsutsugamushi*; and the last two of *R. typhi* and only IgM of *O.tsutsugamushi*; two *R. norvegicus* of Udon Thani had IgG of *R. typhi* and only IgM of *O.tsutsugamushi* (Table 2).

Detection of *R. typhi* and *O. tsutsugamushi* in field-collected flea specimens by PCR

R. typhi DNA was detected in thirteen flea samples among the total of 504 fleas: four from Prachuap Kirikhan and nine from Udon Thani province (Table 1, Figure 3). The four positive detections of *R. typhi* in fleas collected from Prachuap Kirikhan were removed from two *R. norvegicus* rodents. While, the nine positive detections of *R. typhi* in fleas collected from Udon Thani province were removed from nine *Rattus* rodents: four *R. exulans* and five *R. norvegicus*. None of the 504 field-collected flea specimens were positive for *O. tsutsugamushi* DNA. For the PCR examinations on tick and mite specimens; they were all negatives for *R. typhi* and *O. tsutsugamushi*.

Genotype characteristic of rickettsial species using PCR/RFLP technique

Both the 17 kDa antigen gene and *glt*A genes were found in *R. typhi* infected- *X. cheopis* fleas removed from four field-collected rodents of Prachuap Kirikhan province (all from *R. norvegicus*: Collection # PK7 (one), PK7 (two), PK8 (one), PK8 (two)), and nine field-collected rodents of Udon Thani province (from five *R. norvegicus* and four *R. exulans*: Collection# UD10, UD12, UD29, UD36, UD37, UD55, UD64, UD65 and UD69). The PCR-assay products of 17 kDa gene had amplicon sizes about 450 bp and about 380 bp for citrate synthase (*glt*A). Electrophoretic analysis of undigested PCR products of the *glt*A PCR-amplified products from the 13 *R. typhi*-infected *X. cheopis* fleas showed similarity in size.

In an attempt to estimate the smaller DNA fragment size, the Φ (Phi) X 174 double-stranded replicative form DNA (New England BioLabs) cleaved with *Hin*fI restriction endonuclease was used for DNA fragment size standard, as described by by Regnery et al. 1991, in order to obtain accurate DNA fragment sizes. PCR products of the 17 kDa gene from five representative *R. typhi*-infected fleas removed from two *R. norvegicus* rodents from Prachuap Kirikhan and three rodents from Udon Thani (two of *R. exulans* and one of *R. norvegicus*) were subjected to direct automate sequencing. Amplicons from the five *X. cheopis* fleas exhibited sequence homology from 98% to 100% nucleotides pair-wise similarities. The amplicon sequences were identical to *R. typhi* 17 kDa gene of AE017197.1 (Wilmington strain, complete genome) or M28481.1 (17K genus-common antigen gene, complete cds).

DISCUSSION

Murine typhus is an important zoonotic disease present in many parts of the world, and is endemic in tropical areas, including Southeast Asia (Sankasuwan et al. 1969, Azad 1988, Silpapojakul et al. 1991, Wilde et al. 1991, Bassett et al. 1992, Takada et al. 1993, Loh et al. 1996). Murine typhus patients are either residents of endemic areas or travelers who have visited and/or travelled through an endemic area, as reported by health officials in European countries such as Norway (Jensenius 1997). In Thailand, the murine typhus pathogen was first isolated from rodents in 1964 and its presence was reported by Sangkasuwan in 1969 (Sankasuwan et al. 1969). The first outbreak of this febrile disease in Southeast Asia was reported in 1988 (Brown et al. 1988) along the Thai-Cambodia border, where its presence continued to be reported until 1991. Most cases involved Khmer refugees encamped in evacuation sites. Rising antibody titers against *R.typhi* were found to coincide with areas where *R. exulans* and *X. cheopis* were also collected (Wilde et al. 1991).

Our results confirmed the important role of rodents in the genus *Rattus* as the primary reservoir of the rickettsial pathogens identified in this study. More than 93% of the field-collected rodents belonged to the subfamily *Murinae* and represent five species in the genus *Rattus* (*R. bukit*, *R. exulans*, *R. koratensis*, *R. norvegicus,* and *R. rattus*). Among them, *R. exulans* and *R. norvegicus* were the two dominant species, accounting for 86% of all rodents captured. Both species are considered urban dwellers and are commonly found in association with humans. All collected-fleas belonged to one species, *X. cheopis*, the most common rat flea found in Thailand.

Xenopsylla cheopis is closely associated with peridomestic or urban rodents such as *R. norvegicus* and *R. rattus*, where they are commonly found in cities and port areas but their range can also extend into suburban areas (Civen and Ngo 2008). This study demonstrated that of the nine positive samples of *R. typhi* in *X. cheopis* fleas, all were removed from *R. norvegicus* collected from overcrowded and undeveloped areas of Muang District of Udon Thani province. Our findings agreed with the report of Siritantikorn et al. (2003). They also observed that *R. typhi* antibody was found in *R. norvegicus* more frequently than in *R. rattus*. However, another urban rat, *R. exulans*, is also considered a significant reservoir host for *R. typhi*-infected fleas. This was confirmed in the current study that demonstrated that four samples of *X. cheopis* tested positive for *R. typhi* and all infected fleas were removed from *R. exulans*.

After performing the PCR/RFLP technique using the 17 kDa region, we found that most *R. typhi*-infected *X. cheopis* fleas removed from either *R. exulans* or *R. norvegicus* rats had identical sequences, with the 17 kDa gene in the *R. typhi* Wilmington genomes. This is the most common strain isolated from human and animals (McLeod et al. 2004, Ammerman et al. 2008). Murine typhus is an important disease which poses a significant threat to many communities throughout the world, especially in areas closely associated with agricultural activities where animal reservoirs and ectoparasites are abundant. Although flea-borne typhus is considered to be a milder, shorter-lasting disease with low fatality rates as compared to other members of the Typhus group of rickettsial pathogens, it is often an overlooked febrile illness with limited and inadequate laboratory diagnosis available.

Results from the detection of specific antibodies against murine typhus pathogen by the ELISA method revealed that the highest percentage (23.7%) of positive samples was from *R. norvegicus* species. Sera testing positive for antibody were from five different rodent species (*C. murina*, *M. muscurus*, *R. norvegicus*, *R. exulans*, and *T. glis*). A total of 13 flea specimens was found positive for *R. typhi* by PCR detections, nine of which were removed from *R. norvegicus* rodents and four from *R. exulans* rodents. The ELISA detection of specific antibodies against the scrub typhus pathogen showed that positive samples came from three different rodent species: *R. norvegicus*, *B. indica* (rice-field rat), and *R. rattus* and represent all ten trapped-provinces. The highest percentages of samples were from *R. norvegicus* species collected in Udon Thani. However, none of flea samples obtained

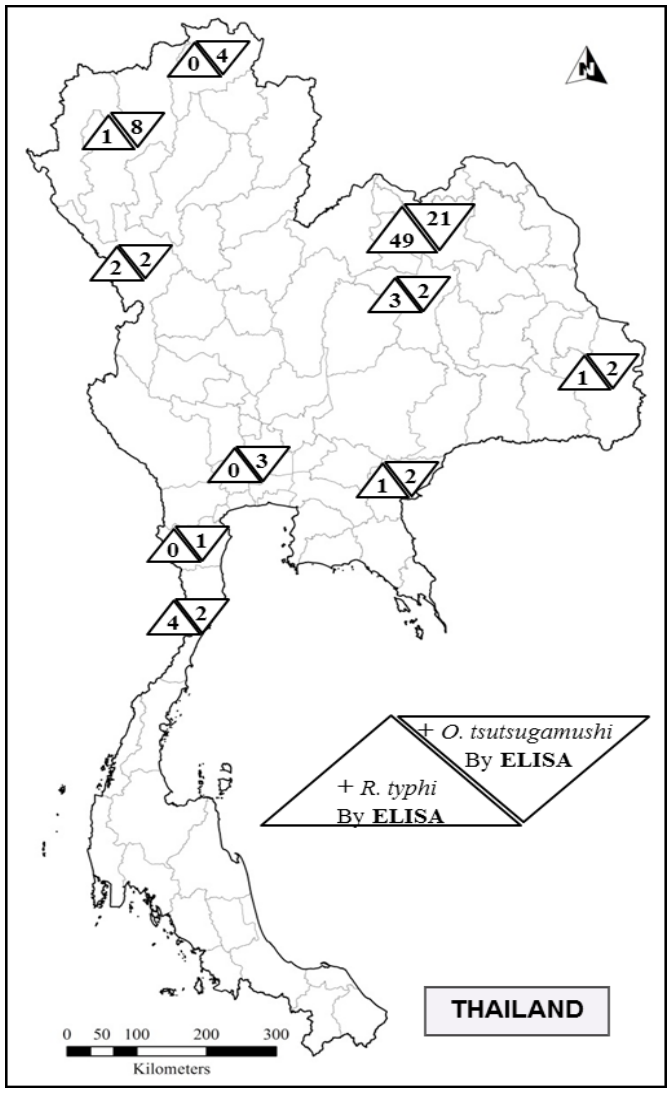


Figure 2. Number of field-collected rodent serum samples, by province, with positive detections of specific antibodies against *Rickettsia typhi* and *Orientia tsutsugamushi* pathogens by the ELISA method.

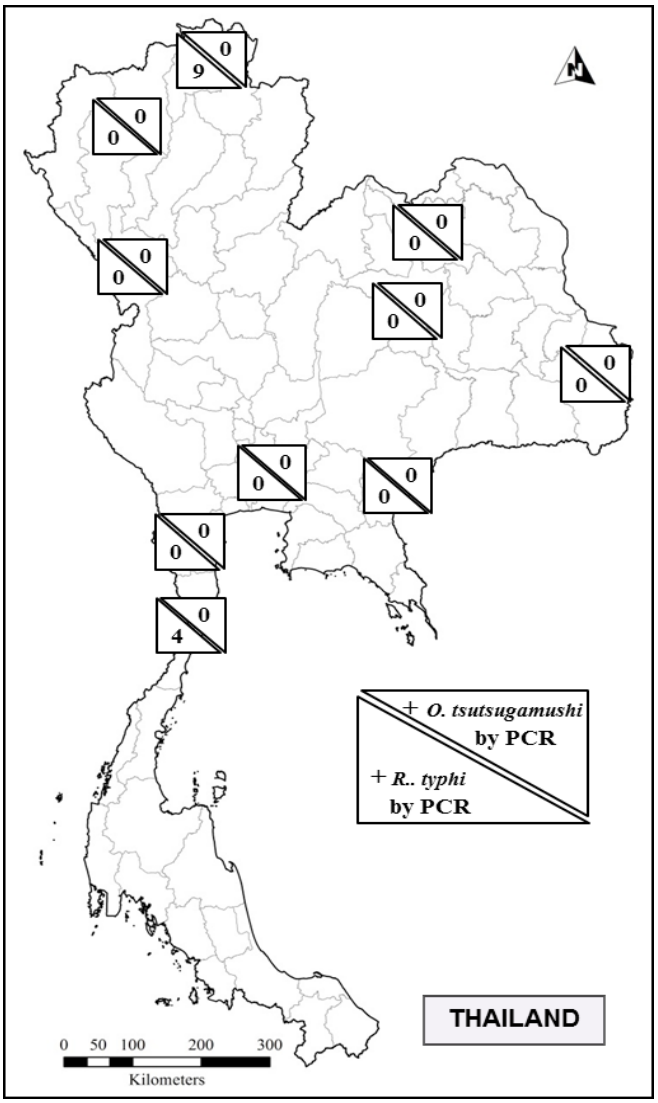


Figure 3. Number of positive samples, by province, of *Rickettsia typhi* and *Orientia tsutsugamushi* by the PCR method in *Xenopsylla cheopis* fleas.

from field-collected rodents were found positive for scrub typhus pathogen. This was anticipated because chigger mites, not fleas, are the vectors of *Orientia*.

The most significant finding in this study was the detection of antibodies in the same animal host reactive to the recombinant proteins derived from two typhus pathogens: murine typhus(*R. typhi*) and scrub typhus (*O. tsutsugamushi*). It is also interesting to note that all positive samples belonged to one rodent species, *R. norvegicus*. Eight of the samples were both IgM and IgG positive for *R. typhi* and *O. tsutsugamushi*. It is interesting to note regarding this co-infection, that the typical hosts for scrub typhus are more rural field-inhabiting species such as *R. rattus*, *B. indica,* and *T. glis*. *R. norvegicus* is generally found associated with more urban settings, near human habitats or in the areas linked to man-made environments, such as in sewage systems. However, this finding would not be as interesting if this dual infection of murine and scrub typhus were found in the *R. rattus,* as it is considered more of a generalist. This species consumes a wide range of foods, and has adapted well to a wide range of settings to include both human settlements and agricultural fields. Therefore, they are behaviorally capable of moving between urban and rural habitats, which enhance their chance of acquiring ectoparasites (fleas and mites) capable of transmitting both murine typhus and scrub typhus. The presence of specific antibodies to both pathogens in *R. norvegicus* has suggested that there is also a possibility for dual exposure in febrile patients to both *R. typhi* and *O. tsutsugamushi*.

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Excito-repellency of essential oils against an *Aedes aegypti* (L.) field population in Thailand

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ABSTRACT: An investigation of the behavioral responses of *Aedes aegypti* (= *Stegomyia aegypti*) to various concentrations of essential oils (2.5, 5, and 10%) extracted from hairy basil (*Ocimum americanum* Linn), ginger (*Zingiber officinale* Roscoe), lemongrass (*Cymbopogon citratus* Stapf), citronella grass (*Cymbopogon nardus* Rendle), and plai (*Zingiber cassumunar* Roxb) were performed using an excito-repellency test chamber. Results showed that *Ae. aegypti* exhibited varying levels of escape response in both the contact and noncontact chambers in response to different essential oils. The magnitude of the behaviors changed in a dose-response fashion depending on the percent volume to volume concentration of oil used. A 2.5% concentration of hairy basil oil produced a significantly greater escape response compared to the other extracts at the same concentration ($P< 0.05$). Oils of ginger, lemongrass, and citronella produced stronger irritant and repellent responses at the median 5% concentration compared to the lowest and highest concentrations. There was marked suppression of escape for both contact and noncontact tests using 10% concentrations of hairy basil, lemongrass, and citronella, with high knockdown for all three oils after 30 min. Hairy basil and lemongrass had the highest insecticidal activity to *Ae. aegypti*, with LC_{50} values of 6.3 and 6.7 percent, respectively. We conclude that the essential oils from native plants tested, and likely many other extracts found in plants, have inherent repellent and irritant qualities that should to be screened and optimized for their behavior-modifying properties against *Ae. aegypti* and other biting arthropods of public health and pest importance. ***Journal of Vector Ecology* 39 (1): 112-122. 2014.**

Keyword Index: Behavior, excito-repellency, *Aedes aegypti*, plant extracts, Thailand.

INTRODUCTION

Dengue remains one of the most important vector-borne diseases endemic to most countries of the tropical and subtropical zones. An average of 50 million cases are documented annually, resulting in tens of thousands of deaths. The viruses responsible for dengue are spread by the bite of an infected *Aedes* mosquito (Gubler 1997). *Aedes aegypti* (*Stegomyia aegypti*) is the primary urban and epidemic vector of dengue fever viruses and is highly anthropophilic in its feeding behavior. It is often found in close association with humans and prefers to live in and around human dwellings (Chan et al. 1971). Despite major advances in dengue research, an effective and reliable dengue vaccine is not yet available. Prevention of this disease remains almost entirely dependent on vector control, which is considered to be the most effective method for reducing virus transmission in dengue endemic areas of the world (Grieco et al. 2007).

One of the most successful methods for mosquito vector control is the use of chemicals, especially the use of synthetic pyrethroids. However, long-term use of pyrethroids to control the vectors has resulted in the development of insecticide resistance in mosquito populations (Chandre et al. 1998, Penilla et al. 1998, Misni et al. 2009). To delay or avoid insecticide resistance in mosquito populations, one could adopt an approach in which protection from the bite of the mosquito could be achieved through behavioral modification rather than toxicity. Protection from mosquito bites could be achieved by avoiding physical contact with mosquitoes using insect repellents. However, little is known of how these chemical actions function to repel insects. In general, there are two different types of behavioral responses found in mosquitoes in response to exposure to chemicals: irritancy and repellency (Roberts et al. 2000). Irritancy results only after physical contact with chemically-treated surfaces. Repellency, however, is a response that results from an insect detecting and avoiding the treated surface without physical contact (Chareonviriyaphap et al. 1997, Roberts et al. 1997, Potikasikorn et al. 2005). Using the excito-repellency test system developed by Chareonviriyaphap et al. (2002), it is now possible to observe both irritant and repellent responses and thus quantitatively measure behavioral responses of mosquitoes following exposure to test compounds. N, N-diethyl-3-methylbenzamide (DEET), the most effective repellent compound, is widely used to prevent the bite of mosquitoes around the world (McCabe et al. 1954, Yap 1986). This compound has generally been regarded as safe, but adverse effects when misapplied can be severe (Yang and Ma 2005). To avoid these harmful effects, several researchers are now focusing on the repellent qualities of products derived from natural plant extracts. This work has recently led to a number of essential oils being recommended as

mosquito repellents due to their eco-friendly and biodegradable nature (Adeniran and Fabiyi 2012). In Thailand, extracts of several plants, including lemongrass oil (*Cymbopogon citratus* Stapf) (Phasomkusolsil and Soonwera 2011a, Adeniran and Fabiyi 2012), citronella grass (*Cymbopogon nardus* Rendle) (Phasomkusolsil and Soonwera 2010, Suwansirisilp et al. 2012), plai (*Zingiber cassumunar* Roxb) (Phasomkusolsil and Soonwera 2010), ginger (*Zingiber officinale* Roscoe) (Tawatsin et al 2006), Makaen (Thai name) (*Zanthoxylum limonella*), clove [*Syzygium aromaticum* (L.) Merrill & Perry] (Trongtokit et al. 2005), and hairy basil (*Ocimum americanum* Linn) (Tawatsin et al. 2001) were explored and tested as insect repellents against several species of mosquitoes. While a great number of essential oils from native plants are known to repel mosquitoes, there has been little research to understand the magnitude of the behavioral response to varying concentrations of the essential oils.

In this study, we evaluated a dose-response relationship for the two nontoxic (contact irritant and noncontact repellent) behavioral responses and contact toxicity of *Ae. aegypti* to five essentials oils extracted from Thai plants using the excito-repellency (ER) test system. DEET was used as a standard reference baseline for comparison purposes.

MATERIALS AND METHODS

Mosquitoes

Aedes aegypti were collected as immatures from PuTeuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (420 m above sea level, 14º 79º N, 99º 17º E).

Ae. aegypti mosquitoes were reared and maintained under laboratory-controlled conditions (27±5º C and 80±10% RH with a 12 h-day/night cycle) at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Following free mating, three to five-day-old female *Ae. aegypti* were allowed to feed on a live guinea pig. Two days after feeding, a plastic bowl containing clean water and filter paper was placed inside the cage overnight for females to lay their eggs. Immature stages were reared in plastic pans under identical population density and provided sufficient tropical fish food (0.5 g/ per day) until reaching the pupal stage. Pupae were transferred into cups containing clean water and were placed in screened cages (30 x 30 x 30 cm^3). The adult mosquitoes were provided cotton pads soaked with 10% sugar solution (carbohydrate meal). Three to five-day-old, nulliparous females were starved for 12 h and provided only water prior to testing. To ensure a test cohort that was genetically similar to natural field populations, only F1-F3 progeny were utilized for testing.

Essential oils and DEET

Essential oils were obtained by the following means: 1) Hairy basil oil (*Ocimum americanum* Linn) by steam distillation of leaves (Chemical constituents: Methyl cinnamate, d-camphor, citral), 2) Ginger oil (*Zingiber officinale* Roscoe) by cool expression of roots (Chemical constituents: α-pinene, camphene, β-pinene, 1, 8-cineole, linalool, borneol, α-terpineol, nerol, neral, geraniol, geranial, geranyl acetate, β-bisabolene, and zingiberene), 3) Lemongrass oil (*Cymbopogon citratus* Stapf) by water distillation of dry leaves (Chemical constituents: myrcene, citronellal, geranyl acetate, nerol, geraniol, neral, and traces of limonene and citral), 4) Citronella oil (*Cymbopogon nardus* Rendle) extracted by steam distillation of citronella grass (Chemical constituents a combination of citronellic acid, borneol, citronellol, geraniol, nerol, citral, citronellal, camphene, dipentene, and limonene), and 5) Plai oil (*Zingiber cassumunar* Roxb) by steam distillation of roots (Chemical constituents: Terpinene, Sabinene, (E)-1(3, 4 dimethoxyphenyl) butadiene) were used for the ER assay. These essential oils were obtained from Thai-China Flavours and Fragrances Industry Co., Ltd., PhraNakhon Si Ayutthaya, Thailand. These oils were kept at 4º C. DEET was used as the "gold-standard" repellent.

Impregnated papers

Three concentrations (2.5, 5, and 10% (v/v)) of each essential oil were diluted from individual stock solutions in absolute ethanol and were impregnated onto a filter paper measuring 14.5 x 17.5 cm (Whatman® No.1), following the World Health Organization procedure for testing papers with insecticidal compounds. All papers were treated at the rate of 2 ml of test solution per 180 cm^2 surface area. Control (blank) papers were treated separately with absolute ethanol only. All tested papers were prepared just prior to testing and were allowed to air dry for 1 h before use in the assays. Papers were used only once per test series (Grieco et al. 2007).

Laboratory test procedures

Fifteen, three to five-day-old, starved nulliparous *Ae. aegypti* mosquitoes were introduced into each test chamber, in which the outer rear door was closed and completely secured. For each trial, four test chambers were set up; two as contact chambers (one treatment and one blank control), whereby the mosquito could have direct tarsal exposure to the respective paper and two chambers as noncontact designs (treatment and blank control) in which papers were protected by a fine-mesh screen barrier denying the mosquito physical contact to the treated surface. A receiving cage was connected to the exit portal for collecting escaping mosquitoes. Once introduced, mosquitoes were allowed a 3-min resting period to adjust to the test chamber conditions. After the resting period, the escape funnel was opened and the observation period was begun. Mosquitoes escaping from the chamber into the receiving cage were recorded at 1-min intervals for a period of 30 min. All tests were performed during daytime hours between 08:00 and 16:00 in a well-lighted environment and under identical laboratory conditions (temperature, humidity, illumination). All trials were replicated four times for each particular test combination. After the 30-min observation time, the number of knocked down/dead mosquitoes, the number of mosquitoes remaining inside the chamber, and the number that escaped to the receiving cage were recorded for each treatment and control chamber. Additionally, all living mosquitoes inside the chamber, for both control and treatment chambers, were removed and maintained in individual plastic holding cups. Plastic cups were provided with a 10% sugar solution and were maintained in the insectary at 27±5º C and 80±10% RH for 24 h to monitor mortality.

Data analysis

For excito-repellency data, the Kaplan-Meier survival

analysis method was used to analyze and interpret mosquito behavioral response data (Kleinbaum 1995). Mosquitoes that escaped the test chamber were treated as "deaths" and those that remained in the test chamber were labeled as "survivals" (Roberts et al. 1997, Chareonviriyaphap et al. 1997). A log-rank method (Mantel and Haenzel 1959) was used to compare the patterns of escape behavior between different treatment designs (contact and noncontact). The data was analyzed to show the treatment effects in terms of essential oil-induced escape rates via the percentage of total mosquitoes that were collected from the receiving box during the 1-min intervals. Escape rates from the treatment chamber were adjusted based on the responses from the control pair. The discriminating level of statistical significance for all tests was set at 95% (P <0.05). Mean percent mortality was calculated per test chamber. Abbott's formula was applied to adjust escape responses based on paired control escape (Abbott 1925). The LC_{50} was calculated based on only those mosquitoes that failed to escape the contact chambers, thus measuring excess mortality (minus controls) that resulted from exposure to active ingredient volatile molecules only using PROC PROBIT, SAS Software version 9 (SAS Institute, Cary, NC, U.S.A.). Non-overlapping, 95% confidence intervals were used to determine significant differences among treatments ($P < 0.05$).

RESULTS

Behavioral avoidance and toxicity response of *Ae. aegypti* to three different concentrations of five essential oils and DEET was documented. Overall, higher escape responses and mortality rate were seen from the contact chambers than those from paired noncontact chambers, regardless of test compounds and concentrations. Among five oils, essential oil from hairy basil produced the strongest irritant and repellent escape responses at the lowest concentration (2.5%).

Escape responses and mortality after a 24-h holding period of *Ae. aegypti* (Kanchanaburi) to essential oils from hairy basil (*Ocimum americanum* Linn), ginger (*Zingiber officinale* Roscoe), lemongrass (*Cymbopogon citratus* Stapf), citronella (*Cymbopogon nardus* Rendle), and plai (*Zingiber cassumunar* Roxb) were characterized in contact and noncontact exposure chambers compared to DEET and presented in Table 1. In general, higher escape responses were observed in contact trials than noncontact trials and in paired controls. Higher mortalities were observed at higher concentrations than those at lower. *Ae. aegypti* showed higher degrees of contact escape responses to 2.5% hairy basil oil with 71.4% escaped and to 5% lemongrass oil, citronella oil, and ginger oil with 69.1, 66.1, and 61.8% escaped, respectively. In contact trials, no mortality was observed from escaped mosquitoes for all three concentrations of hairy basil and plai oil. Similarly, low mortality was also observed from noncontact trials for chambers treated with hairy basil (0-2.4), plai (0-6.7), and citronella oil (0-4.2). Percent mortalities of non-escaped specimens were observed, ranging from 0-89.3% for hairy basil oil, 0-32.1% for ginger oil, 0-33.3% for DEET, and 6.9-15.1% for citronella. In noncontact trials, higher mortality was observed from escaped specimens for all five oils compared to non-escaped. The percent mortalities of non-escaped specimens were low, ranging from 2.9-8.8% for citronella oil and 0-2.3% for plai oil. However, no mortality occurred for escaped and non-escaped specimens in any control trials. Total knockdown (100%) was observed in those non-escaped mosquitoes with 10% hairy basil oil and lemongrass oil in both trials with subsequent mortalities of 68.8% and 98.2%, respectively. Percent knockdown of non-escaped specimens in contact and noncontact trials for 10% citronella oil were 86.8% and 94.2%, respectively. Low mortality was observed in non-escaped specimens with plai oil (0-4%), whereas the escaped specimens ranged from 0 to 6.7% mortality. High mortality was observed in non-escaped mosquitoes in chambers treated with lemongrass oil (92-98.2%).

The LC_{50} values based on 24-h mortality for the five essential oils and DEET ranged from 6.28 to 4,023% (Table 2). Lemongrass oil (LC_{50} 6.28%) followed by hairy basil oil (LC_{50} 6.73%) were the most toxic compounds, followed by ginger oil (LC_{50} 13.5%) and DEET (LC_{50} 13.63%), with no overlapping LC_{50} confidence intervals. Citronella oil was the least toxic compound (LC_{50} 4,023%) tested.

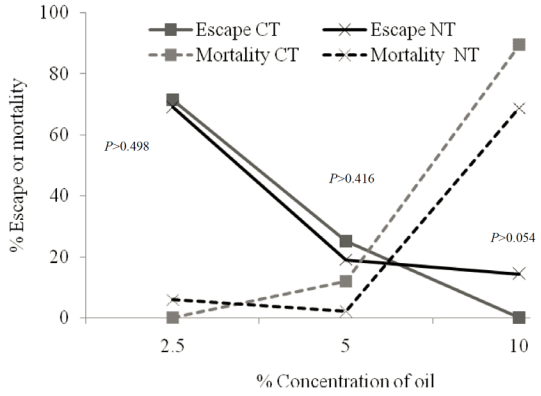
Multiple log-rank comparisons for contact, noncontact and control trials for each essential oil and DEET were conducted (Table 3). Interestingly, significant differences in escape patterns by nulliparous females were observed in paired comparisons between control and noncontact ($P < 0.05$), except for 2.5% ginger oil, lemongrass oil, and 10% lemongrass oil, plai oil, and citronella oil. Significant differences in escape responses were also found when contact was compared to all paired controls ($P < 0.05$), except for 10% hairy basil oil and citronella oil. Likewise, marked differences in escape response were present in paired contact and noncontact trials ($P < 0.05$), with the lowest (2.5%) and highest (10%) concentration of ginger oil and plai oil, respectively, and all three concentrations of lemongrass oil and DEET (Figure 1).

Log-rank statistical comparisons between percent concentrations of each essential oil in either contact or noncontact trials were analyzed. There were marked differences in the behavioral response among different concentrations of oils. Significant differences in escape patterns were found with the lowest dose (2.5%) of ginger oil, citronella oil, and DEET compared to higher (10%) concentrations ($P < 0.05$), except lemongrass oil, in both contact and noncontact trials. Significant differences in escape patterns were also found with the lowest dose (2.5%) of hairy basil oil compared to other concentrations ($P < 0.05$) (Table 4).

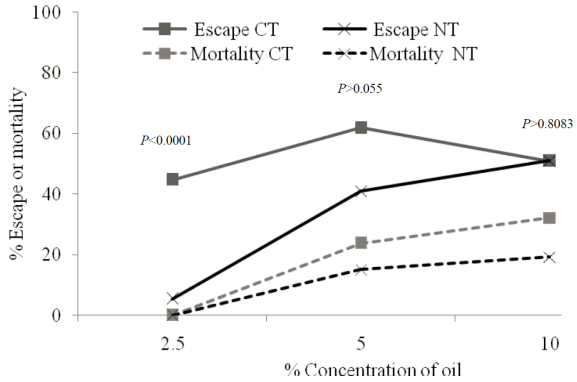
Statistical comparisons of escape responses for *Ae. aegypti* exposed to each essential oil compared to DEET at a concentration of 2.5% are presented in Table 5. In both contact and noncontact trials, there were significant differences in escape response between DEET and hairy basil. There were also significant differences between the responses recorded for citronella oil and DEET in the noncontact assay. Comparisons of escape responses for *Ae. aegypti* exposed to essential oils demonstrated that, when comparing hairy basil to others at the lowest dose (2.5%), the escape response of test cohorts was significantly different in both contact and noncontact trials (Table 6).

Figures 2-4 (A-F) are survival analysis curves that compare the patterns of escape and the percentage of mosquitoes remaining in the test chambers under contact and noncontact trials against hairy basil oil, ginger oil, lemongrass oil, citronella oil, plai oil, and DEET at different concentrations over a 30 min test period.

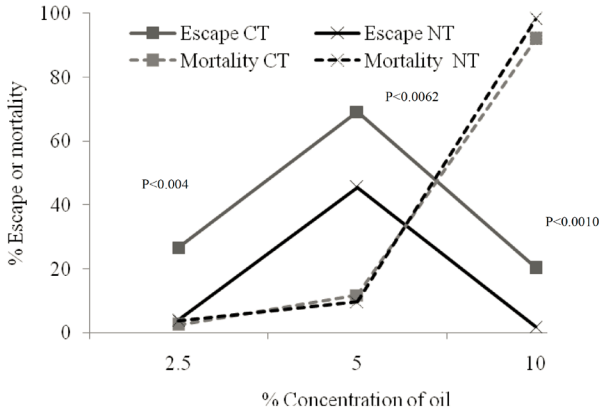
Hairy basil oil (1A)



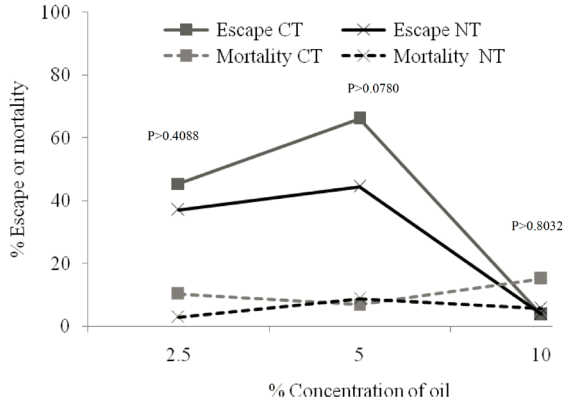
Ginger oil (1B)



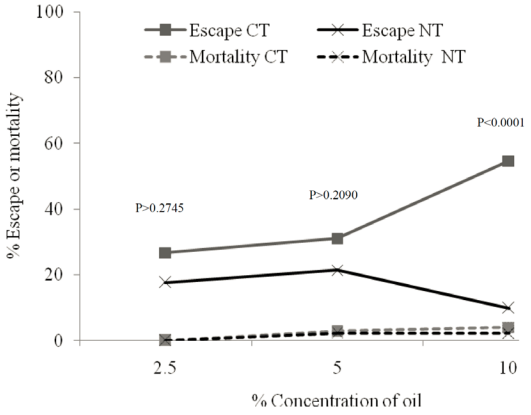
Lemongrass oil (1C)



Citronella oil (1D)



Plai oil (1E)



DEET (1F)

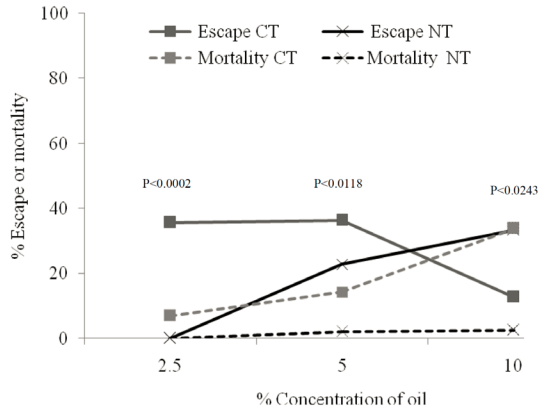


Figure 1. Percent escape response and mortality of female *Ae. aegypti* at concentrations of 2.5, 5, and 10% for the essential oils (Hairy basil, 1A), (Ginger, 1B), (Lemongrass, 1C), (Citronella 1D), (Plai, 1E), and (DEET, 1F) at 30-min contact and noncontact exposures. Log-rank comparison was performed on escape responses to determine differences between contact and noncontact by concentration ($P<0.05$).

Table 1. Escape response and mortality of a Kanchanaburi population of *Ae. aegypti* females after a 24-h holding period following exposure in excito-repellency contact and noncontact trials.

Compounds	Conditions	Dose % (v/v)	Number Tested	Esc. (%*)	% Knockdown		% Mortality	
					Esc	NonEsc	Esc	NonEsc
Hairy basil	CT	2.5	60	44 (71.4)	0	0	0	0
		5	58	16 (25.1)	0	66.7	0	11.9
		10	60	4 (1.7)	0	100	0	89.3
	NT	2.5	58	41 (69.1)	0	0	2.4	5.9
		5	60	13 (19.0)	0	53.2	0	2.1
		10	59	11 (14.)	0	100	0	68.8
Ginger	CT	2.5	57	26 (44.7)	0	0	0	0
		5	59	38 (61.8)	5.3	76.2	5.3	23.8
		10	60	32 (50.9)	0	64.3	9.	32.1
	NT	2.5	59	6 (5.4)	0	0	0	0
		5	60	27 (40.9)	0	66.7	7.4	15.2
		10	56	30 (51.0)	0	34.6	16.7	19.2
Lemongrass	CT	2.5	60	19 (26.6)	0	0	10.5	2.4
		5	57	40 (69.1)	0	0	2.5	11.8
		10	60	13 (20.3)	40	100	0	92
	NT	2.5	58	6 (3.9)	0	0	16.7	37
		5	60	29 (45.5)	0	0	6.9	9.7
		10	57	3 (1.8)	0	100	0	98.2
Citronella	CT	2.5	58	29 (45.4)	0	10.3	6.9	10.3
		5	56	38 (66.1)	0	6.9	0	6.9
		10	59	6 (3.)	50	86.8	16.7	15.1
	NT	2.5	58	24 (37.0)	4.2	14.7	4.2	2.9
		5	58	28 (44.5)	0	8.8	0	8.8
		10	57	5 (4.0)	40	94.2	0	5.8
Plai	CT	2.5	60	19 (26.7)	0	0	0	0
		5	59	20 (31.0)	0	0	0	2.8
		10	58	33 (54.6)	0	0	0	4
	NT	2.5	60	13 (17.6)	0	0	0	0
		5	58	15 (21.4)	0	0	6.7	2.3
		10	60	9 (9.8)	0	0	0	2.1
DEET	CT	2.5	60	24 (35.6)	0	0	0	0
		5	57	24 (36.4)	0	74.3	0	22.9
		10	58	10 (12.7)	10	89.6	20	33.3
	NT	2.5	59	6 (7.0)	0	0	0	0
		5	60	12 (14.3)	0	0	0	2.1
		10	60	21 (33.9)	0	20.5	4.7	2.6

Esc.: Escaped mosquitoes that leave the chamber; NonEsc.: Non-escaped mosquitoes that remain in the chamber; CT: contact treatment; NT: noncontact treatment.

*Adjusted (corrected) percent escape based on paired control response. Percentage escape rounded to nearest tenth.

Table 2. Probit analysis lethal concentration (LC_{50}) percent values at 24 h for nulliparous *Ae. aegypti* adult mosquitoes to five essential oils and DEET.

Treatment	N	LC_{50} (%)	95% CL	Slope±SE	Chi-Square	*P*>ChiSq
Hairy basil	104	6.73	5.98-7.46	7.8380±1.1737	44.59	0.3407
Ginger	80	13.50	9.26-50.66	2.5949±0.8397	9.55	0.1400
Lemongrass	109	6.28	4.07-8.45	6.2844±1.6988	13.68	0.0003
Citronella	100	4,023	-[a]	0.3997±0.6221	0.41	0.2179
Plai	105	158.55	-[a]	1.2851±1.1792	1.19	0.5529
DEET	119	13.63	9.88-33.35	2.4719±0.6880	12.91	0.3364

[a] Not determined.

Table 3. Log-rank comparisons of the escape responses between paired control and contact trials, control and noncontact trials, and paired contact and noncontact trials for *Ae. aegypti* nulliparous females exposed to five essential oils and DEET.

Compound	Dosage	Control vs Contact	Control vs Noncontact	Contact vs Noncontact
Hairy basil	2.5%	<0.0001*	<0.0001*	0.4987
	5%	0.0003*	0.0024*	0.4160
	10%	0.7171	0.0232*	0.0540
Ginger	2.5%	<0.0001*	0.7387	<0.0001*
	5%	<0.0001*	<0.0001*	0.0550
	10%	<0.0001*	<0.0001*	0.8083
Lemongrass	2.5%	0.0008*	0.6994	0.0040*
	5%	<0.0001*	<0.0001*	0.0062*
	10%	0.0009*	0.5625	0.0010*
Citronella	2.5%	<0.0001*	<0.0001*	0.4088
	5%	<0.0001*	<0.0001*	0.0780
	10%	0.4861	0.4276	0.8032
Plai	2.5%	0.0007*	0.0083*	0.2745
	5%	0.0001*	0.0023*	0.2090
	10%	<0.0001*	0.1238	<0.0001*
DEET	2.5%	<0.0001*	0.1460	0.0002*
	5%	<0.0001*	0.0351*	0.0118*
	10%	0.0402*	<0.0001*	0.0243*

*Log-rank tests with statistically significant ($P<0.05$) differences in escape response between pairs.

Table 4. Multiple paired log-rank comparisons of escape responses between three different concentrations of essential oil in nulliparous *Ae. aegypti* exposed to contact and noncontact trials in the excito-repellency test system.

Essential oil	Dosage	Contact trial (*P*)	Noncontact trial (*P*)
Hairy basil	2.5% vs 5%	<0.0001*	<0.0001*
	2.5% vs 10%	<0.0001*	<0.0001*
	5% vs 10%	0.0027*	0.6888
Ginger	2.5% vs 5%	0.0889	<0.0001*
	2.5% vs 10%	<0.0001*	<0.0001*
	5.0% vs 10%	0.2650	0.3089
Lemongrass	2.5% vs 5%	<0.0001*	<0.0001*
	2.5% vs 10%	0.1908	0.0570
	5% vs 10%	<0.0001*	<0.0001*
Citronella	2.5% vs 5%	0.0554	0.4068
	2.5% vs 10%	<0.0001*	<0.0001*
	5% vs 10%	<0.0001*	<0.0001*
Plai	2.5% vs 5%	0.4497	0.6143
	2.5% vs 10%	0.0012*	0.4822
	5% vs 10%	0.0200*	0.2267
DEET	2.5% vs 5%	0.9716	0.1441
	2.5% vs 10%	0.0061*	0.0012*
	5% vs 10%	0.0054*	0.0517

*Log-rank tests with statistically significant ($P<0.05$) differences in escape response between pairs.

Table 5. Log-rank comparison of escape responses of nulliparous *Ae. aegypti* exposed to five essential oils and DEET at a concentration of 2.5%.

Insect repellent	Essential oil	Contact	Noncontact
DEET	Hairy basil	0.0003*	<0.0001*
DEET	Ginger	0.6439	0.9752
DEET	Lemongrass	0.3206	0.9898
DEET	Citronella	0.3049	0.0001*
DEET	Plai	0.2063	0.0973

*Log-rank tests with statistically significant ($P<0.05$) differences in escape response between pairs.

Table 6. Log-rank comparison of escape responses of nulliparous *Ae. aegypti* exposed to Hairy basil oil compared to four other essential oils at a concentration of 2.5%.

Essential oils	Contact	Noncontact
Hairy basil vs Ginger	0.0007*	<0.0001*
Hairy basil vs Lemongrass	<0.0001*	<0.0001*
Hairy basil vs Citronella	0.0066*	0.0029*
Hairy basil vs Plai	<0.0001*	<0.0001*

*Log-rank tests with statistically significant ($P<0.05$) differences in escape response between pairs.

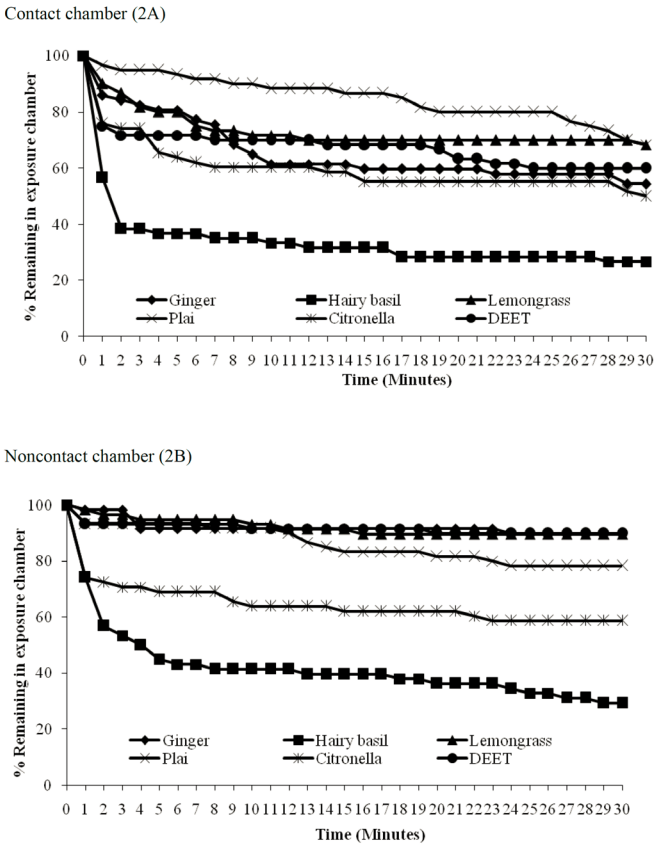


Figure 2. Survival analysis showing cumulative percent of *Ae. aegypti* escaping the treated test chambers at a 2.5% concentration of five essential oils and DEET during a 30-min contact (2A) and noncontact (2B) exposure.

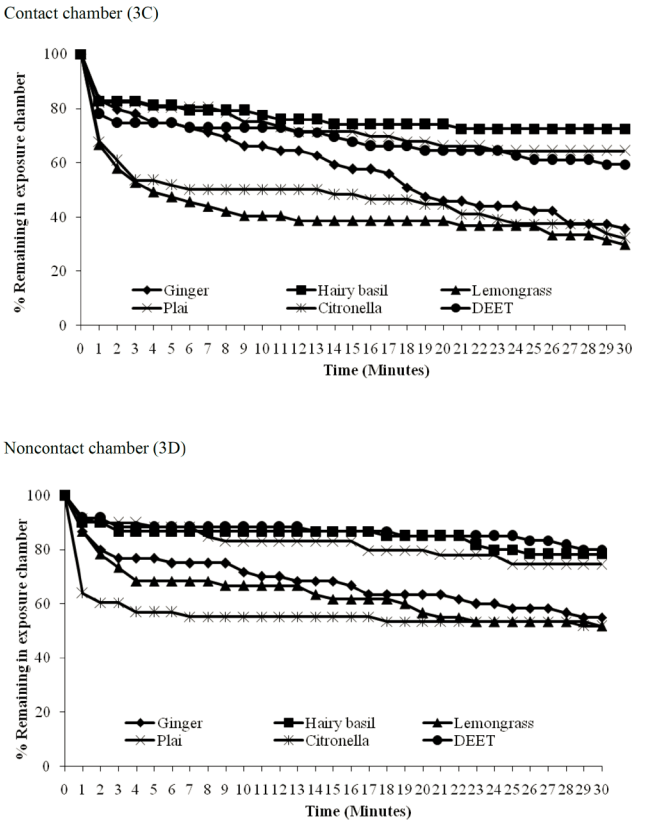


Figure 3. Survival analysis showing cumulative percent of *Ae. aegypti* escaping the treated test chambers at a 5% concentration of five different essential oils and DEET during a 30-min contact (3C) and noncontact (3D) exposure.

In both contact and noncontact trials, escape responses of *Ae. aegypti* females exposed to hairy basil oil at 2.5% were higher than other test combinations (Figure 2). With lemongrass oil, strong contact escape patterns were seen when using 5% concentrations compared to the other essential oils at the same dose (69%) (Figure 3).

DISCUSSION

Several studies have shown that various plant species contain a number of phytochemical substances that can be used as repellents against mosquitoes or other blood-sucking pests (Pålsson and Jaenson 1999, Trongtokit et al. 2004, Odalo et al. 2005, Zhu et al. 2006, Noosidum et al. 2008, Polsomboon et al. 2008, Kumar et al. 2011, Suwansirisilp et al. 2012). However, comparatively few studies have identified the precise chemical actions (contact irritant and noncontact repellent actions) of plant derivatives to mosquitoes using the ER system. In Thailand, Polsomboon et al. (2008) first observed the strong irritancy responses of field-collected *Ae. aegypti* to catnip oil using the ER system. Subsequently, Noosidum et al. (2008) investigated the excito-repellent responses of three wild herb species, *Melaleuca leucadendron* L., *Litsea cubeba* (Lour.) Persoon, and *Litsea salicifolia* (Nees) on *Ae. aegypti*. Results showed that wild herbs elicited strong irritant actions in *Ae. aegypti*. Recently, Suwansirisilp et al. (2012) observed the behavioral action of four commercially available essential oils, including orange oil, cinnamon oil, citronella oil, and clove oil on two field populations of *Ae. aegypti* and *Culex quinquefasciatus*. Results demonstrated that oils from citronella and clove yielded a stronger irritancy response than repellency.

In this study, we investigated the two nontoxic properties of five different botanicals that have been extensively used in Thailand for personal protection from mosquito bites. Essential oils extracted from native plants included hairy basil leaf (*Ocimum americanum* Linn), ginger (*Zingiber officinale* Roscoe), lemongrass (*Cymbopogon citratus* Stapf), citronella (*Cymbopogon nardus* Rendle), and plai (*Zingiber cassumunar* Roxb). However, no data exists to support the use of these oils for personal use. This investigation is the first to quantify the behavior modifying properties of these natural compounds and further highlights the need to optimize and standardize their use to achieve maximum impact. The ER test system demonstrated its value in assessing behavioral responses in mosquitoes and is a valuable tool for screening these fundamental activities for public health purposes. In addition, oils from other botanical sources should be screened for protective properties against a variety of mosquito species. This study revealed that significant behavioral avoidance responses were observed in contact trials compared to paired controls for all oils tested and that the responses were similar for all compounds. The most striking escape response in *Ae. aegypti* occurred after

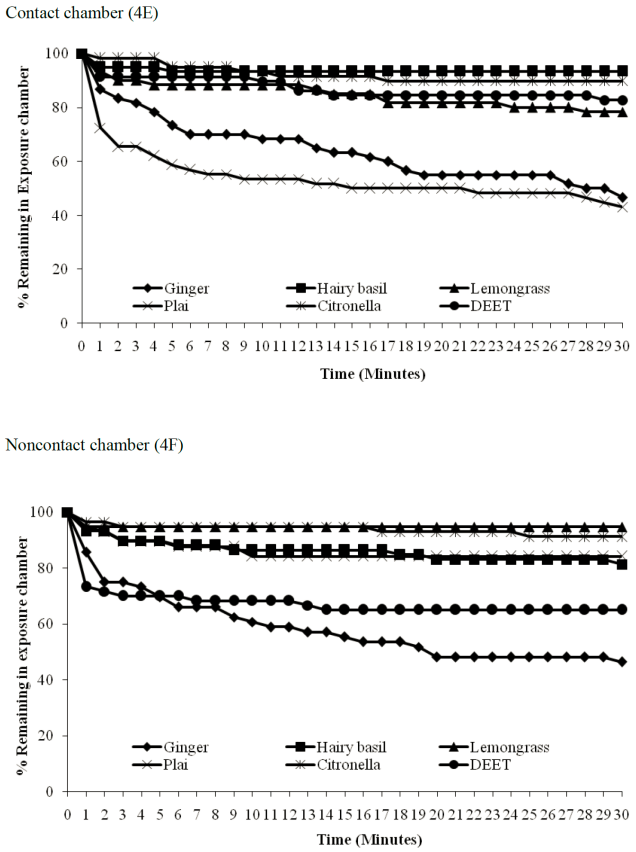


Figure 4. Survival analysis showing cumulative percent of *Ae. aegypti* escaping the treated test chambers at a 10% concentration of five different essential oils and DEET during a 30-min contact (4E) and noncontact (4F) exposure.

physical contact with hairy basil oil. The use of native plant oils traditionally used to repel or prevent the bites of mosquitoes as alternatives to the current commercially available synthetic ingredients has been greatly understudied (Walker et al. 1996, Moore et al. 2007).

Information obtained from this study will help to better understand how essential oils affect mosquito behavior. The 2.5% hairy basil oil induced the maximum escape response with approximately 70% of *Ae. aegypti* successfully escaping within 30 min in both contact and noncontact tests. Lemongrass and hairy basil showed the highest toxicity against *Ae. aegypti* (LC_{50} values, 6.28% and 6.73%, respectively). Citronella oil produced the least toxicity based on a LC_{50} of 4,023. However, Lemongrass gave poor goodness-of-fit for the model, accounted for by multiplying the variances by a heterogeneity factor (χ^2/k - 2), where k is the number of concentrations to account for extra-binomial variations that creates poor fit (Schleier III and Peterson 2010). Phasomkusolsil and Soonwera (2011b) showed Lemongrass, *C. citratus* (Stapf) and Citronella grass, *C. nardus* (Rendle) oils produced toxicity against *Ae. aegypti* with LC_{50} values of <0.10% and 0.26%, respectively. A knockdown effect against *Ae. aegypti* population was observed with hairy basil (*Ocimum americanum* Linn) and Lemongrass oil (*Cymbopogon citratus* Stapf) and at least 85% knockdown for citronella at the highest dose (10%) (Table 1). This indicated that the volatilized test compound overwhelmed the mosquitoes resulting in knockdown. Suwansirisilp et al. (2012) reported that citronella oil acts as a knockdown agent for *Ae. aegypti* in both contact (50%) and noncontact (70%) tests. In Sri Lanka, Lemongrass has also shown knockdown effects on other insects such as adult *Musca domestica,* wherein Samarasekera et al. (2006) reported good knockdown and mortality activity at LD_{50} of 1.71 µg/insect, compared to higher doses required for citronella oil (LD_{50} 3.10 µ/insect). However, in our study a high percentage of recovery from initial knockdown was observed at 10% citronella oil for both contact (71.7%) and noncontact (88.4%) trials. Another interesting point is that hairy basil and plai did not affect the mortality rate of escaped mosquitoes from the contact trials even at the higher concentrations.

Duration of efficacy of the essential oils on mosquito behavior was not measured in this study, but it is important to note that citronella oil from different sources may result in different protection times. Recently, Phasomkusolsil and Soonwera (2010) reported that essential oils from citronella grass (*Cymbopogon nardus* Rendle) provided protection times of 130 min, 140 min, and 115 min against *An. minimus, Cx. quinquefasciatus,* and *Ae. aegypti*, respectively. Tawatsin et al. (2001) reported that citronella and hairy basil oil provided repellency against *Ae. aegypti* for approximately 3 h.

Currently, there is no effective vaccine against dengue, and due to insecticide resistance, many of our chemical control strategies are no longer effective for controlling the vector populations. Despite this fact, vector control remains a critical means of fighting this mosquito-borne disease. One alternate method of chemical control is the use of repellents for personal protection. Prevention of mosquito bites by natural insect repellents has become more popular, and there are many commercially available repellents on the market. In Thailand, many botanical plants hold the potential as spatial repellents for use against disease-carrying mosquito species. Evaluations of the anti-biting activity of these plant species are required, however, to determine the effectiveness and proper dosing of the essential oils derived from these plants to develop effective repellents for protection against vector-borne diseases.

Acknowledgments

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Pyrethroid susceptibility and behavioral avoidance in *Anopheles epiroticus*, a malaria vector in Thailand

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ABSTRACT: The physiological susceptibility to insecticides and the behavioral responses of four wild-caught populations of female *Anopheles epiroticus* to synthetic pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin) were assessed. Test populations were collected from different localities along the eastern coast, Trat (TR), Songkhla (SK), and Surat Thani (ST) and one population from the western coast, Phang Nga (PN). Results showed that all four populations of *An. epiroticus* were susceptible to all three synthetic pyrethroids tested. Behavioral responses to test compounds were characterized for all four populations using an excito-repellency test system. TR displayed the strongest contact excitation ('irritancy') escape response (76.8% exposed to deltamethrin, 74.1% permethrin, and 78.4% alpha-cypermethrin), followed by the PN population (24.4% deltamethrin, 35% permethrin, and 34.4% for alpha-cypermethrin) by rapidly escaping test chambers after direct contact with surfaces treated with each active ingredient compared with match-paired untreated controls. Moderate non-contact repellency responses to all three compounds were observed in the TR population but were comparatively weaker than paired contact tests. Few mosquitoes from the SK and ST populations escaped from test chambers, regardless of insecticide tested or type of trial. We conclude that contact excitation was a major behavioral response in two populations of *An. epiroticus*, whereas two other populations showed virtually no escape response following exposure to the three pyrethroids. The explanation for these large unexpected differences in avoidance responses between pyrethroid-susceptible populations of the same species is unclear and warrants further investigation. ***Journal of Vector Ecology* 39 (1): 32-43. 2014.**

Keyword Index: *Anopheles epiroticus*, behavior avoidance, pyrethroids, excito-repellency.

INTRODUCTION

Malaria is one of the most important mosquito-borne diseases in Thailand with approximately 30,000 reported cases each year. The disease is relatively common in hilly-forested areas, especially near the Thai-Myanmar and Thai-Malaysia national borders where favorable environmental conditions and efficient malaria vectors are present. Malaria transmission also remains prevalent in some foci along the coastal zones of Thailand where *Anopheles epiroticus* Linton & Harbach is active (Manguin et al. 2010, Dusfour et al. 2007). *Anopheles epiroticus* (formerly *Anopheles sundaicus* species A) is a member of the *Anopheles sundaicus* complex and has been shown epidemiologically to be an important vector of human plasmodia on the Southeast Asian mainland (Dusfour et al. 2004). In Thailand, this species has been regarded as a secondary or focally important vector of malaria in insular and coastal areas of the country (Gould et al. 1966, Harinasuta et al. 1974, Prasittisuk 1985, Sumruayphol et al. 2010).

DDT was the chemical of choice in the 1950s and was used extensively in malaria endemic areas. Its use was gradually phased out in Thailand between 1995 and 2000 and replaced by two pyrethroids, deltamethrin and permethrin (Chareonviriyaphap et al. 1999, 2000). Deltamethrin has been used primarily for indoor residual spraying (IRS) of house surfaces and permethrin for treatment of bed-nets and window and door curtains (Chareonviriyaphap et al. 2004). Additionally, alpha-cypermethrin has gradually become more common for protection against indoor and outdoor biting mosquitoes, including *Anopheles* (Grieco et al. 2007, Mongkalangoon et al. 2009). As pyrethroids have begun to lose effectiveness against mosquitoes that have developed significant resistance against them in many places worldwide (Corbel and N'Guessan 2013), it is crucial to continue monitoring how mosquitoes respond to synthetic pyrethroids, including behavioral responses to sub-lethal concentrations.

Understanding the behavioral responses of different mosquito populations to insecticides can facilitate vector control by selecting and implementing the most effective interventions that better target the primary mosquito vectors. In general, two types of stimulus-mediated responses by mosquitoes to insecticides have been recognized, excitation ('irritancy') and repellency, which disrupt normal patterns of host-seeking or resting behavior (Roberts et al. 2000). As defined, irritancy occurs following physical (tarsal) contact with chemical residues, whereas spatial repellency acts at a distance from an insecticide-treated surface

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that effectively deters insects from entering a treated area. The system for describing and quantifying the excitatory effects of insecticides on mosquitoes was first developed in the early 1960s and has been modified over subsequent decades (Rachou et al. 1963, Evans 1993, Chareonviriyaphap et al. 1997, Roberts et al. 2000). An improved excito-repellency chamber (ER) system for testing both contact irritancy and non-contact repellency has been modified further into a collapsible chamber designed for greater ease of use and set-up (Chareonviriyaphap et al. 2002).

Although studies have shown clear behavioral avoidance in various *Anopheles* species (Sungvornyothrin et al. 2001, Chareonviriyaphap et al. 2002, 2006, Pothikasikorn et al. 2005, Muenworn et al. 2006), no observations have been made on the responses of *An. epiroticus* to synthetic pyrethroids. Described herein are observations using an excito-repellency test system to quantitatively measure behavioral responses between four wild-caught populations of *An. epiroticus* against the recommended operational dosage of deltamethrin, permethrin, and alpha-cypermethrin.

MATERIALS AND METHODS

Anopheles epiroticus test populations

The Trat population (TR) was obtained using a live buffalo as bait and a mouth aspirator to collect resting mosquitoes off nearby netting in Klong Yuan Village, Ko Chang District, Trat Province, southeastern Thailand (12 º 02' N, 102 º 23' E). The Songkhla population (SK) was obtained from a cow-bait collection method using mouth aspirators to capture mostly blood-fed resting mosquitoes in Bang Not Nai Village, Hat Yai District, Songkhla Province, southern Thailand (7° 46' N, 100° 28' E). The Surat Thani population (ST) was obtained using a cow-bait method and mouth aspirator in Laem Sui Village, Chaiya District, Surat Thani Province, southern Thailand (9° 24'N; 99° 18'E). The Phang Nga population (NG) was also collected using cattle as bait and mouth aspirator in Laem Pakarang Village, Takua Pa District, Phang Nga Province, southern Thailand (8° 43'N; 98° 14'E). The buffalo/cow-baited capture method involved using one or more animals to attract host-seeking mosquitoes, allowing them to first blood feed and subsequently rest on netting set up 0.3 m from ground level and placed around the periphery of the animal holding areas. Collections were conducted 15 min each hour from 18:00-06:00. All female mosquitoes were held in a plastic cup covered with netting and provided a cotton pad saturated with 10% sugar solution while awaiting species identification and processing for testing.

Morphological and molecular anopheline species identification

Female mosquitoes were identified to either species or species group using the morphological keys of Rattanarithikul et al. (2006) before testing and subsequently, all test specimens initially identified as *Anopheles sundaicus* complex and used in the experiments were individually identified using DNA extraction procedures (Linton et al. 2001). Molecular analysis for sibling species identification within the *An. sundaicus* complex was performed by allele-specific polymerase chain reaction assay (AS-PCR) described by Dusfour et al. (2007). Following each bioassay, mosquitoes were frozen using liquid nitrogen and stored individually in cryo-tubes to prevent cross-contamination until tested.

Insecticides

The three insecticides selected for susceptibility assays and excito-repellency tests belong to the pyrethroid class of chemicals: 1. Deltamethrin [(*S*)-alphacyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2 dimethylcyclopropanecarboxylate] provided by BASF, Aktiengescllshaft Bangkok, Thailand in August, 2008; 2. Permethrin [(3-phenoxybenzyl (1 *RS*, 3 *RS*, 1*RS*, 3 *SR*)-3 -(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate)] provided by Ladda Company, Bangkok, Thailand in October 2010; and 3. Alpha-cypermethrin ((*R*)-cyano (3-phenoxyphenyl) methyl (1*S*,3*S*)-*rel*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) received from BASF Aktiengescllshaft, Thailand in August 2010.

Insecticide treated papers

Insecticide treated papers were produced at the Department of Entomology, Kasetsart University, based on World Heath Organization procedures and specifications. For susceptibility tests, test papers of each active ingredient were prepared using Whatman® No. 1 paper at 12 x 15 cm size using the standard operational diagnostic doses of 0.05% deltamethrin and 0.75% permethrin. Alpha-cypermethrin was tested at a concentration of 0.082%. For excito-repellency tests, all test papers (15x 17.5 cm size) were individually and uniformly treated with standard field doses of 0.02 g/m^2 deltamethrin, 0.3 g/m^2 permethrin, and 0.03 g/m^2 of alpha-cypermethrin. Control papers were impregnated with solvent only (acetone mixed with silicon oil).

Insecticide susceptibility tests

The World Health Organization commercial test kits were used in this study. Twenty-five wild-caught, predominantly blood-fed female mosquitoes were introduced into each respective holding tube configuration containing either an insecticide-treated paper and non-treated holding paper or a control with a non-insecticide paper treated with diluents carrier only and a non-treated holding paper. Following a brief holding period (~5 min), all mosquitoes were exposed for 60 min to either treated or control paper surfaces. Immediately after exposure, the number of knockdown mosquitoes in each test was recorded and all specimens transferred into corresponding clean holding tubes and provided with 10% sucrose cotton pads. The mortality of test and control mosquitoes was recorded after 24 h post-exposure. If the matched control mortality was greater than 5%, the final percent test mortality would be adjusted using Abbott's formula (Abbott 1925). Four replicates of paired controls and treatments for each insecticide were performed. The susceptibility to each insecticide was evaluated by the WHO revised criteria.

Excito-repellency tests

An excito-repellency test system was used to evaluate the behavioral responses of *An. epiroticus* to a discriminating concentration of each insecticide. The system consisted of two treatment chambers containing insecticide-treated papers (one chamber for contact and another for non-contact treatment) and two matched control chambers containing only oil-based carrier

(non-insecticidal) treated papers Chareonviriyaphap et al. (2002). One test design allows the mosquitoes the opportunity to make direct contact with the treated paper and therefore measures a combined 'excito-repellency' response, excitation (irritancy) as a primary action and spatial repellency as a secondary action. The non-contact test design provides a screened barrier between mosquito and treated surface and thus assesses repellency alone.

All tests were performed between 08:00-16:30 under natural field conditions. For each test, 15 (most recently blood-fed) female mosquitoes were released into each of the four chambers. Mosquitoes were allowed to acclimate to the inside of the chamber for approximately three minutes. At the beginning of the test, the number of mosquitoes that escaped from the respective chambers into the attached receiving cage was recorded at 1 min intervals for a period of 30 min. All mosquitoes escaping during each 1 min interval were transferred to clean cups. At the end of 30 min, the number of dead or knockdown mosquitoes was recorded separately from inside each exposure chamber and external holding cage for both treatment and control set-ups. All live mosquitoes from each chamber were held separately and provided with 10% sugar solution as nutrition. After 24 h, mortality was recorded and corrected as above, when applicable. All assays were replicated four times per treatment for a total 240 mosquitoes (60 contact and 60 non-contact with matched controls) for each insecticide.

Data analysis

The susceptibility to each insecticide was assessed according to the following WHO revised criteria: Final mortality between 98% and 100%, a test population is considered 'susceptible'; mortality between 90% and 97% is either treated as 'suggestive of the existence of resistance' and requiring further investigation (i.e., additional bioassays or determination of mechanism(s); and mortality below 90% is an indication of outright 'resistance' in the population tested. If the observed mortality (corrected if necessary) is between 90% and 97%, the presence of resistant genes in the vector population should be confirmed (not conducted in this study). If at least two additional tests consistently show mortality below 98%, then resistance is confirmed. Lastly, if mortality is less than 90%, confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary, provided a minimum of 100 mosquitoes of each species (population) is tested.

Percentage of escape obtained from the treated exposure chambers was adjusted based on matched-paired control escape responses using Abbott's formula. Likewise, when control mortality was between 5% and 20%, the final mortality percent in insecticide exposed samples was also adjusted accordingly. Kaplan-Meier survival (life table) analysis was used to estimate mosquito escape rates over time for each test configuration (i.e., by chemical, contact, and non-contact configurations, respectively). (Kleinbaum 1996, Roberts et al. 1997). Survival analysis was also used to estimate the escape time (ET) at which 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of the test populations escaped from the chamber. Patterns of escape behavior were evaluated within the test cohorts and among different treatment groups using a log-rank method (Mantel and Haenzel 1959). Statistical significance for all tests was set at $P < 0.05$.

RESULTS

All four populations demonstrated complete (100%) susceptibility to all three compounds. Matched control mortalities were negligible (0-4%), therefore final percent test mortalities did not require a calculated correction.

The percentage of *An. epiroticus* that escaped within a 30 min period exposed to either deltamethrin, permethrin, or alpha-cypermethrin in contact and non-contact trials are presented in Tables 1 and 2. Among the four populations, TR demonstrated the strongest escape responses in both contact (78.4% alpha-cypermethrin, 76.8% deltamethrin, and 74.1% permethrin) and non-contact trials (46.3% for alpha-cypermethrin,17.5% deltamethrin, and 37.6% permethrin), followed by the PN population in contact trials (34.4% alpha-cypermethrin, 24.4% deltamethrin, and 35% permethrin). Conversely, the ST displayed no or low escape responses to all three compounds in both contact (0.1% alpha-cypermethrin, 3.4% deltamethrin, and 0% permethrin) and non-contact trials (1.7% alpha-cypermethrin, 0% deltamethrin, and permethrin) trials. SK showed no responses to deltamethrin (0%) in contact trials and to permethrin (0%) in non-contact trials. There was a relatively low number of mosquitoes that escaped from the control chambers in both the contact (0-16.1%) and non-contact configurations (0-17.0%).

The mortality of mosquitoes after a 24 h holding period from treated and untreated chambers with matched controls is shown in Tables 1 and 2. In general, for both TR and PN populations, higher mortalities were observed in contact vs non-contact trials as well as higher mortalities in non-contact tests compared to matched controls (control data not shown). Higher mortalities were typically observed in contact tests among mosquitoes remaining in chambers compared to those that escaped; the lone exception was the PN population exposed to alpha-cypermethrin. Among remaining mosquitoes in contact trials, SK population gave the highest mortalities (84% alpha-cypermethrin, 61% deltamethrin, and 65% permethrin). Lower percent mortalities were observed from escaped mosquitoes in all populations, except the SK population in which 61% succumbed to alpha-cypermethrin and 67% to permethrin (albeit total escape numbers were low that influenced final percent kill). In non-contact trials, mortalities of escaped and remaining mosquitoes were generally low, ranging between 0-40% for escaped and between 0-37% for remaining specimens. The control percent mortality in contact trials was generally low but appears inflated in a few instances because of low numbers escaping the chambers. In the non-contact trials the control mortalities of both escaped and remaining mosquitoes were generally much lower than the treatment mortalities, except in the SK alpha-cypermethrin and permethrin trials and in the PN deltamethrin trial where the control mortalities appeared elevated (Tables 1 and 2). Notable exceptions included SK alpha-cypermethrin control mortality (17% and 65%), permethrin (40% and 33.3%), and PN deltamethrin (5.1% and 30.5%), in escaped and remaining mosquitoes, respectively.

Times in minutes for mosquitoes to escape from treated chambers are given in Table 3. Escaped mosquitoes that departed from insecticide-treated chambers within 30 min are grouped as 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of total test sample escaping. In general, ET_{25}, ET_{50}, and ET_{75} values of TR population

Table 1. Percentage escape and 24 h mortality of *Anopheles epiroticus* exposed to alpha-cypermethrin (Acyp 0.03 g/m^2), deltamethrin (Delta 0.02 g/m^2), and permethrin (Perm 0.3 g/m^2) in contact trials.

Chemical	Population	No. Mosquitoes	% Escaped*	% Mortality*	
				Escaped	Remain
Acyp	TR	Treatment (59)	78.4	22.9	36.4
		Control (58)	13.8	0	0
	SK	Treatment (59)	1.8	61.4	84
		Control (58)	16.1	22.2	46.8
	PN	Treatment (59)	34.4	25.4	17.8
		Control (57)	7.0	0	8.8
	ST	Treatment (58)	0.1	0	62.1
		Control (60)	3.3	1.7	0
Delta	TR	Treatment (60)	76.8	10.6	60.8
		Control (59)	6.8	0	1.8
	SK	Treatment (60)	0	4.0	60.9
		Control (59)	10.0	16.7	14.8
	PN	Treatment (60)	24.4	22.3	49.4
		Control (58)	5.2	3.4	27.6
	ST	Treatment (60)	3.4	5.0	53.5
		Control (62)	1.6	0	3.2
Perm	TR	Treatment (59)	74.1	0	13.7
		Control (60)	15.0	11.1	2.0
	SK	Treatment (59)	14.3	66.7	64.5
		Control (60)	1.7	0	41.4
	PN	Treatment (60)	35.0	15.0	28.1
		Control (60)	0	0	5.0
	ST	Treatment (60)	0	0	37.3
		Control (60)	1.7	0	1.7

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani
*Treatment percent escape and mortality adjusted based on paired control responses.

from contact trials were comparatively low, consisting of 1, 5, and 17 min for alpha-cypermethrin, 1, 2, and 16 min for deltamethrin, and 1, 4, and 16 min for permethrin, respectively. For non-contact trials, ET_{25} values for TR population were 10, 29, and 19 min for alpha-cypermethrin, deltamethrin, and permethrin, respectively. The PN population showed ET_{25} on contact trials only of 18, 6, and 10 min for alpha-cypermethrin, deltamethrin and permethrin, respectively. Escape times could not be determined for SK and ST populations due to insufficient numbers of mosquitoes escaping during the test period (Table 3).

Log-rank comparisons between the three insecticides and test conditions (contact and non-contact trials) are presented in Tables 4 and 5. The escape responses in the TR population were significantly greater in the contact and non-contact trials compared to the matched controls, and also between contact vs the non-contact trials (Table 4). The same patterns were observed for the PN population, except in the non-contact vs control trial (Table 4). Generally, for the SK and ST populations there were no significant differences. For non-contact trials, the escape responses within populations comparing insecticides showed no significant difference in the contact and non-contact trials, except for deltamethrin compared to alpha-cypermethrin and permethrin in the TR population, and deltamethrin and permethrin in the PN population (Table 5).

The comparisons of escape probabilities based on survival analysis showing proportions of mosquitoes remaining in treatment and control chambers at 1 min intervals over a 30 min period in response to three insecticides in contact and non-contact trials are presented in Figures 1-3. There were significant differences in excitation seen in all contact and paired controls for TR and PN populations exposed to deltamethrin, permethrin, and alpha-cypermethrin (Table 4). The SK population showed a significant difference when tested against permethrin only. In non-contact and paired controls significant differences was observed in the TR population exposed to all compounds and deltamethrin in SK. Patterns of escape were not significantly different between contact and controls for SK and ST populations for deltamethrin ($P = 0.7687$ of SK, $P = 0.3014$ of ST) and alpha-cypermethrin ($P = 0.7902$ of SK, $P = 0.9691$ of ST).

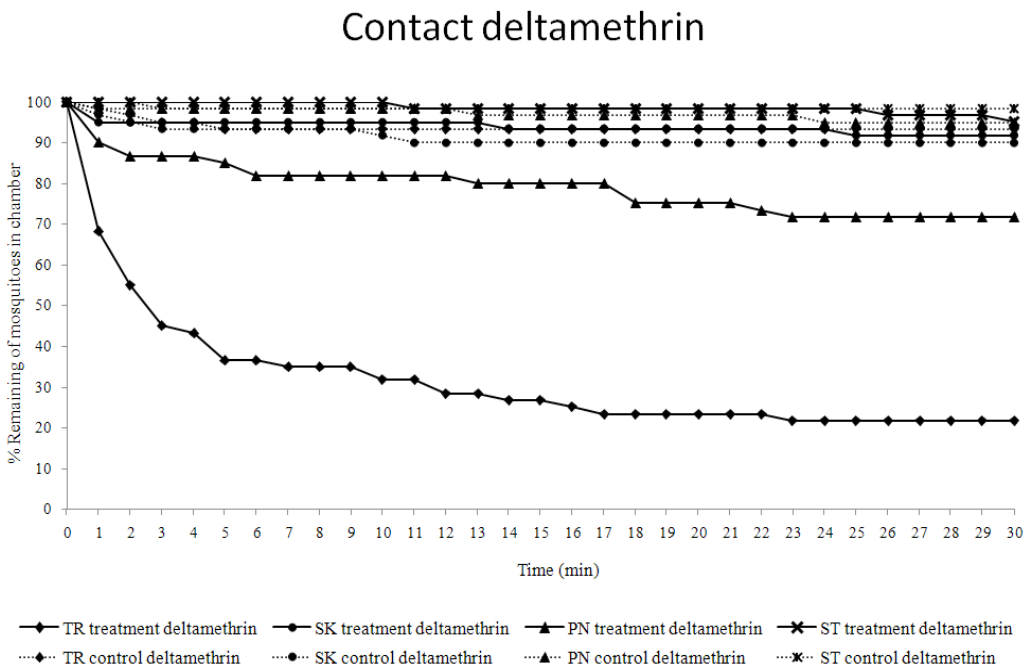


Figure 1A. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.02 g/m^2 deltamethrin in treatment and control contact trials.

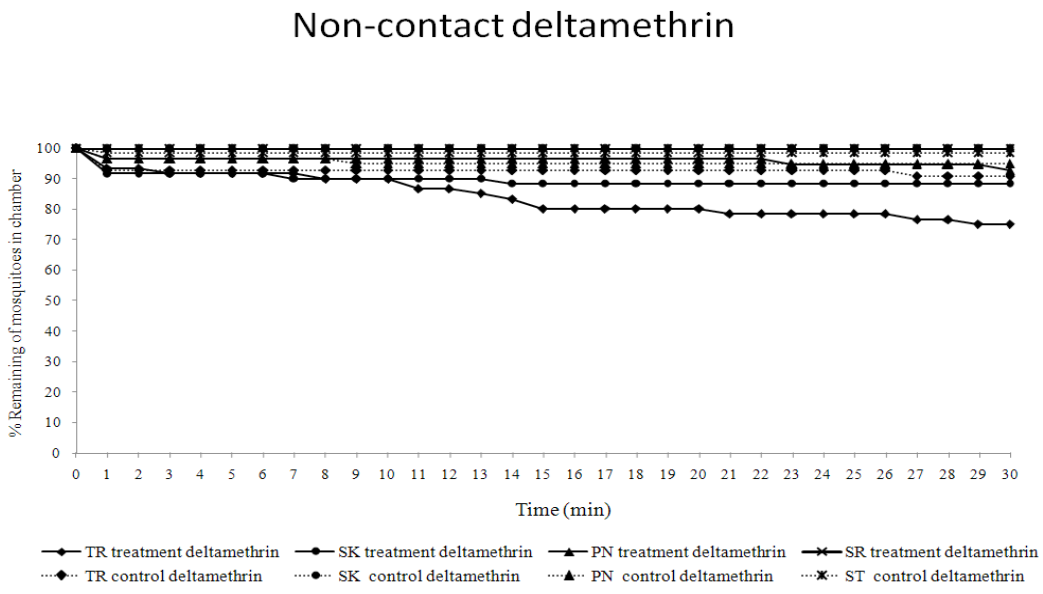


Figure 1B. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.02 g/m^2 deltamethrin in treatment and control non-contact trials.

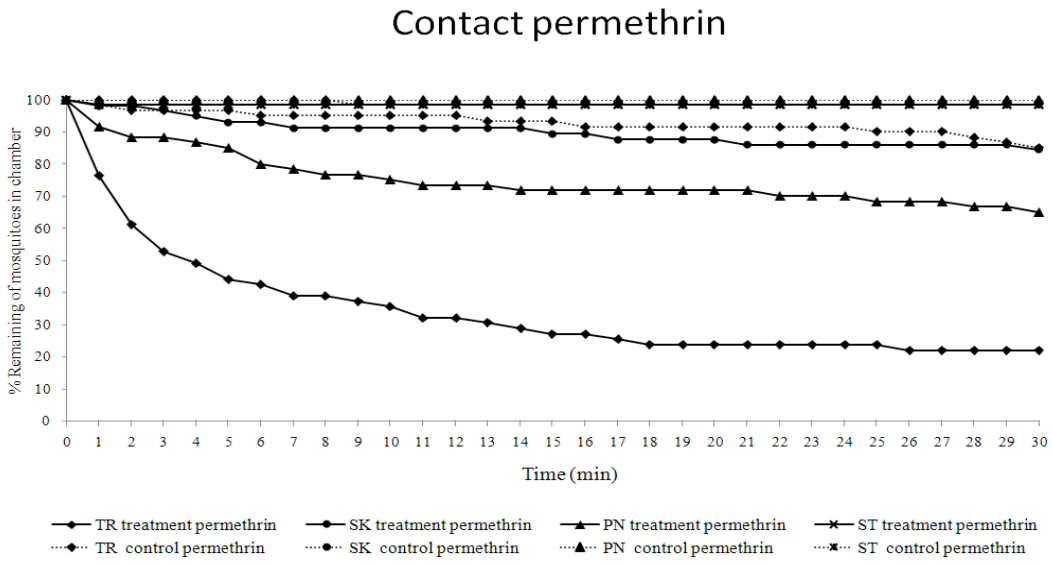


Figure 2A. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.3 g/m^2 permethrin in treatment and control contact trials.

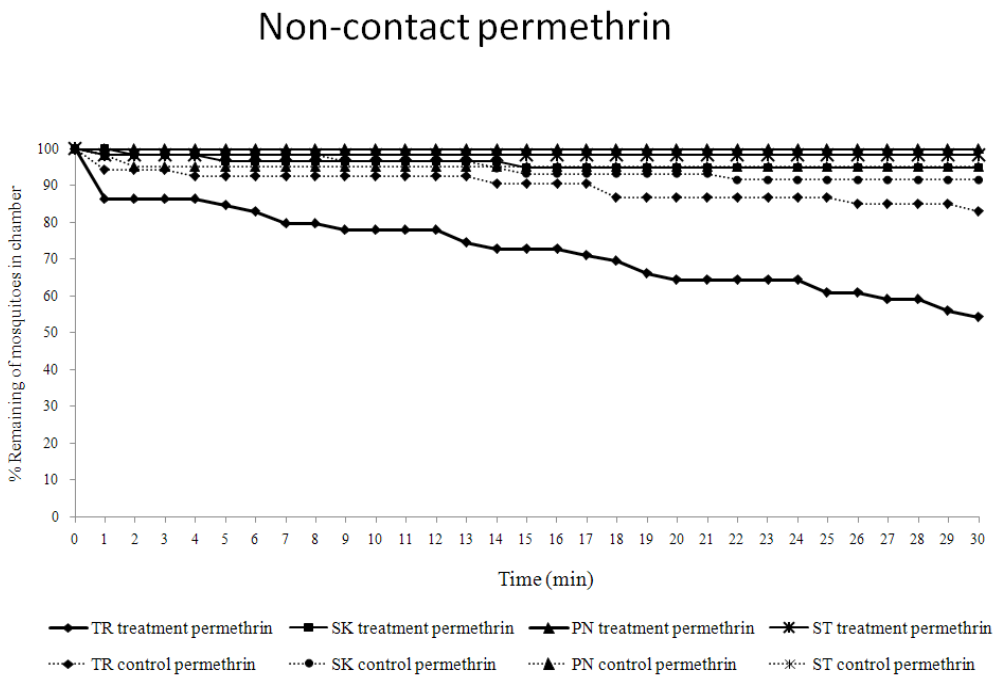


Figure 2B. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.3 g/m^2 permethrin in treatment and control non-contact trials.

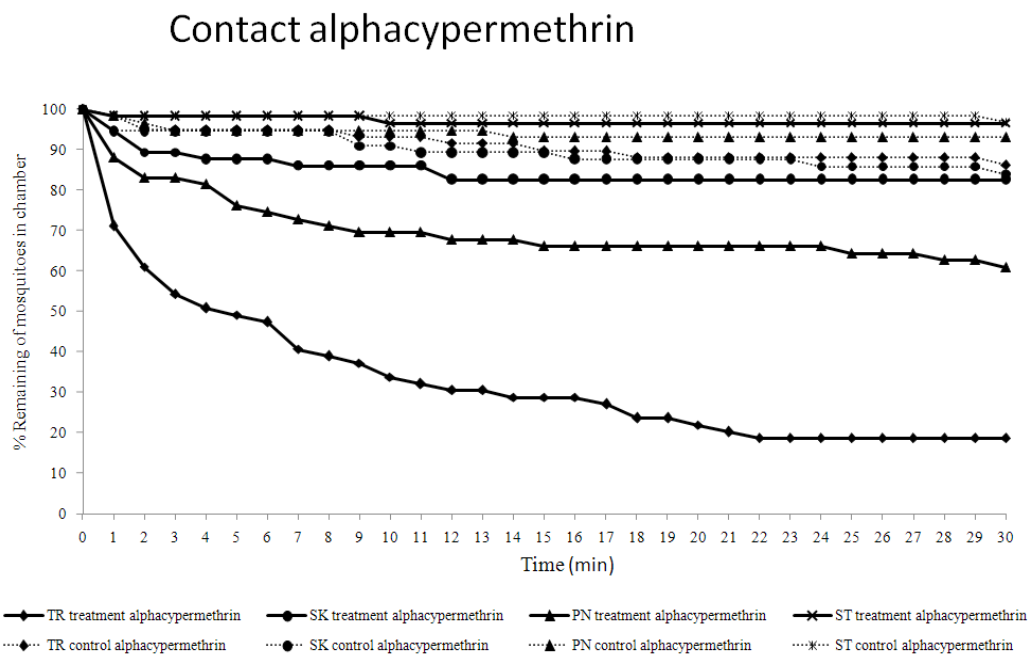


Figure 3A. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.03 g/m^2 alpha-cypermethrin in treatment and control contact trials.

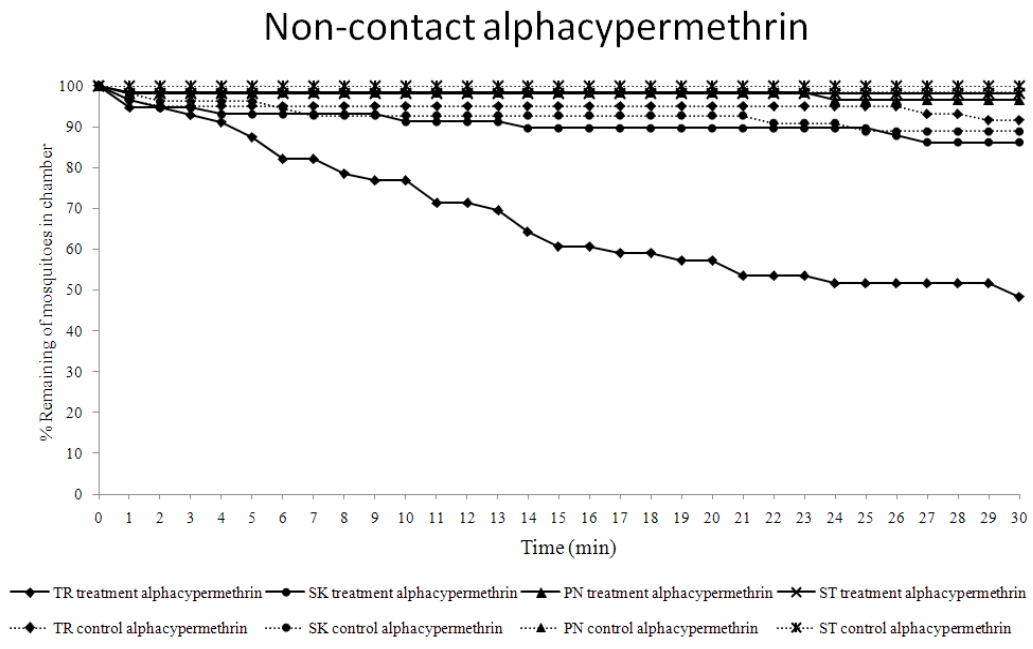


Figure 3B. Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.03 g/m^2 alpha-cypermethrin in treatment and control non-contact trials.

Table 2. Percentage escape and 24-h mortality of *Anopheles epiroticus* exposed to alpha-cypermethrin (Acyp 0.03 g/m^2), deltamethrin (Delta 0.02 g/m^2), and permethrin (Perm 0.3 g/m^2) in combined non-contact trials.

Chemical	Population	No. Mosquitoes	% Escaped*	% Mortality*	
				Escaped	Remain
Acyp	TR	Treatment (56)	46.3	0	0
		Control (59)	10.2	0	1.9
	SK	Treatment (56)	3.2	40.0	36.6
		Control (59)	10.9	16.7	65.3
	PN	Treatment (60)	1.7	0	15.5
		Control (62)	1.6	0	11.3
	ST	Treatment (58)	1.7	0	0
		Control (60)	0	0	0
Delta	TR	Treatment (60)	17.5	0	0
		Control (56)	9.1	0	2.0
	SK	Treatment (60)	11.7	28.5	11.8
		Control (56)	0	0	3.7
	PN	Treatment (58)	2.2	0	7.5
		Control (59)	5.1	5.1	30.5
	ST	Treatment (60)	0	0	8.7
		Control (59)	1.7	0	5.1
Perm	TR	Treatment (59)	37.6	0	0
		Control (53)	17.0	0	0
	SK	Treatment (59)	0	0	0
		Control (53)	8.5	40.0	33.3
	PN	Treatment (60)	0	0	22.2
		Control (60)	5.0	0	10.0
	ST	Treatment (60)	0	0	0
		Control (60)	1.7	0	3.3

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani

* Treatment percent escape and mortality adjusted based on paired control responses.

DISCUSSION

In this study, four field-collected populations of *An. epiroticus* displayed complete susceptibility to deltamethrin, permethrin, and alpha-cypermethrin even though deltamethrin and permethrin in various forms have been used to control malaria since 1990 and 1992, respectively (Chareonviriyaphap et al. 1999). Deltamethrin has been used primarily for indoor residual spraying of homes and both deltamethrin and permethrin for individual and factory treatment of bednets to combat malaria transmission in Thailand. Two of the study locations, Trat (TR) and Phang Nga (PN) provinces are designated areas that experience 'periodic' malaria transmission and have had pyrethroids used in anti-malarial campaigns (TR= permethrin, deltamethrin, and alpha-cypermethrin, and PN= bifenthrin). By the same criteria, both Songkhla (SK) and Surat Thani (ST) provinces areas are regarded as 'low risk' locations and have had no insecticide-based malaria control activities in the collected locations. To date, insecticide resistance has not been reported in *An. epiroticus* in Thailand (Chareonviriyaphap et al. 1999, Dusfour et al. 2004). Recently, Van Bortel et al. (2008) reported that *An. epiroticus* in Vietnam was resistant to four different pyrethroids (permethrin, deltamethrin, alpha-cypermethrin, and lambda-cyhalothrin).

This study is the first report to distinguish two types of behavioral responses, contact irritancy and non-contact repellency, of *An. epiroticus* to deltamethrin, permethrin, and alpha-cypermethrin. We found that wild-caught populations of *An. epiroticus* from TR and PN rapidly escaped from direct contact with surfaces treated with each compound compared to paired untreated controls. Alpha-cypermethrin exhibited the strongest excitatory effect on *An. epiroticus* followed by deltamethrin and permethrin. These findings indicate that contact irritancy is the primary behavioral response of all three compounds. Similar findings of strong contact excitatory action of pyrethroids have been observed in other *Anopheles* species (*An. dirus*, *An. minimus*, *An. harrisoni*, *An. maculatus,* and *An. sawadwongporni* in Thailand (Chareonviriyaphap et al. 2004, Tisgratog et al. 2011, Tananchai et al. 2012).

The most striking escape response following physical contact with each of three synthetic pyrethroids was observed in *An. epiroticus* from Ko Chang Island population from in Trat Province (TR). TR showed the strongest contact and non-contact responses of the four populations tested. The combined 'excito-repellent' effect was strongest with alpha-cypermethrin (78.4%

Table 3. Escape time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of *Anopheles epiroticus* to escape from chambers treated with insecticides during 30 min of exposure.

Populations	Test condition	Alpha-cypermethrin			Deltamethrin			Permethrin		
		ET25	ET50	ET75	ET25	ET50	ET75	ET25	ET50	ET75
TR	Contact	<1	5	17	<1	2	16	1	4	16
	Non-contact	10	29	-	29	-	-	19	-	-
SK	Contact	-	-	-	-	-	-	-	-	-
	Non-contact	-	-	-	-	-	-	-	-	-
PN	Contact	18	-	-	6	-	-	10	-	-
	Non-contact	-	-	-	-	-	-	-	-	-
ST	Contact	-	-	-	-	-	-	-	-	-
	Non-contact	-	-	-	-	-	-	-	-	-

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani
(-) Insufficient number of mosquitoes escaped from test chamber.

contact escape and 46.3% repellency) followed by permethrin and deltamethrin, respectively. In all cases, each contact-non-contact paired comparison was significantly ($P < 0.0001$) different (Table 4). Additionally, TR control escape in both contact and non-contact tests were relatively high compared to the other three populations but were significantly ($P < 0.0001$) different between test-control pairings. In all cases, final percent escape responses in each treatment test were corrected based on paired control escape responses. In the final analyses, we did not attempt to correct or make a distinction between excitation irritancy and spatial repellency effects in the contact tests by comparing paired contact and non-contact tests.

More moderate or intermediate contact escape responses were observed with the population from Phang Nga Province (PN) and were significantly different from control responses but not different in non-contact pairings. As seen in the TR population, all three pyrethroids produced significantly different escape responses between PN contact and non-contact tested females. Post-exposure mortality was low in mosquitoes escaping the treated chambers in virtually all contact and non-contact trials, suggesting that TR and PN populations exhibiting behavioral avoidance rather than toxicity may play a more significant role in control of human-vector transmission. On the other hand, the relatively non-responsive ST and SK populations would presumably be controlled by contact toxicity from indoor residual insecticide applications.

Interestingly, TR produced a strong overall 'excito-repellency' to all three chemicals, while PN reactions were more modest in excitation and low for repellency. On the other hand, no or very minimal escape responses to both contact and non-contact exposures to the three pyrethroids were observed in the ST and SK populations These two populations were collected from cattle near rice fields along the eastern coastline of southernmost Thailand. It is unclear why there were such poor responses in these populations, especially in the contact tests. Similar investigations with other anopheline species in Thailand and elsewhere have shown consistent strong excitatory responses compared to paired controls and non-contact tests (Pothikasikorn et al. 2005, 2007, Tisgratog et al. 2011). All tests took place during daytime hours. All four populations were wild-caught and predominately freshly blood-fed at time of testing. Age-grading (e.g., parity) was not conducted so the relative age of each tested population is not known. Therefore, differences between these two 'refractory' populations and TR and PN may have possibly been due to age or influenced by some other aspect of physiological status of the female mosquitoes at the time of testing (Sungvornyothin et al. 2001, Chareonviriyaphap et al. 2006). Dates of experimentation for each population may have played a role but an association is not readily apparent. The TR population was tested in March, 2012, SK in November, 2012, and both ST and PN in February, 2013. Moreover, testing of populations was done independently by two persons, each observing a responsive and non-responsive population each (TR and SK, and ST and PN, respectively) thus making it unlikely the results are due to experimental error. Previous history of exposure to residual pyrethroids used in the national malaria control program does show a difference between responsive (TR and PN) and non-responsive (SK and ST) populations. Both refractory populations came from non-control areas, whereas both populations showing either strong or moderate escape responses have presumably been exposed to pyrethroids in the recent past.

It is also possible there might be some unique natural genetic pre-disposition in the ST and SK populations regarding relative insusceptibility to the excitatory and repellency effects of the pyrethroids and/or concentrations used in this study. Interestingly, it has been shown that a population maintained as an inbred insecticide-susceptible colony in a laboratory setting for over 20 years demonstrated much less avoidance behavior to insecticides compared to wild-caught populations of the same species (Chareonviriyaphap et al. 1997, 2004). Moreover, observations on anopheline response to insecticides have shown that different species or different populations of the same species can produce markedly different behavioral responses to the same active ingredients and test systems. For example, a relatively strong repellency response to bifenthrin was present in a laboratory colony of *Anopheles minimus*, whereas a field population provided only a weak escape reaction (Tisgratog et al. 2011). Similarly, relatively weak repellency responses in *Anopheles harrisoni* were seen compared to its sibling species, *An. minimus* that showed strong repellency to pyrethroids (Pothikasikorn et al. 2005, 2007).

The use of wild-caught mosquitoes carries both advantages and disadvantages for interpretation of data. One advantage with natural populations is for indicating changes in insecticide susceptibility that more closely reflect the changes in intervention efficacy seen in the field. Moreover, the age distribution of the vectors should be representative of the wild vector population at a given time and location as well as being more genetically representative of the population at large compared with F1 progeny that may be more restricted genetically because of inadvertent selective rearing. The primary disadvantage with using wild-caught specimens is age distribution and physiological condition of vectors that will vary between samples reducing the comparability of results from one period to the next. Use of laboratory-reared F1 progeny allows age of vectors to be uniform between tests, thus allowing results from different times and places to be compared.

Previous studies have clearly demonstrated that behavioral responses to DDT and other chemicals used in IRS occur in many malaria vector species that raise an issue of the influence of avoidance behavior in disease transmission control (Chareonviriyaphap et al. 2001, Sungvornyothin et al. 2001, Pothikasikorn et al. 2005). As pyrethroids have dominated as chemicals used against malaria vectors in Thailand, detailed monitoring and awareness of behavioral responses of mosquitoes to these chemicals is of importance for estimating impact on vector populations and transmission. To our knowledge there has been no published information on behavioral avoidance of *An. epiroticus* (or formerly *Anopheles sundaicus* A) to insecticidal compounds in Thailand and only limited information on physiological (toxic) responses. Van Bortel et al. (2008) described two Thai populations of *An. epiroticus* being susceptible to DDT and permethrin from 2003-2005. Therefore, this study represents the first full characterization of the physiological and behavioral responses of *An. epiroticus* to deltamethrin, permethrin, and alpha-cypermethrin.

Behavioral responses by mosquitoes to insecticides have been recognized for many decades (Kennedy 1947, Davidson 1953). In the past, chemicals that produced avoidance responses in mosquitoes were often regarded as inferior attributes when selecting compounds for vector control programs. Virtually all work focused on the direct insecticidal (toxic) action on insect populations as the primary, if not only, means to control vectors and transmission. Until recently, relatively few investigations have concentrated on behavioral responses, specifically avoidance or deterrence of mosquitoes to chemicals. Since the development of two independent test systems (Chareonviriyaphap et al. 1997,

Table 4. Comparison of escape patterns of *Anopheles epiroticus* between test conditions (paired contact, non-contact, and control trials).

Population	Insecticide	*P* values of treatment pairs		
		Control vs Contact	Control vs Non-contact	Contact vs Non-contact
TR	Alpha-cypermethrin	**< 0.0001**	**< 0.0001**	**< 0.0001**
	Deltamethrin	**< 0.0001**	**0.0282**	**< 0.0001**
	Permethrin	**< 0.0001**	**0.0012**	**< 0.0001**
SK	Alpha-cypermethrin	0.7902	0.6467	0.5505
	Deltamethrin	0.7687	**0.0066**	0.5561
	Permethrin	**0.0070**	0.4732	0.0617
PN	Alpha-cypermethrin	**< 0.0001**	0.5434	**< 0.0001**
	Deltamethrin	**0.0008**	0.9301	**0.0029**
	Permethrin	**< 0.0001**	0.1521	**< 0.0001**
ST	Alpha-cypermethrin	0.9691	0.3091	0.5626
	Deltamethrin	0.3014	0.3132	0.0807
	Permethrin	1.0000	1.0000	1.0000

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani
Significant effects in bold type.

Table 5. Comparison of escape responses of *Anopheles epiroticus* between insecticides in paired contact and non-contact trials.

Chemicals	Contact				Non-contact			
	TR	SK	PN	ST	TR	SK	PN	ST
Acyp vs Delta	0.9112	0.1461	0.2132	0.6941	**0.0034**	0.7604	0.3566	0.3091
Acyp vs Perm	0.8160	0.7333	0.5955	0.5427	0.4959	0.1077	0.1556	0.9808
Delta vs Perm	0.7040	0.2397	0.4611	0.3173	**0.0209**	0.1908	**0.0358**	0.3173

Significant effects in bold type.

Grieco et al. 2007) and a mathematical framework for data analysis and interpretation (Roberts et al. 2000), reports on mosquito behavioral responses to public health insecticides and screening of topical repellent compounds have progressively increased (Sungvornyothin et al. 2001, Kongmee et al. 2004, Chareonviriyaphap et al. 2001, 2004, Grieco et al. 2005, 2007, Pothikasikorn et al. 2005, 2007, Polsomboon et al. 2008, Thanispong et al. 2010, Tisgratog et al. 2011, Tananchai et al 2012). Moreover, specially constructed experimental huts have been used to better approximate insect behaviors under more natural settings (Chareonviriyaphap et al. 2013). The aim was to confirm chemical actions identified in laboratory tests are similar to responses of wild mosquito populations in the field. For instance, Grieco et al. (2007) demonstrated that all three chemical actions in the laboratory could be observed in experimental huts using *Ae. aegypti*.

The World Health Organization has guidelines for standardized efficacy testing of spatial repellents. There remains a need for the optimization and standardization of ER test systems to assess and compare behavioral responses of mosquitoes to public health insecticides. Despite tremendous progress in insecticide avoidance studies, there remains much to understand about insecticidal influence on vector biology and behavior that in turn affect malaria transmission. Clarification of normal responses assists in the rationale for mosquito control activities and expenditures. Excito-repellency assays should be an integral component of any evaluation of an insecticide's full capabilities and potential to suppress disease transmission.

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Synergistic repellent and irritant effect of combined essential oils on *Aedes aegypti* (L.) mosquitoes

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ABSTRACT: This study was designed to compare the behavioral responses of *Aedes aegypti* to a single essential oil and to a mixture of two or three essential oils using an excito-repellency test chamber. Mixtures were prepared from essential oils extracted from *Litsea cubeba* (LC), *Litsea salicifolia* (LS), and *Melaleuca leucadendron* (ML). In general, the mixture of essential oils produced a much stronger escape response by *Ae. aegypti*, regardless of the test conditions. No significant difference in escape responses was seen when the mixture of oils was compared with a standard commercial product containing DEET. Greater contact irritancy was seen from mixed oils of LC and LS than with other mixed oils. Mixtures of LC and LS at 0.075% showed the highest synergistic action (65.5% escaped) compared to that with unmixed oil alone at the same concentration (LC=20% and LS=32.2%). In addition, mixtures of LC and LS at 0.075% demonstrated the highest non-contact repellency (62.7%) and showed a greater effect than the use of LC (20%) or LS (20.3%) alone. We conclude that mixtures of two essential oils show potential as active ingredients for mosquito repellents. ***Journal of Vector Ecology* 39 (2): 298-305. 2014.**

Keyword Index: Excito-repellency, irritancy, essential oils, *Melaleuca leucadendron*, *Litsea cubeba*, *Litsea salicifolia, Aedes aegypti*, DEET.

INTRODUCTION

For decades, synthetic insecticidal compounds within different chemical classes have been used by the private and public health sectors. Synthetic pyrethroids have become ubiquitous due to their relatively low mammalian toxicity and ability to rapidly immobilize invertebrates at low levels (Jirakanjanakit et al. 2007, Thanispong et al. 2008). Because of the frequent use of pyrethroids, selection pressure for insecticide resistance has become a major concern (Roberts et al. 1997, Jirakanjanakit et al. 2007, Thanispong et al. 2008). In addition to toxicants, effective repellents such as DEET (*N*, *N*-diethyl-*meta*-toluamide) are readily available and commonly used (Fradin and Day 2002). DEET is used extensively to protect against biting mosquitoes, however it has been shown to have significant adverse or toxic effects to humans (Brown and Hebert 1997, Odalo et al. 2005). Consequently, there is interest in the development of insect repellents from natural plant extracts that might be less toxic to humans, such as thyme (Park et al. 2005) and citronella (Yang and Ma 2005).

Recently, several plant species were evaluated as potential repellents or toxicants against blood-feeding invertebrates and pestiferous flies in Lao PDR (De Boer et al. 2010). Although several essential oils have exhibited significant repellent activity against target insects (Noosidum et al. 2008, Kim et al. 2012, Suwansirisilp et al. 2013), comparatively little has been done to identify the degree of behavioral responses using the mixtures of at least two essential oils. In this study, we compared the behavioral responses of *Aedes aegypti* (L.) to a single essential oil and mixtures of essential oils from *Litsea cubeba* (Lour.) Persoon (LC), *Litsea salicifolia* (Roxburgh ex Nees) Hook. f. (LS), and *Melaleuca leucadendron* L. (ML) using an excito-repellency test chamber.

MATERIALS AND METHODS

Mosquitoes

Aedes aegypti populations were collected from Pu Teuy Village, Sai Yok District, Kanchanaburi Province, Thailand (14°17'N, 99°1'E). All female mosquitoes were identified to species and colonies were established using modified methods from Kongmee et al. (2004). Two days post-emergence, mosquitoes were blood-fed on live guinea pigs held for three days until oviposition occurred on filter paper contained in dishes with tap water. Egg and larval stages were reared in plastic pans and pupae were maintained in plastic cups, which were held at 25 ± 5° C, 12:12 (L:D) photoperiod, and 80 ± 10% relative humidity. Adults were held in 30×30×30 cm screened holding cages and provided with cotton pads soaked with 10% sugar solution. Only three to five day-old female *Ae. aegypti* were used and were sugar-starved (water only) for 12 h before testing.

Oil extraction

Fresh ML leaves were collected from Chanthaburi Province, Thailand, in October, 2010 (13°98'N, 48°18'E). Mature fruits of LC and LS were collected from Doi Ang-khang, Fang District, Chiang Mai Province in November, 2010 (19°54'N, 99°2'E). Essential oils from the plants were extracted over 6 h by water distillation using a Clevenger-type apparatus. The superior phase was collected from the condenser, dried over anhydrous sodium sulfate, and stored in amber-colored vials at 0° C until testing (Noosidum et al. 2008).

Behavioral tests

This experiment was designed to compare the repellent (non-contact response) and irritant response (contact response) from a single oil and oil mixture in various concentrations using excito-repellency assays described in Noosidum et al. (2008). Based on our previous study (Noosidum et al. 2008), LC and LS at 0.025, 0.05, 0.075, 0.1, 0.125, and 0.15 (v/v), and ML at 0.3 and 0.5 (v/v) were diluted in absolute ethanol and impregnated onto a (15×17.5 cm) filter paper. All filter papers were treated with 2.75 ml of test solution per 180 cm^2. DEET (15% w/w) and 11 concentrations of the oil mixture were also evaluated. The control papers were treated with absolute ethanol only. All tests were performed during 08:00-16:30 under laboratory-controlled conditions.

Each experimental series consisted of two test chambers treated with essential oil, one contact and one non-contact, and two identical untreated control chambers. Female mosquitoes were sugar starved and held in cups approximately eight to ten h before testing. Fifteen mosquitoes were placed in each test chamber (23×23×23 cm stainless steel box) using a mouth aspirator. Mosquitoes were allowed a 3-min resting period in the test chamber, after which the escape tunnel was opened to begin the observation period. A receiving cage (23×23×23 cm paper box) was connected to the exit portion for collecting and all escaped mosquitoes were recorded at 1-min intervals for a period of 30 min. After test completion, the number of dead or knockdown mosquitoes in each treatment test chamber and the escape-receiving cage were recorded. All knockdown and live specimens were transferred separately to clean holding cups and a cotton pad with 10% sugar solution was provided for 24 h. Mortality was recorded at the end of the period. All experimental treatments were replicated four times.

Data analysis

Kaplan-Meier survival analysis was used to analyze and interpret the behavioral response data. Survival analysis was used to estimate the probability of escape time (ET) and to compare differences in mosquito response between the three oils (Kleinbaum 1995) based on mosquito escape patterns at 1-min intervals. For purposes of analysis, mosquitoes that escaped were treated as 'deaths' and those remaining in the chambers as 'survivals' (Chareonviriyaphap et al.1997). The escape time (ET) in minutes for 30% (ET_{30}) and 50% (ET_{50}) was calculated using survival analysis and patterns of escape response were determined using the log-rank method (Mantel and Haenzel 1959). For those tests in which the control mortality exceeded 5%, a Abbott's correction factor was applied to the data (Abbott 1925). Statistical significance for all tests was set at $P < 0.05$.

RESULTS

Escape responses of *Ae. aegypti* that were exposed to a single or a mixture essential oil from LC, LS, or ML were defined in contact and non-contact trials. A commercial DEET (15% w/w) was used as the standard repellent and irritant. Knockdown of escaped and unescaped *Ae. aegypti* females were observed during the exposure period (30 min). Overall, no knockdown specimen was recorded from chambers treated with all treatments, except LC 0.175+LS 0.175 (Tables 1-4). Also, mortalities of *Ae. aegypti* females after 24 h post-exposure were obtained.

The percentage and rate of escape response during the 30 min exposure to contact and non-contact chambers treated with LC, LS, ML, DEET, and from LC, LS, and ML combinations in different concentrations are illustrated in Figures 1-4. The rates represent the probability of escape from a chamber with a given oil and concentration. Strong escape patterns were observed with concentrations of 0.125% LC and 0.15% LC in both contact and non-contact trials, in comparison to other doses (Figure 1). Similarly, 0.125% LS and 0.15% LS showed marked escaped responses in contact and non-contact trials, respectively (Figure 2). With LC+LS, 0.075% LC + 0.075% LS showed strong escape patterns in both contact and non-contact trials compared to other doses (Figure 3). Other combination treatments and DEET also showed strong escape patterns (> 30%) (Figure 4).

Low mortality (0-7.02%) was observed in all contact, non-contact, and control trials. For contact trials of a single oil, mortalities ranged between 0 and 5.1% in chambers treated with 0.1% LS, 0.15% LS, 0.3% ML, 0.5% ML, and DEET (Table 1). Mortality rates of <7% were observed in non-contact trials in 0.1% LS, 0.3% ML, and DEET and other experimental trials did not result in mortality (Table 2). In contact trials of oil mixture treatments, mortality rates from escape and not escape specimens varied between 0 and 6.7% (Table 3). The percent mortality for non-contact trials of combined treatments was 6.7% or less in escape and not escape specimens (Table 4).

The escape patterns from chambers treated with test compounds were defined as an escape time for 30% (ET_{30}) and 50% (ET_{50}) of the test population to leave the treated chambers in 19 min (Table 5). In contact trials, the greatest ET_{50} was found at 0.075% LC+0.075% LS at 4 min, followed by 0.05% LC+0.05% LS at 6 min (Table 5). In non-contact trials, ET_{30} and ET_{50} values for all three pure compounds and DEET were over 3 min (Table 5). The number of escaped specimens from LC and LS were low, with no ET_{50} values observed in any concentration (Table 5). The LC+LS combinations at 0.075% and 0.1% had ET_{50} values of 7 and 28 min, respectively. The mixture of essential oils combined from two plants, LC, LS and ML, showed ET_{50} values from 9 to 29 min (Table 5). However, the results of this study showed that combined essential oils in each concentration typically had higher effectiveness than pure essential oils alone, with the greatest efficacy at 0.075% LC+0.075% LS in contact and non-contact trials. Most of the mixture oils increased the percentage of repellent and irritant behaviors more than pure oils in both contact and non-contact trials.

Multiple log-rank comparisons were made in contact, non-contact, and control trials for the three compounds by concentration. Significant differences in escape patterns were observed in comparison with contact vs non-contact trials ($P < 0.05$) for 0.05% LC, 0.05% LS, 0.5% ML, and 0.025% LC+LS. Statistical differences in escape responses were found in all

Table 1. Mortality of *Ae. aegypti* females after a 24-h holding period following exposure in excito-repellency contact trials of pure compounds.

Treat[1]	Dose (%)[2]	Number		% Knockdown		% Mortality[3]	
		Tested	Esc (%)	Esc	Not esc	Esc	Not esc
LC	0.025	56	3 (5.4)	0	0	0	0
	0.05	58	10 (17.2)	0	0	0	0
	0.075	60	12(20.0)	0	0	0	0
	0.1	59	13 (22.0)	0	0	0	0
	0.125	60	30 (50.0)	0	0	0	0
	0.15	58	30 (51.7)	0	0	0	0
LS	0.025	58	5 (8.6)	0	0	0	0
	0.05	59	17 (28.8)	0	0	0	0
	0.075	59	19 (32.2)	0	0	0	0
	0.1	58	17 (29.3)	0	0	3.5	5.1
	0.125	60	25 (41.7)	0	0	0	0
	0.15	60	26 (43.3)	0	0	0	2.3
ML	0.3	60	10 (16.7)	0	0	1.7	0
	0.5	60	17 (28.3)	0	0	0	1.7
15% DEET		60	26 (43.3)	0	0	1.7	1.7

[1]LC: *Litsea cubeba*; LS: *Litsea salicifolia*, ML: *Melaleuca leucadendron*, DEET: a commercial DEET 15% w/w. [2]Essential oil dosages: 0.025%, 0.05%, 0.075%, 0.1%, 0.125%, 0.15%, 0.3%, 0.5%. [3]Mortalities based on number dead following 24-h holding period after specimens were removed from exposure chamber or females escaped from exposure chambers. Esc: escape mosquitoes, Not esc: non-escaped mosquitoes.

Table 2. Mortality of *Ae. aegypti* females after a 24-h holding period following exposure in excito-repellency non-contact trials of pure compounds.

Treat [1]	Dose (%)[2]	Number		% Knockdown		% Mortality[3]	
		Tested	Esc (%)	Esc	Not esc	Esc	Not esc
LC	0.025	60	2 (3.3)	0	0	0	0
	0.05	57	3 (5.3)	0	0	0	0
	0.075	60	12 (20.0)	0	0	0	0
	0.1	60	14 (23.3)	0	0	0	0
	0.125	60	29 (48.3)	0	0	0	0
	0.15	58	28 (48.3)	0	0	0	0
LS	0.025	60	7 (11.7)	0	0	0	0
	0.05	55	7 (12.7)	0	0	0	0
	0.075	59	12 (20.3)	0	0	0	0
	0.1	57	15 (26.3)	0	0	3.51	7.02
	0.125	60	20 (33.5)	0	0	0	0
	0.15	58	27 (46.6)	0	0	0	0
ML	0.3	59	8 (13.6)	0	0	0	6.8
	0.5	59	6 (10.2)	0	0	0	0
15% DEET		59	27 (45.8)	0	0	0	1.7

[1]LC: *Litsea cubeba*; LS: *Litsea salicifolia*, ML: *Melaleuca leucadendron*, DEET: a commercial DEET 15% w/w. [2]Essential oil dosages: 0.025%, 0.05%, 0.075%, 0.1%, 0.125%, 0.15%, 0.3%, 0.5%. [3]Mortalities based on number dead following 24-h holding period after specimens were removed from exposure chamber or females escaped from exposure chambers. Esc: escape mosquitoes, Not esc: non-escaped mosquitoes.

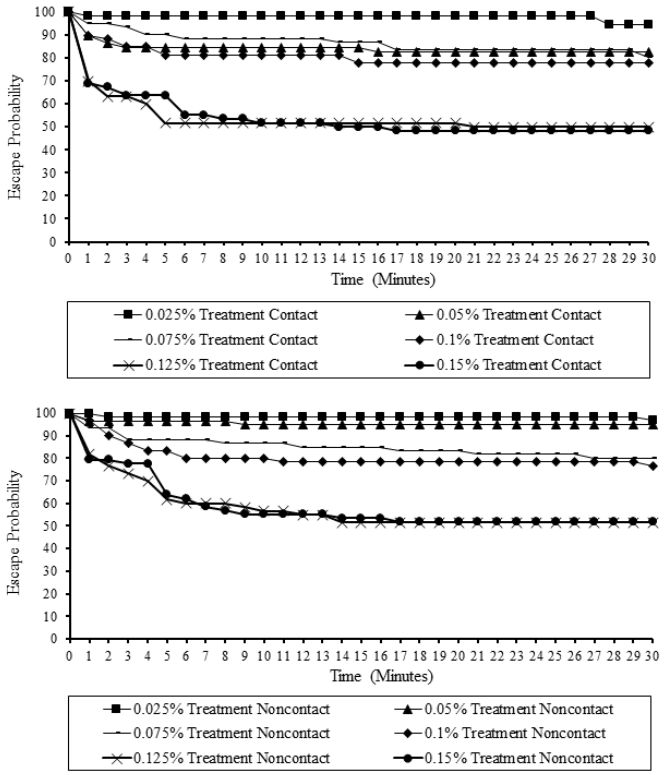


Figure 1. Escape probability of *Ae. aegypti* females in contact (top) and non-contact (bottom) trials using six different concentrations of *Litsea cubeba* essential oil extract.

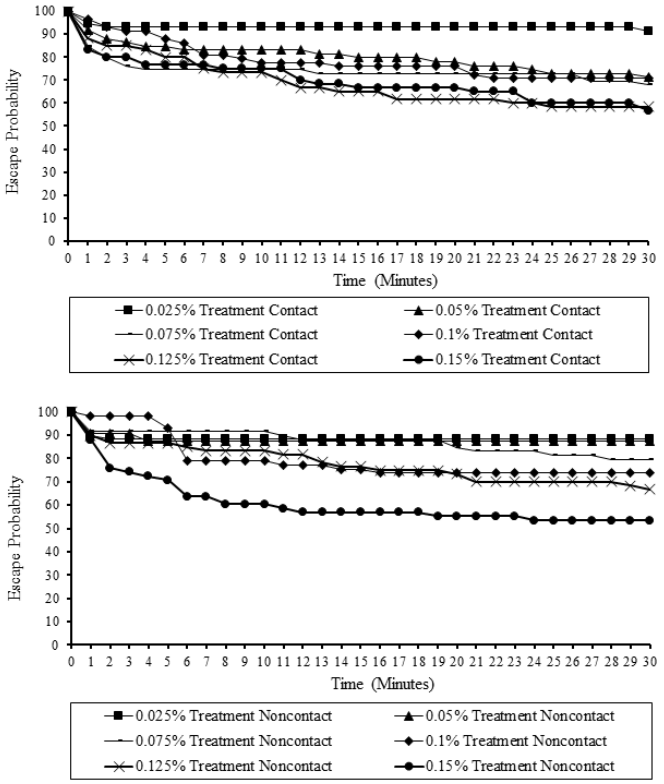


Figure 2. Escape probability of *Ae. aegypti* females in contact (top) and non-contact (bottom) trials using five different concentrations of *Litsea salicifolia* essential oil extract.

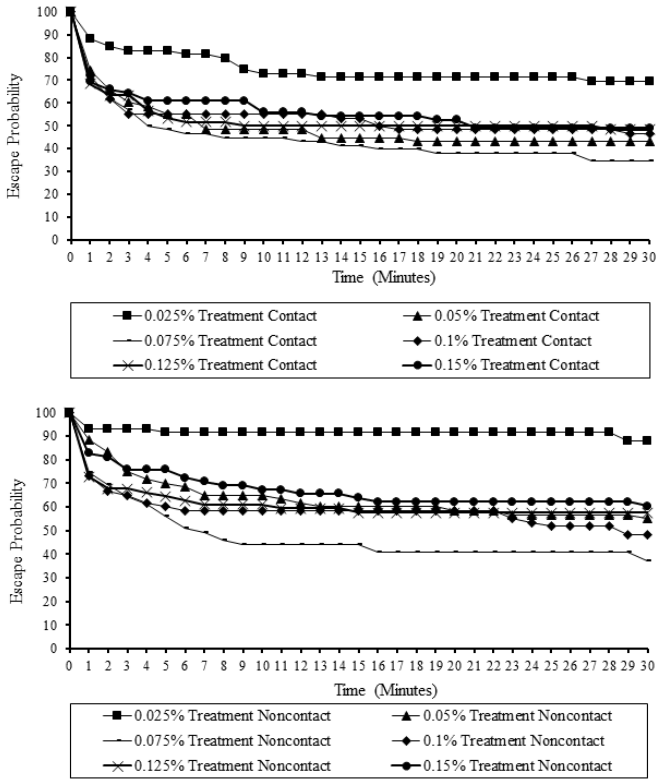


Figure 3. Escape probability of *Ae. aegypti* females in contact (top) and non-contact (bottom) trials using five different concentrations of combination between *Litsea cubeba* and *Litsea salicifolia* essential oil extracts.

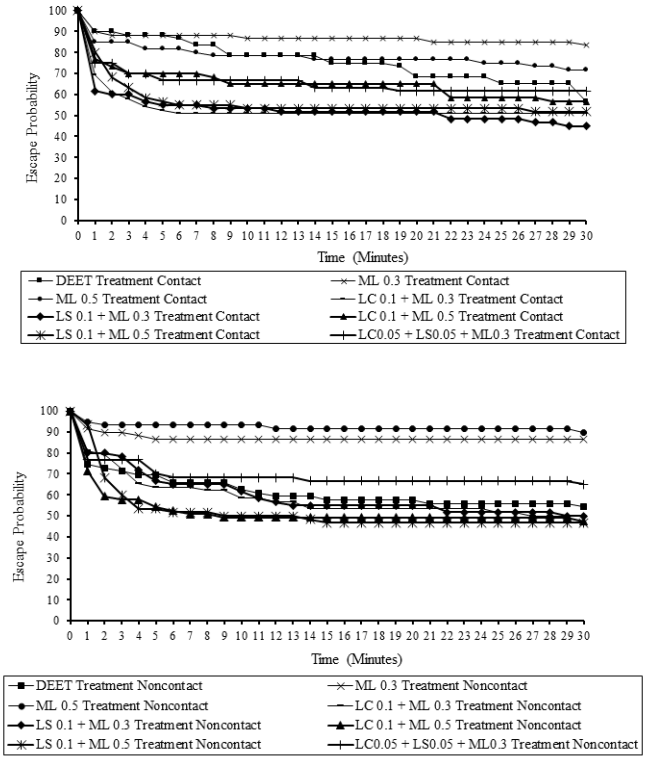


Figure 4. Escape probability of *Ae.* aegypti females in contact (top) and non-contact (bottom) trials using different concentrations of DEET, *Melaleuca leucadendron* essential oil extract and various combinations.

Table 3. Mortality of *Ae. aegypti* females after a 24-h holding period following exposure in excito-repellency contact trials of combined compounds.

Treat # Dose (% v/v) [1]	Number		% Knockdown		% Mortality [2]	
	Tested	Esc (%)	Esc	Not esc	Esc	Not esc
LC 0.025 + LS 0.025	59	18 (30.5)	0	0	0	0
LC 0.05 + LS 0.05	58	33 (56.9)	0	0	0	0
LC 0.075 + LS 0.075	58	38 (65.5)	0	0	5.2	5.2
LC 0.1 + LS 0.1	60	32 (53.3)	0	0	3.3	1.7
LC 0.125 + LS 0.125	60	31 (51.7)	0	0	1.7	0
LC 0.15 + LS 0.15	59	30 (50.9)	0	0	0	0
LC 0.175 + LS 0.175	59	15 (25.4)	0	67.8	1.7	15.3
LC 0.1 + ML 0.3	59	29 (49.2)	0	0	0	2.3
LS 0.1 + ML 0.3	60	33 (55.0)	0	0	0	0
LC 0.1 + ML 0.5	60	26 (43.3)	0	0	5.0	2.2
LS 0.1 + ML 0.5	60	29 (48.3)	0	0	3.3	6.7
LC 0.05 + LS 0.05 + ML 0.3	60	23 (38.3)	0	0	5.0	5.0

[1]LC: *Litsea cubeba*; LS: *Litsea salicifolia*, ML: *Melaleuca leucadendron*. [2]Mortalities based on number dead following 24-h holding period after specimens were removed from exposure chamber or females escaped from exposure chambers. Esc: escape mosquitoes, Not esc: non-escaped mosquitoes.

Table 4. Mortality of *Ae. aegypti* females after a 24-h holding period following exposure in excito-repellency non-contact trials of combined compounds.

Treat # Dose (% v/v)[1]	Number		% Knockdown		% Mortality[2]	
	Tested	Esc (%)	Esc	Not esc	Esc	Not esc
LC 0.025 + LS 0.025	58	7 (12.1)	0	0	0	0
LC 0.05 + LS 0.05	60	27 (45.0)	0	0	0	0
LC 0.075 + LS 0.075	59	37 (62.7)	0	0	3.4	3.4
LC 0.1 + LS 0.1	60	31 (51.7)	0	0	6.7	0
LC 0.125 + LS 0.125	59	25 (42.4)	0	0	0	0
LC 0.15 + LS 0.15	58	23 (39.7)	0	0	0	0
LC 0.175 + LS 0.175*	59	19 (32.2)	0	39.0	1.7	1.7
LC 0.1 + ML 0.3	58	29 (50.0)	0	0	0	4.44
LS 0.1 + ML 0.3	60	30 (50.0)	0	0	0	0
LC 0.1 + ML 0.5	59	31 (52.5)	0	0	5.1	1.7
LS 0.1 + ML 0.5	60	32 (53.3)	0	0	5.0	5.0
LC 0.05 + LS 0.05 + ML 0.3	60	21 (35.0)	0	0	5.0	3.3

[1]LC: *Litsea cubeba*; LS: *Litsea salicifolia*, ML: *Melaleuca leucadendron*. [2]Mortalities based on number dead following 24-h holding period after specimens were removed from exposure chamber or females escaped from exposure chambers. Esc: escape mosquitoes, Not esc: non-escaped mosquitoes.

Table 5. Estimated time in min for 30% (ET_{30}) and 50% (ET_{50}) of *Ae. aegypti* females to escape from exposure chambers in excito-repellency of contact and non-contact trials of the oil compounds.

Treat # Dose (% v/v)[1]	Contact		Non-contact	
	ET_{30}[2]	ET_{50}	ET_{30}[2]	ET_{50}
15% DEET	19		3	
0.125% LC	1	20	3	
0.15% LC	1	14	4	
0.075% LS	26			
0.125% LS	11		21	
0.15% LS	12		5	
0.05% LC + 0.05% LS	2	6	5	
0.075% LC + 0.075% LS	1	4	1	7
0.1% LC + 0.1% LS	1	16	1	28
0.125% LC + 0.125% LS	1	9	1	
0.15% LC + 0.15% LS	1	20	7	
0.1% LC + 0.3% ML	1		3	27
0.1% LS + 0.3% ML	1	21	4	29
0.1% LC + 0.5% ML	3		1	8
0.1% LS + 0.5% ML	1		1	9
0.05% LC + 0.05% LS + 0.3% ML	3		5	

[1]LC: *Litsea cubeba*; LS: *Litsea salicifolia*, ML: *Melaleuca leucadendron*: DEET: a commercial DEET 15% w/w.
[2]Survival analysis estimate time in min for 30 and 50% of test populations to escape from exposure chambers. (-) indicates less than 30% of test specimens escaped from exposure chambers, therefore ET_{30} and ET_{50} estimates could not be calculated for 30 min exposure period.

treatments when contact was compared to the controls ($P < 0.05$), except 0.025% LC, 0.075% LC, 0.025%-0.075% LS, 0.5% ML, and 0.025% LC+0.025% LS. Likewise, marked differences in escape responses were observed in paired non-contact and control trials ($P < 0.05$) for several treatments, except 0.025% LC, 0.075% LC, and 0.025% LS.

DISCUSSION

In this study, the essential oils which demonstrated repellent activities were tested against *Ae. aegypti*, a vector of the dengue pathogen. The importance of the synergistic effects between essential oils from different plants (LC, LS, and ML) was the main focus of this study. Although synthetic chemicals repellents are more frequently used than essential oils, natural products have been shown to provide effective and relatively safer repellency against insect vectors (Fradin and Day 2002, Sritabutra et al. 2011). In general, the use of natural products that possess reliable efficacy, safety, and are environmentally friendly have been become more popular. Essential oils from numerous plant species have been extensively tested to assess their repellent properties (Noosidum et al. 2008, De Boer et al. 2010, Sritabutra et al. 2011, Suwansirisilp et al. 2013).

In a previous study, essential oils extracted from ML, LC, and LS had shown high irritant and repellent properties against *Ae. aegypti* females (Noosidum et al. 2008). In this study, the combinations of mixture oils demonstrated the capability to repel *Ae. aegypti*, which is similar to Suwonkerd and Tantrarongroj (1994). They reported that repellent cream made from 2.5% citronella oil, 5% galingale oil, and 0.5% vanillin prevented *Anopheles minimus* Theobald biting for 6 h, while a cream containing 10% citronella oil prevented mosquito biting for only 2 h. Sritabutra et al. (2011) reveal that the mixtures of essential oils derived from 5% citronella oil + 5% eucalyptus oil, and 5% eucalyptus oil + 5% sweet basil oil also showed greater than 98 h of protection time against *Ae. aegypti*, compaired to 65-81 h of protection with 10% of pure oils alone. Both formulations had an additive effect, however, some combinations had a reduction (antagonistic effect) in protection time when compared with pure compound. Similar to this study, the synergistic effect was also found in a combination of 5% eucalyptus oil + 5% sweet basil oil which could provide 210 h of protection time for *Anopheles dirus* Peyton & Harrison, while pure compounds only had 95 and 45 h of protection time, respectively (Sritabutra et al. 2011).

In summary, combine-extracted oils produced vigorous contact irritancy and non-contact repellency at low concentrations, suggesting that combinations of two essential oils may serve as a more effective mosquito repellent than each separate essential oil. However, combinations of different oils will decrease or increase their efficacy depending upon plant-oil species and concentration and will also give us more knowledge of alternative chemicals for mosquito control. Plant extracts and essential oils can be an effective alternative to synthetic insecticides for vector control. Additional studies are recommended to determine the secondary metabolites of effective plant extracts against mosquitoes to further develop new and safe insecticides. Development of better formulations, such as using a fixative component, could improve efficacy (Tawatsin et al. 2001, Tuetun et al. 2005, Kim et al. 2012).

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Comparative Behavioral Responses of Pyrethroid–Susceptible and –Resistant *Aedes aegypti* (Diptera: Culicidae) Populations to Citronella and Eucalyptus Oils

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ABSTRACT The objective of this study was to compare the behavioral responses (contact irritancy and noncontact spatial repellency) between susceptible and resistant populations of *Aedes aegypti* (L.) (=*Stegomyia aegypti*) to essential oils, citronella, and eucalyptus, *Eucalyptus globulus*, extracts, using an excito-repellency test system. N, N-diethyl-meta-toluamide (DEET) was used as the standard reference repellent. Mosquitoes included two long-standing insecticide susceptible colonies (U.S. Department of Agriculture and Bora Bora) and two pyrethroid-resistant populations recently obtained from Phetchabun and Kanchanaburi provinces in Thailand. Both DEET and citronella produced a much stronger excitation ("irritancy") and more rapid flight escape response in both pyrethroid-resistant populations compared with the laboratory populations. Noncontact repellency was also greater in the two resistant populations. Eucalyptus oil was found to be the least effective compound tested. Differences in responses between long-established pyrethroid-susceptible colonies and newly established and naturally resistant colonies were clearly demonstrated. These findings also demonstrate the need for further comparisons using natural pyrethroid-susceptible populations for elucidation of factors that might contribute to different patterns of escape behavior.

KEY WORDS *Aedes aegypti*, behavior, DEET, citronella oil, eucalyptus oil

Dengue viruses are responsible for one of the most prevalent and important vector-borne diseases (dengue fever and dengue hemorrhagic fever) of public health importance in the tropical and subtropical world (Gubler 1998). It is estimated that ≈400 million dengue infections occur annually, of which close to one-quarter will manifest with some form of clinical or subclinical severity (Bhatt et al. 2013). This represents a threefold increase in the number of estimated cases by the World Health Organization (WHO 2012), but likely still represents half of the world population live in the dengue-endemic countries (WHO 2012). Thailand has long had persistent dengue transmission with all four serotypes circulating in urban and rural settings. The principal vector of dengue viruses is *Aedes aegypti* (L.) (=*Stegomyia aegypti*), which is a common resident in urban areas, whereas *Aedes albopictus* (Skuse) (=*Stegomyia albopicta*), a dengue vector of generally secondary importance, is more prevalent in more open suburban and rural settings (Pethuan et al. 2007). Both species have proven difficult to control because of their bionomics and behavioral traits in close association with human activities. Years of research and significant financial support have yet to provide an effective vaccine to protect against dengue. Thus, the prevention and control of dengue remains almost exclusively based on vector control and personal protection practices to reduce transmission risk. In some instances, this requires the use of various insecticides applied as larvicides, outdoor space sprays, and indoor aerosol applications (Reiter and Gubler 1997, Grieco et al. 2007).

The intensive use of insecticides has led to high levels of chemical resistance in certain pests and disease vectors (Chareonviriyaphap et al. 1999, Thanispong et al. 2008) resulting in reduced effectiveness of more traditional chemical-based prevention and control methods. There have been numerous reports of dengue vector mosquitoes developing resistance to pyrethroids in Thailand (Jirakanjanakit et al. 2007, Thanispong et al. 2008, Chuaycharoensuk et al. 2012). An alternative measure of bite prevention is the use of topical (skin) insect repellents. One of the most common synthetic insect repellents is N, N-diethyl-meta-toluamide (DEET), an ingredient that has been used for >50 yr by tens of millions of people

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(Katz et al. 2008). DEET has had a relatively good safety record but on rare occasion can result in significant adverse health effects in humans, especially when misapplied at higher concentrations or for prolonged periods of time (Roland et al. 1985, Brown and Hebert 1997, Petrucci and Sardini 2000). For these reasons, there has been a continuing interest in developing insect repellents from a wide variety of natural plant extracts, such as thyme, clove, celery, citronella, and others (Barnard 1999, Trongtokit et al. 2004, Tuetun et al. 2005).

Botanicals or plant-based compounds have long been used as personal mosquito repellents. Citronella (*Cymbopogon nardus* (L.) Rendle) is widely used and has been shown to effectively repel *Anopheles minimus* Theobald, *Culex quinquefasciatus* (Say), and *Ae. aegypti* (Thomas et al. 2009, Phasomkusolsil and Soonwera 2010). Eucalyptus (*Eucalyptus globulus* Labillardière) extracts are capable of killing the egg, nymphal, and adult stages of head lice (Choi et al. 2010) and larval and pupal stages of the housefly (Kumar et al. 2012). Although several essential oils have exhibited significant repellent activity against a variety of insects, little is known about the two different forms of behavioral responses, contact excitation ("irritancy") and noncontact spatial repellency, of insects to these compounds.

Contact irritancy occurs when insects make a direct contact with chemical residues on a treated surface, resulting in excitation-mediated response. Spatial repellency responses occur when an active ingredient is detected from a distance without making physical contact. The combined effects of both irritancy and repellency have been shown repeatedly to drive sensitive insects away from areas with treated surfaces (e.g., escape or deterred entry into houses with insecticide sprayed walls), thereby disrupting normal patterns of flight and host-seeking behavior (Chareonviriyaphap et al. 1997, 2004; Roberts et al. 1997, 2000). In this study, we compared these two nontoxic responses between susceptible and resistant populations of *Ae. aegypti* exposed to citronella or eucalyptus oil using an excito-repellency test system.

Materials and Methods

Mosquitoes. Four populations of *Ae. aegypti* were used to compare the excito-repellency responses. Two laboratory populations originally from the United States (U.S. Department of Agriculture [USDA]) and Bora Bora Island (BB) in French Polynesia, and two field populations collected from Phetchabun (PB) and Kanchanaburi (KB) provinces, Thailand. The USDA test population is a long-standing (≈70 yrs.) laboratory colony originating from the USDA, Gainesville, FL. This insecticide-susceptible colony has been maintained continuously at the Department of Entomology, Kasetsart University, Bangkok, Thailand for >10 yr. The BB insecticide-susceptible population had been maintained for at least 10 yr at the Department of Disease Control, Ministry of Public Health, Thailand, before eggs were obtained in September 2012 for rearing at Kasetsart University. The PB population was collected as larvae from Maung District (16° 766′ N, 101° 100′ E, 78 m elevation above sea level) in July 2011 and returned to Kasetsart University for colonization. The KB population larvae collected from Pu Teuy Village, Sai Yok District (14° 20′ N, 98° 59′ E, 281 m above sea level) in January 2012 and returned to Kasetsart University for colonization.

All mosquitoes used in this study were uniformly reared at the Kasetsart University Department of Entomology. Three- to 5-d-old female mosquitoes were used in the susceptibility and behavioral tests. For behavioral tests, mosquitoes were provided no access to sugar solution for ≈24 h before conducting the assays. For the recent field-derived populations, all testing was conducted using F2 and F3 generations of *Ae. aegypti*.

Essential Oils and Synthetic Repellent. Citronella oil, *C. nardus*, (product code: MK-40012; lot: NO5410008-1/1910) and eucalyptus oil, *Eucalyptus globulus*, the southern blue gum (product code: MK-20014; lot: NO5405017/0206) were supplied from Thai-China Flavors and Fragrances Industry Company (TCFF, Lat Bua Luang, Ayutthaya, Thailand). Oil is extracted from leaves using steam distillation. Light-proof bottles were received tightly sealed from the manufacture and were used according to the shelf life characteristics and prescribed product storage criteria. Technical DEET (99.13% purity) was obtained from Sherwood Chemical (Thailand) Ltd. Company (Lot: 0326/2009). The required doses of plant extract oils and DEET were diluted in absolute ethanol to produce a 2.5% final concentration for each and prepared according to the method described by Polsomboon et al. (2008).

Chemical Treatment. The recommended WHO diagnostic dosages of 0.05% deltamethrin, 0.75% permethrin, and 0.05% cypermethrin impregnated papers were purchased from the Vector Control Research Unit, University Sains Malaysia (Penang, Malaysia) for insecticide susceptibility tests. For behavioral tests, 2.5% of each active ingredient diluted in absolute ethanol was used to treat test surfaces. Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, the United Kingdom; Lot No: FS001745) was cut into 14.7 by 17.5 cm size (257.3 cm^2) and impregnated with 2.8 ml of test solution over the entire surface area by a method adapted from WHO (WHO 1998).

Insecticide Susceptibility Tests. Groups of 25 female mosquitoes were exposed to diagnostic doses of three insecticides according to the WHO bioassay test procedures (WHO 1998). The mosquitoes were allowed to contact insecticide-treated paper in the tube for 1 h. Exposed mosquitoes were subsequently transferred to clean holding tubes and a cotton pad soaked with 10% sugar solution was placed on top of the tube as sustenance. Four replicates per treatment (25 mosquitoes per test, a total of 100 mosquitoes) were performed, including matched controls with filter paper without insecticide of 100 females each. Knockdown of mosquitoes after 60 min exposure and mortality 24 h postexposure were recorded.

Behavioral Assays. The excito-repellency system was used to investigate the behavioral responses

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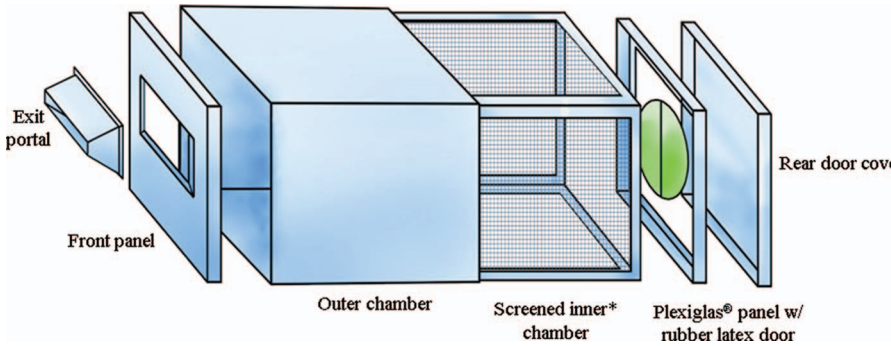


Fig. 1. Blow-up diagram of the excito-repellency test chamber showing key components in apparatus design, including the screened inner chamber to measure spatial repellency. (Online figure in color.)

(flight and escape) of *Ae. aegypti* (Chareonviriyaphap et al. 2004, Polsomboon et al. 2008). Briefly, the complete test system trial consists of four chambers (23.5 by 23.5 by 23.5 cm^3) in which two chambers were designed for the respective treatments exposure (with active ingredient) and other two were set up as matched controls with blank papers (no active ingredient; Figs. 1 and 2). The treatment and control trials were designed for two conditions: a contact design whereby mosquitoes have the opportunity of direct contact with the chemical residue on four treated papers affixed inside of each wire mesh screen of the inner chamber insert, and a noncontact design, with the wire mesh screen serving as a barrier between treated paper and mosquito when placed on the outside of the inner chamber preventing direct contact with the chemical. Fifteen, 3–5-d-old, sugar-starved females were released into each of four chambers. After a brief 3-min resting period, the exit door was open to allow test specimens to depart the test chamber to an exterior receiving box attached to the test chamber. All escaped mosquitoes were recorded at 1-min intervals for a period of 30 min. After the test, the number of knockdown mosquitoes inside the test chamber and those having escaped into the receiving box were recorded. All mosquitoes were subsequently transferred separately by test chamber into clean plastic cups covered with netting, provided with a 10% sugar solution on a cotton pad, and held for 24 h when mortality was recorded. The test design was replicated four times for each treatment and mosquito population.

Data Analysis. For contact susceptibility tests the final percentage mortality was corrected using Abbott's formula if matched control mortality was between 5 and 20% (Abbott 1925). For behavioral response data, a Kaplan–Meier survival analysis was used to describe and predict behavioral escape time (ET) responses of mosquitoes exposed to the compounds (Roberts et al. 1997, Kongmee et al. 2004). The survival analysis is the most appropriate statistical treatment for excito-repellency data. This statistical method can determine the estimated rate of mosquitoes remaining in the chamber over a 30-min period. Mean ET values, measured as minutes from beginning of test required for 25 (ET_{25}), 50 (ET_{50}), and 75% (ET_{75}) of the test mosquito population escaped was calculated. A log-rank test was conducted for comparison of the differential escape rates among the four mosquito populations and two plant oils and synthetic repellent (Mantel and Haenszel 1959). Statistical significance was set at $P < 0.05$ for all paired comparisons.

Results

Results of insecticide susceptibility tests of each mosquito population using a single diagnostic dose of 0.05% deltamethrin, 0.75% permethrin, and 0.05% cypermethrin are presented in Table 1. In general, strong physiological resistance to all synthetic pyrethroids was observed in recent field-derived populations PB (61% survival to deltamethrin, 100% for permethrin, and 98% for cypermethrin), whereas a comparably more moder-

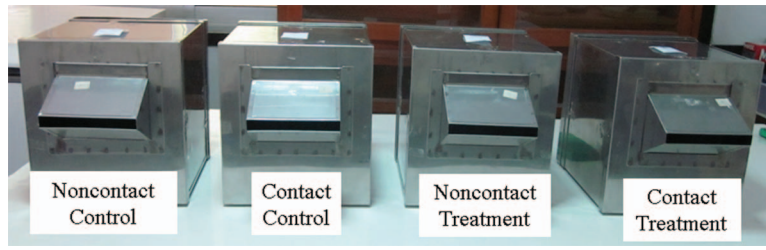


Fig. 2. Excito-repellency test system showing the four chambers aligned in sequence for each trial run, one pair as contact and matched control, the other set as noncontact and control chamber. Each chamber has an open exit portal without an attached external receiving box. (Online figure in color.)

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Table 1. Percent 24-h mortality and SD of four populations of *Ae. aegypti* at WHO-recommended diagnostic concentrations of pyrethroids: 0.05% deltamethrin, 0.75% permethrin, and 0.05% cypermethrin after a 1-h exposure at four replicates per treatment (25 mosquitoes per test)

Chemical	Population	Replication	*N*	% Mortality ± SD[a]
0.05% Deltamethrin	PB	4	95	38.95 ± 14.2a
	KB	4	93	86.02 ± 5.7b
	USDA	4	100	100 ± 0.0c
	BB	4	100	100 ± 0.0c
0.75% Permethrin	PB	4	99	0a
	KB	4	103	82.52 ± 7.1b
	USDA	4	100	100 ± 0.0c
	BB	4	100	100 ± 0.0c
0.05% Cypermethrin	PB	4	100	2.0 ± 2.3a
	KB	4	101	64.36 ± 7.5b
	USDA	4	100	100 ± 0.0c
	BB	4	100	100 ± 0.0c

USDA; BB, Bora-Bora; PB, Phetchabun; KB, Kanchanaburi.

[a] Different letters indicate significant differences ($P < 0.05$) by one-way analysis of variance (ANOVA) using Duncan's multiple range test.

ate resistance was observed from KB (14% survival to deltamethrin, 17% to permethrin, and 36% to cypermethrin). Complete knockdown and mortality was observed from the two insecticide-susceptible populations (Table 1). Control mortalities were low and did not exceed 5% in any test (data not shown).

Escape responses of the two pyrethroid-resistant (PB and KB) and two -susceptible *Ae. aegypti* populations (USDA and BB) to 2.5% concentration DEET, citronella, or eucalyptus in either contact or noncontact excito-repellency trials are shown in Tables 2 and 3. Overall, stronger escape responses of *Ae. aegypti* were seen with DEET (27–53% for contact escape and 13–42% for noncontact escape) and citronella (42–84% for contact and 36–52% for noncontact) compared with eucalyptus (10–23% for contact and 2–27% for noncontact), regardless of test configuration. The highest escape responses in contact trials using DEET and citronella were observed in the highly pyrethroid-resistant PB population with 53.3 and 84.4% escape, respectively, whereas the lowest escape percentages were seen in pyrethroid-susceptible USDA and BB populations. In noncontact trials, PB and KB displayed a stronger escape reaction to citronella (52 and 40%, respectively) than USDA and BB (36 and 39%, respectively). All *Ae. aegypti* populations exhibited low escape response to eucalyptus extract in both contact (10–23%) and noncontact (2–27%) tests. In all tests and test compounds, very low percent mortality was observed from escape and nonescape mosquitoes, ranging between 0 and 2% (Tables 2 and 3).

The time required in minutes for 25, 50, and 75% of test specimens to escape from treated chambers during the 30-min period is shown in Table 4. Overall, PB and KB insecticide-resistant populations demonstrated a fastest escape response in time and percent escape compared with the two laboratory populations. In contact trials, PB- and KB-resistant populations had the more rapid ET_{25} escape values against DEET and citronella, ranging between 1 and 3 min. The BB laboratory population also showed a comparatively fast ET_{25} (4 min) against citronella compared with USDA (15 min). Because of the low percentage of total escape mosquitoes in the allotted time by the four populations exposed to eucalyptus oil, the ET values could not be calculated. In noncontact trials, the most rapid ET_{25} value (4 min) was seen from PB and citronella oil. Statistical comparisons between strain response and chemical at ET_{25} only detected significant differences ($P < 0.05$) with DEET in both contact and noncontact tests and citronella in contact tests (Table 4).

Multiple log-rank comparisons between the four test populations and each of the three compounds were conducted. In contact trials with DEET, statistical differences in mosquito escape responses during the exposure period were observed in all instances

Table 2. Percent escape response and percent mortality of female *Ae. aegypti* exposed to DEET, citronella oil, or eucalyptus oil in contact excitation ("irritancy") trials

Compound	Test population	Replication	Treatment		Control		% Mortality			
							Treatment		Control	
			N	% Esc. ± SD[a]	*N*	% Esc. ± SD[a]	Esc[b]	NEsc[c]	Esc.	NEsc.
DEET	USDA	4	60	26.7 ± 9.4a	60	6.7 ± 5.4a	0	0	0	0
	BB	4	60	28.3 ± 11.4a	60	11.7 ± 8.4a	0	0	0	0
	PB	4	60	53.3*† ± 12.2b	61	13.3 ± 14.4a	0	0	0	1.6
	KB	4	58	46.6*† ± 10.7b	59	5.0 ± 3.4a	0	0	0	0
Citronella	USDA	4	60	41.7 ± 29.0a	60	5.0 ± 3.4a	0	0	0	0
	BB	4	58	41.1 ± 22.5a	60	6.7 ± 7.7a	0	0	1.7	0
	PB	4	59	84.4*† ± 13.8b	59	8.5 ± 8.4a	0	0	0	0
	KB	4	61	49.1 ± 7.3a	60	3.4 ± 3.9a	0	0	0	0
Eucalyptus	USDA	4	60	15.0 ± 8.4a	60	5.0 ± 3.4ab	0	0	0	0
	BB	4	60	10.0 ± 11.6a	59	5.0 ± 6.4ab	0	0	0	0
	PB	4	60	18.3 ± 11.4b	60	0a	0	0	0	0
	KB	4	60	23.3† ± 20.7b	60	9.7 ± 8.1b	0	0	0	0

USDA; BB, Bora-Bora; PB: Phetchabun; KB, Kanchanaburi.

[a] Different letters indicate significant differences in each test compound at ($P < 0.05$) by one-way ANOVA using Duncan's multiple range test.

[b] Esc., escaped mosquitoes.

[c] NEsc., not escaped mosquitoes.

* Log-rank tests with statistically significant differences ($P < 0.05$) in percentage escape between USDA and two field strains.

† Log-rank tests with statistically significant differences ($P < 0.05$) in percentage escape between BB and two field strains.

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Table 3. Percent escape response and percent mortality of female *Ae. aegypti* exposed to DEET, citronella oil, or eucalyptus oil in noncontact (spatial repellency) trials

Compound	Test pop	Replication	Treatment		Control		% Mortality			
							Treatment		Control	
			N	% Esc. ± SD[a]	*N*	% Esc. ± SD[a]	Esc.[b]	NEsc.[c]	Esc.	NEsc.
DEET	USDA	4	60	13.3 ± 9.4a	60	5.0 ± 10.0a	0	0	0	0
	BB	4	59	37.6 ± 18.9ab	60	6.7 ± 9.4a	0	0	0	0
	PB	4	59	30.7* ± 20.8ab	58	8.3 ± 9.7a	0	0	0	0
	KB	4	60	41.7* ± 6.4b	60	6.7 ± 5.4a	0	1.7	0	0
Citronella	USDA	4	60	35.9 ± 29.1a	59	6.8 ± 5.4a	0	0	0	0
	BB	4	59	39.3 ± 35.4a	60	8.4 ± 3.3a	0	0	0	0
	PB	4	58	51.6* ± 10.5a	58	1.8 ± 3.6a	0	0	0	0
	KB	4	60	40.0 ± 10.3a	59	3.5 ± 4.0a	0	0	0	0
Eucalyptus	USDA	4	60	1.7 ± 3.4a	59	0a	0	0	0	0
	BB	4	61	9.6 ± 10.7ab	59	1.7 ± 3.6ab	0	0	0	0
	PB	4	59	5.0 ± 6.4ab	56	5.3 ± 3.6b	0	0	0	0
	KB	4	60	26.7*† ± 19.6b	60	1.7 ± 3.6ab	0	0	0	0

USDA; BB, Bora-Bora; PB, Phetchabun; KB, Kanchanaburi.

[a] Different letters indicate significant differences in each test compound at ($P < 0.05$) by one-way ANOVA using Duncan's multiple range test.

[b] Esc., escaped mosquitoes.

[c] NEsc., not escaped mosquitoes.

* Log-rank tests with statistically significant differences ($P < 0.05$) in percentage escape between USDA and two field strains.

† Log-rank tests with statistically significant differences ($P < 0.05$) in percentage escape between BB and two field strains.

comparing laboratory with field populations ($P < 0.05$). With citronella, a significant difference in escape was seen between PB and the USDA and BB populations ($P < 0.0001$). With eucalyptus oil, only a marginal statistical difference was seen between KB and BB ($P < 0.04$). For noncontact trials, only four pairings demonstrated statistical differences. These included comparisons between USDA and KB for both DEET ($P < 0.0002$) and eucalyptus ($P < 0.0001$), USDA and PB for citronella ($P < 0.02$), and BB and KB with eucalyptus ($P < 0.01$). Only one comparison combination (BB–KB) produced significant differences in both contact and noncontact trials. Using survival statistics, probability patterns of the escape responses from contact and noncontact test chambers during a 30-min exposure are shown in Figs. 3 and 4. These figures show the relationship between the percent of remaining in treatment chamber at 1-min intervals. PB displayed the lowest number of mosquitoes remaining in the test chambers in contact (Fig. 3) and noncontact trials (Fig. 4) for all three compounds tested.

Discussion

In this study, we compared the behavioral responses between insecticide-susceptible and -resistant populations of *Ae. aegypti* to essential oils using an excito-repellency test chamber system. The susceptible populations were two well-established populations found completely susceptible to diagnostic concentrations of deltamethrin, permethrin, and cypermethrin and two resistant populations derived from recent field collections in Thailand that either showed very strong resistance to all three synthetic pyrethroids or a more mod-

Table 4. Time in minutes for 25 (ET_{25}), 50 (ET_{50}), and 75% (ET_{75}) of *Ae. aegypti* to escape from treated chambers containing 2.5% active ingredient of three chemicals in contact and noncontact chambers using four replicate trials of 15 female mosquitoes per chamber

Treatment	Population	Contact			Noncontact		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
DEET	USDA	17	–	–	–	–	–
	BB	24	–	–	12	–	–
	PB	1	12	–	24	–	–
	KB	3	–	–	8	–	–
	χ^2	32.78 (df = 3)*	–	–	9.45 (df = 2)*	–	–
Citronella	USDA	15	–	–	13	–	–
	BB	4	–	–	10	–	–
	PB	1	2	9	4	29	–
	KB	2	–	–	11	–	–
	χ^2	22.73 (df = 3)*	–	–	4.74 (df = 3)ns	–	–
Eucalyptus	USDA	–	–	–	–	–	–
	BB	–	–	–	–	–	–
	PB	–	–	–	–	–	–
	KB	–	–	–	29	–	–

(–) Too few mosquitoes escaped from chambers, therefore, ET could not be calculated.

χ^2 = Chi-square test.

* Significance at $P < 0.05$; ns, no significant difference.

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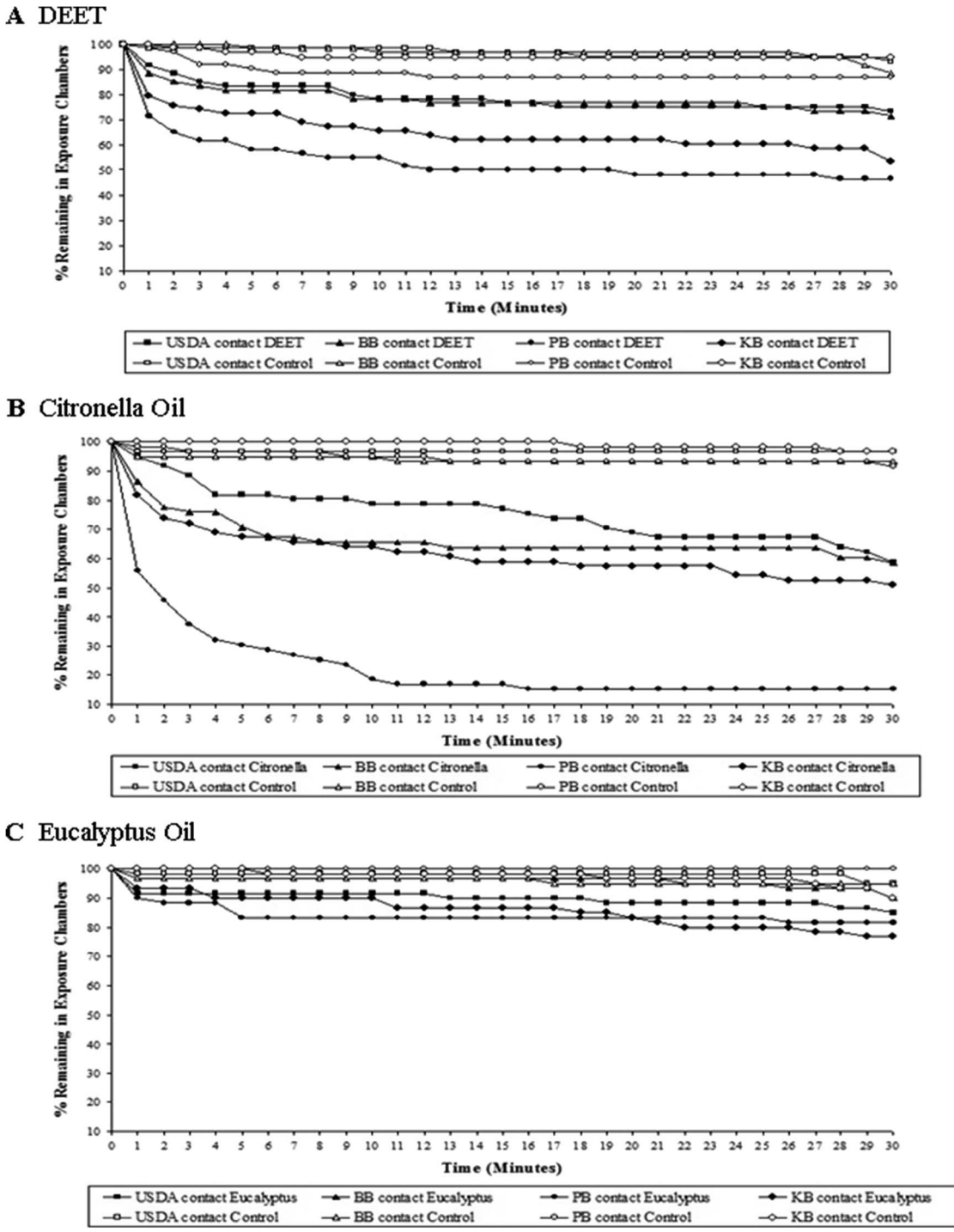


Fig. 3. Kaplan–Meier survival curves of laboratory and field populations of *Ae. aegypti* escaping from paired treated and control contact chambers during the 30 min of exposure to DEET (A), citronella oil (B), and eucalyptus oil (C).

erate resistance response, based on revised WHO criteria (WHO 2013).

The study findings demonstrated a much stronger contact irritant escape response in the pyrethroid-resistant, recent field-derived, populations of *Ae. aegypti* (PB and KB) exposed to citronella oil and DEET compared with the two insecticide-susceptible, long-standing laboratory colonies (USDA and BB). Stronger noncontact

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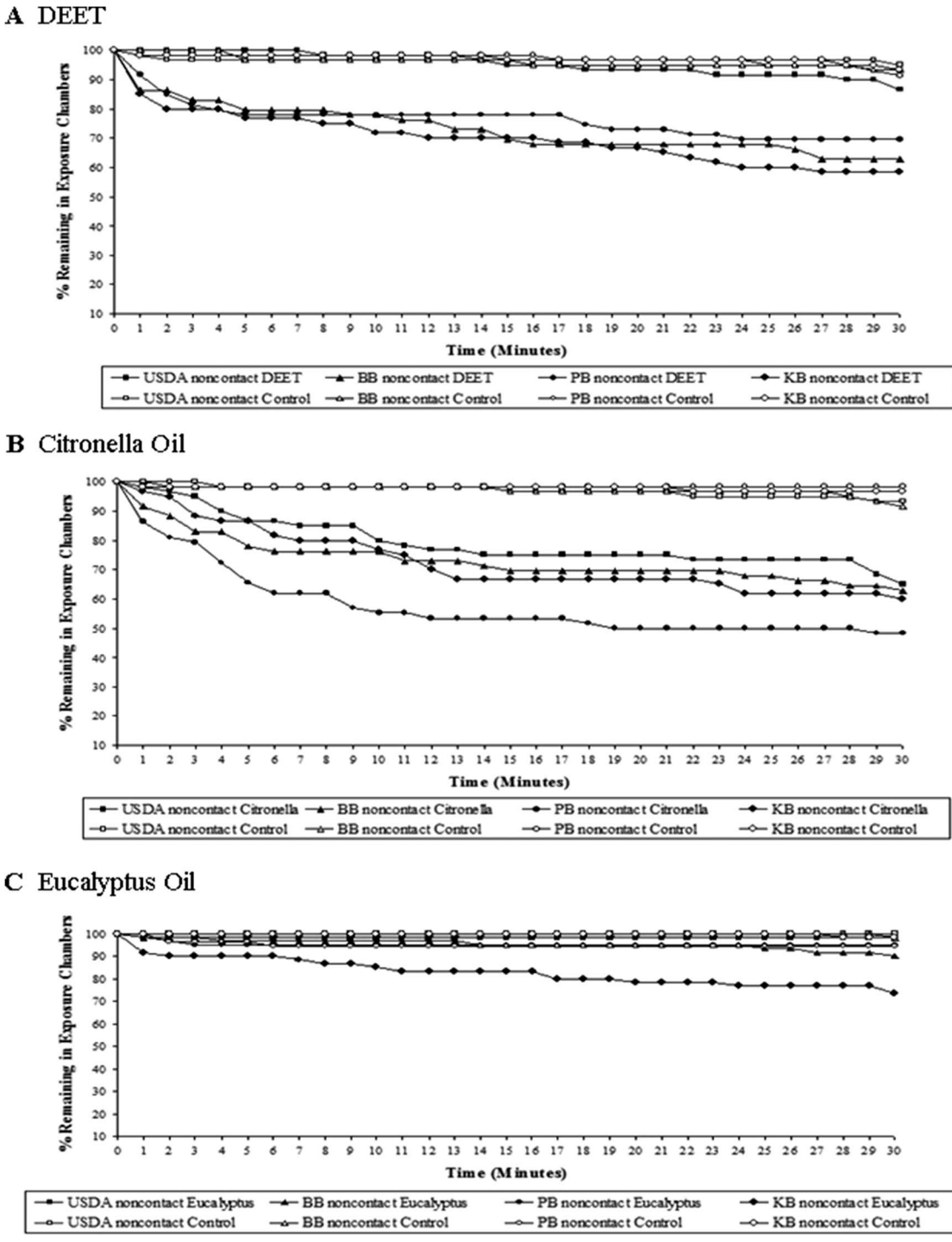


Fig. 4. Kaplan–Meier survival curves of laboratory and field populations of *Ae. aegypti* escaping from paired treated and control noncontact chambers during the 30 min of exposure to DEET (A), citronella oil (B), and eucalyptus oil (C).

repellency was also seen in these low generation (F2 and F3) field populations. Our results showed that DEET and citronella produced the strongest behavioral avoidance response in the PB population, which also had the highest degree of resistance to pyrethroid insecticides compared with the other populations tested. In contrast, USDA and BB showed low to medium escape response to DEET and citronella. This was in agreement with

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Chareonviriyaphap et al. (1997), who showed that a long-established, insecticide-susceptible laboratory colony of *Anopheles albimanus* Wiedemann demonstrated virtually no avoidance behavior to pyrethroids compared with significant escape responses seen in natural populations. Similar findings were reported by Kongmee et al. (2004), who showed a long-established laboratory population of *Ae. aegypti* had much weaker responses to direct, sublethal contact with synthetic pyrethroids compared with recent wild-caught populations. Clark et al. (2011) found that *Ae. aegypti* showed a lower response to host odors after four to six generations of colonization compared with wild-type, field populations. In addition, Stanczyk et al. (2010) proposed that long colonized populations appear to lose a degree of sensitivity in olfactory sensory (receptor) neurones on the insect's antennal flagella that diminish the sensillum functions to more effectively detect volatile chemicals over time. Why chemosensilla might become desensitized while in colony is unclear. Therefore, results from experimentation using or relying on laboratory-reared mosquitoes should be viewed with a degree of caution as it may be likely the responses do not reflect the responses of field mosquitoes (exclusive of insecticide susceptibility status). Our findings also suggest a possible link between background pyrethroid resistance and enhanced sensitivity (avoidance behavior) to DEET and citronella. To show this, further tests will be required comparing insecticide-susceptible and -resistant populations of wild-caught *Ae. aegypti* populations reared to second or third generations that exclude other possible confounding factors (age and physiological conditions) that might influence behavioral responses (Sungvornyothin et al. 2001).

Among the 2.5% concentration of tested compounds, eucalyptus oil was not shown to be an effective excitatory or repellent compound compared with DEET and citronella, showing weak behavioral avoidance responses in both resistant and susceptible mosquito populations. The primary isolate found in eucalyptus oil is eucalyptol, an organic compound that has been shown to have both toxic and repellent properties against various insect species (Klocke et al. 1987, Sfara et al. 2009). Mandal (2011) found that eucalyptus at higher doses of 50 and 100% vol:vol showed excellent repellent action. Yang and Ma (2005) also reported that 15% eucalyptus was able to deter biting of *Ae. albopictus.* Several factors may influence test results, including oil concentration, plant extraction methods to produce the oil, different plant sources, mosquito test species, and repellent methodology used (Barnard et al. 1998, Shaaya and Rafaeli 2007).

This study found citronella as effective as DEET against the insecticide-resistant mosquitoes. Tawatsin et al. (2001) reported that citronella is quite effective at repelling *Ae. aegypti*, *Anopheles dirus* Peyton & Harrison, and *Cx. quinquefasciatus.* Citronella is an effective as well as a safe alternative to synthetic repellents because of its low toxicity to humans and fast biodegradability (Adeyemi 2011). To enhance the protection time (persistence) and effectiveness, it would be useful to test different concentrations that may lead to improvement and optimization in repellency against mosquitoes.

There are several essential oils that have been reported to be effective against various mosquito species, including citronella, catnip, eucalyptus, neem seed, hairy basil, and clove (Tawatsin et al. 2001, Zhu et al. 2006, Suwansirisilp et al. 2012). Citronella is one of the most common ingredients in commercial insect repellents, primarily as a consumer alternative to those containing DEET. However, an improvement in the efficiency of repellent products to enhance persistence to protect effectively protect against mosquito bites is needed. Progress is being made; for example, the development of a microencapsulated citronella oil formulation on treated textile fabrics provides a more potent and longer lasting protection from *Ae. aegypti* (Specos et al. 2010). Nuchuchua et al. (2009) has shown that essential oils in nanoemulsion formulations can improve both physical stability and prolong mosquito protection time.

Understanding the full range of responses by a mosquito population to an insecticide, including toxicity and avoidance behavior, is of great importance to vector control programs. The vast majority of research on synthetic chemicals against insects has focused exclusively on toxicity and mechanisms of resistance (Brogdon and McAllister 1998, Corbel and N'Guessan 2013). However, numerous attempts have been made to investigate the behavioral responses of mosquitoes to test compounds using a variety of excito-repellency test systems (Roberts et al. 1984; Chareonviriyaphap et al. 1997; Rutledge et al. 1999; Grieco et al. 2005, 2007). Historically, most behavioral studies have concentrated on insecticides and common synthetic repellent compounds, whereas investigations on the properties of botanical essential oils have been relatively more recent. More than 90 plant species have been investigated to evaluate repellent or toxic properties against blood-feeding invertebrates and pestiferous insects (De Boer et al. 2010). Although a number of essential oils have known repellent activities against blood-feeding insects, comparatively few studies have been performed to identify and differentiate between the two primary types of excito-repellency actions—contact excitation and noncontact spatial repellency (Polsomboon et al. 2008, Suwansirisilp et al. 2012). A better understanding of the mechanisms of how these essential oil products protect against mosquito attack is important for guiding future research for developing new and better repellent products. The continuing spread and concern on development of resistance to insecticides by mosquitoes has further prompted the search and use of alternative compounds or new approaches to protect humans from mosquito bites (Achee et al. 2012).

Possible alternatives to insecticide use may be found with various botanical compounds that are known to possess varying degrees of repellency that can protect humans from mosquito attack. In the product development phase, an initial screening step should be performed to assure and validate the reputed effects of active ingredients against various mosquito populations before investing time and funds investigating candidate compounds further. We have

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found the excito-repellency test system is a useful tool to perform screening on excito-repellency.

In summary, this study compared the behavioral responses (contact irritancy and noncontact repellency) between susceptible and resistant populations of *Ae. aegypti* to essential oils, citronella and eucalyptus extracts, using an excito-repellency test system. DEET was used as the standard reference repellent. Both DEET and citronella produced much stronger excitation ("irritancy") and flight escape responses in both pyrethroid-resistant field-derived populations compared with the laboratory colonies. Greater noncontact repellency was also seen in the two resistant populations as compared with the laboratory populations. Eucalyptus oil was found to be the least effective compound tested. These finding suggest a possible link between background pyrethroid resistance and enhanced sensitivity (avoidance behavior) to DEET and citronella. Further study is required to establish and understand the association between prolonged and continuous colonization (successive generations) as a possible desensitizing factor in the mosquitoes ability to detect offensive chemicals.

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Diversity of *Anopheles* species and trophic behavior of putative malaria vectors in two malaria endemic areas of northwestern Thailand

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ABSTRACT: We determined the species diversity, blood-feeding behavior, and host preference of *Anopheles* mosquitoes in two malaria endemic areas of Tak (Mae Sot District) and Mae Hong Son (Sop Moei District) Provinces, located along the Thai border with Myanmar, during a consecutive two-year period. Anopheline mosquitoes were collected using indoor and outdoor human-landing captures and outdoor cow-baited collections. Mosquitoes were initially identified using morphological characters, followed by the appropriate multiplex AS-PCR assay for the identification of sibling species within *Anopheles* (*Cellia*) complexes and groups present. Real-time PCR was performed for parasite-specific detection in mosquitoes (*Plasmodium* spp. and *Wuchereria bancrofti*). A total of 7,129 *Anopheles* females were captured, 3,939 from Mae Sot and 3,190 from Sop Moei, with 58.6% and 37% of all anophelines identified as *An. minimus*, respectively. All three malaria vector complexes were detected in both areas. One species within the Minimus Complex (*An. minimus*) was present along with two related species in the Funestus Group, (*An. aconitus*, *An. varuna*), two species within the Dirus Complex (*An. dirus*, *An. baimaii*), and four species within the Maculatus Group (*An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori*, and *An. dravidicus*). The trophic behavior of *An. minimus, An. dirus, An. baimaii, An. maculatus,* and *An. sawadwongporni* are described herein. The highest *An. minimus* densities were detected from February through April of both years. One specimen of *An. minimus* from Mae Sot was found positive for *Plasmodium vivax.* ***Journal of Vector Ecology* 39 (2): 424-436. 2014.**

Keyword Index: *Anopheles dirus*, *Anopheles minimus*, *Anopheles maculatus*, species complexes, malaria, Thailand.

INTRODUCTION

After years of intensive, well-organized malaria control activities in Thailand, malaria still remains prevalent in vulnerable areas, especially along the less developed international borders with Myanmar. Approximately 85% of all reported malaria cases in the country occur in this poorly monitored border region, primarily related to transient employment opportunities or occupational activities including agriculture, hunting, and gem mining. Several primary malaria vector species in Thailand are abundant in forested areas along the international border (Chareonviriyaphap et al. 2003, Corbel et al. 2013). Bancroftian filariasis is also endemic along the Thai-Myanmar border but of low prevalence. However, the detection of this parasite is not uncommon among Burmese working in Thailand (Khamboonruang et al. 1987, Jitpakdi et al. 1998, Bhumiratana et al. 2002). Nocturnally subperiodic (NSP) *Wuchereria bancrofti* is transmitted by a wide variety of mosquito species including anophelines also capable of malaria transmission (Pothikasikorn et al. 2008).

Of the 73 known *Anopheles* species in Thailand, various sibling species in the Minimus and Dirus Complexes and the Maculatus Group have been recognized as the main malaria vectors in the country (Rattanarithikul et al. 2006, Sinka et al. 2011). Molecular techniques based on polymerase chain reaction (PCR) have allowed for precise and reliable differentiation and identification of the sibling species of medical importance (Manguin et al. 2008, Sinka et al. 2011). Among these 73 species, five to six species, depending on the literature, are incriminated as primary malaria vectors in Thailand (Rattanarithikul et al. 2006), including *Anopheles baimaii* (previously *An. dirus* D) (Green et al. 1991) and *Anopheles dirus* (Rosenberg et al. 1990, Green et al. 1991) of the Dirus Complex, *Anopheles minimus* (previously *An. minimus* A) (Rattanarithikul et al. 1996a) of the Minimus Complex, and *Anopheles pseudowillmori* (Green et al. 1991), *An. maculatus* and *An. sawadwongporni* of the Maculatus Group (Saeung 2012). Additionally, *An. campestris* and *An. epiroticus* (Sundaicus Complex) have also been incriminated as potential malaria vectors in Thailand (Apiwathnasor et al. 2002). *Wuchereria bancrofti* develops experimentally in *An. minimus* and *An. maculatus* (Pothikasikorn et al. 2008). Natural infections of this parasite have also been found in *An. minimus, An. maculatus and An. vagus* in Thailand (Pothikasikorn et al. 2008).

While the number of cryptic species has increased, Thailand has been identifying sibling species within the complexes for several decades, yet information on the distribution, ecology and behavior of many of these species remains poor or lacking. This information is quite critical in defining the vector capacity of each species (Takken and Verhulst 2013). Knowledge on mosquito behavior is crucial

to understanding the epidemiology of vector transmission throughout the range of each species and for assisting national and international efforts in vector-borne disease control. Numerous observations on trophic behavioral and seasonal abundance have been conducted on *Anopheles* complexes (*sensu lato*) or related groups (Ismail et al. 1974, Harbach et al. 1987, Ratanatham et al. 1988, Rattanarithikul et al. 1996a, Chareonviriyaphap et al. 2003). Unfortunately, most of these observations relied on species that were identified by morphological characters alone, as molecular assays capable of reliably identifying each sibling species were not readily available or still non-existent. During the past few decades, studies on malaria vectors in Thailand using molecular identification assays have increased and provided precise identification to the species level, as well as the recognition of additional *Anopheles* species and species complexes in Thailand (Baimai et al. 1984, 1988, Baimai 1989, Green et al. 1990, Green et al. 1992, Poopittayasataporn and Baimai 1995, Walton et al. 1999, Garros et al. 2004, Rattanarithikul et al. 2006, Dusfour et al. 2007, Walton et al. 2007, Poolprasert et al. 2008, Saeung et al. 2008, Thongsahuan et al. 2009, Eamkum et al. 2014).

Previous studies have described the biting activity of *An. minimus* and *An. harrisoni* in malaria-endemic Tak (Tisgratog et al. 2012) and Kanchanaburi Provinces (Sungvornyothin et al. 2006), and both biting activity and host preference of *An. dirus* and *An. baimaii* in Kanchanaburi Province (Tananchai et al. 2012). However, additional investigations on the bionomics and blood feeding activities of sympatric malaria vectors belonging to species complexes are needed. In the present study, molecular identification assays were combined with the systematic descriptions of the trophic behavior, biting activity, seasonal abundance, and parasite infections of individual sibling species in the two most malaria endemic areas of Mae Sot (Tak Province) and Sop Moei (Mae Hong Son Province), northwestern Thailand. Additionally, overall anopheline species diversity from these two areas along the Thai-Myanmar border is described.

MATERIALS AND METHODS

Collection sites

Adult mosquito collections were conducted every two months during three consecutive nights, for a period of two years in one village each in Mae Sot District (Tak Province) and Sop Moei District (Mae Hong Son Province) located in northwestern Thailand (Figure 1).

The Mae Sot field site has a western boundary with Kayin State, Myanmar and located at approximately 471 m above sea level. There were five isolated hill tribes living in the area, the most common ethnic group being Karen (> 85%). Rubber plantations, fruit orchards and other agricultural crops surrounded most of the study site. A narrow, slow running stream, approximately 0.5 m in average depth and 2 m wide, ran across the village.

The Sop Moei study site neighbors Myanmar, Shan State to the north and Kayin and Kayah States to the west. The field site is located approximately 50 m from the Ngao River and one kilometer from Mae Ngao National Park. The study site is surrounded by secondary forest at approximately 126 m above sea level. There are several tribal groups in this area in which the most common is Thai.

Collection methods

Standard landing collection techniques were used such as indoor and outdoor human-baited landing captures and cattle-baited outdoor trap collections. Indoor and outdoor human landing collections (HLC) were conducted by two teams of four people each. The first team worked from 18:00 to 24:00, followed by a second team from midnight to 06:00. Two people sat inside the house, while the other two took up a position outside approximately 100 m from the same hut. Human-landing collections occurred for 45 min each hour followed by a 15 min resting period. The collectors rotated hourly between indoor and outdoor collections. Cattle bait collections were conducted by one separate collector for 15 min each hour. Additional details on human landing collection methods were described previously (Tisgratog et al. 2012). Collected mosquitoes were kept in a clean plastic cup covered with netting and provided 10% sugar solution soaked on cotton. The following morning, mosquitoes were taken back to the field laboratory for morphological identification. Hourly ambient outdoor air temperatures and relative humidity were recorded. Rainfall data was collected from the Mae Sot and Sop Moei District meteorological stations, located approximately 10 km from each village. The human and animal use protocols for this study were approved by the Ethical Research Committee, Chulalongkorn University, Bangkok, Thailand (No. 0961/56).

Mosquito species identification

Each female mosquito was intially sorted using morphological keys (Rattanarithikul et al. 2006), individually stored in a clean, labelled 1.5 ml non-silicone tube, and immediately frozen and stored in a liquid nitrogen tank. Mosquito samples were sent to the Department of Entomology, Kasetsart University, in Bangkok. Individual *An. minimus* s.l., *An. dirus* s.l. and *An. maculatus* s.l. were placed in a DNA extraction tube and homogenized in 50 ml of extraction buffer (0.2 M sucrose, 0.1M Tris-HCl at pH 8.0, 50mM EDTA and 0.5% SDS). A volume of 7 µl of 8 mM KOAc (pH 9.0) was added and the tube placed on ice for 30 min. The sample was centrifuged at 12,000 rpm for 20 min and the supernatant was removed to a clean tube. Then, 100 µl of 100% ethanol was added and the samples placed at 4° C for 30 min. Samples were spun at 12,000 rpm for 20 min at 4° C. The supernatant was again cleaned using 150 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4° C, and again with 100% ethanol and centrifuged at 12,000 rpm for 5 min at 4° C. The resultant pellet was dried at room temperature for 20 min before being re-suspended in 100 µl of TE buffer and stored at -20°C, based on Linton et al. (2001) and Manguin et al. (2002).

Species complex or group specific multiplex allele-specific polymerase chain reaction (AS-PCR) assays were used for molecular species identification within the 1) Minimus

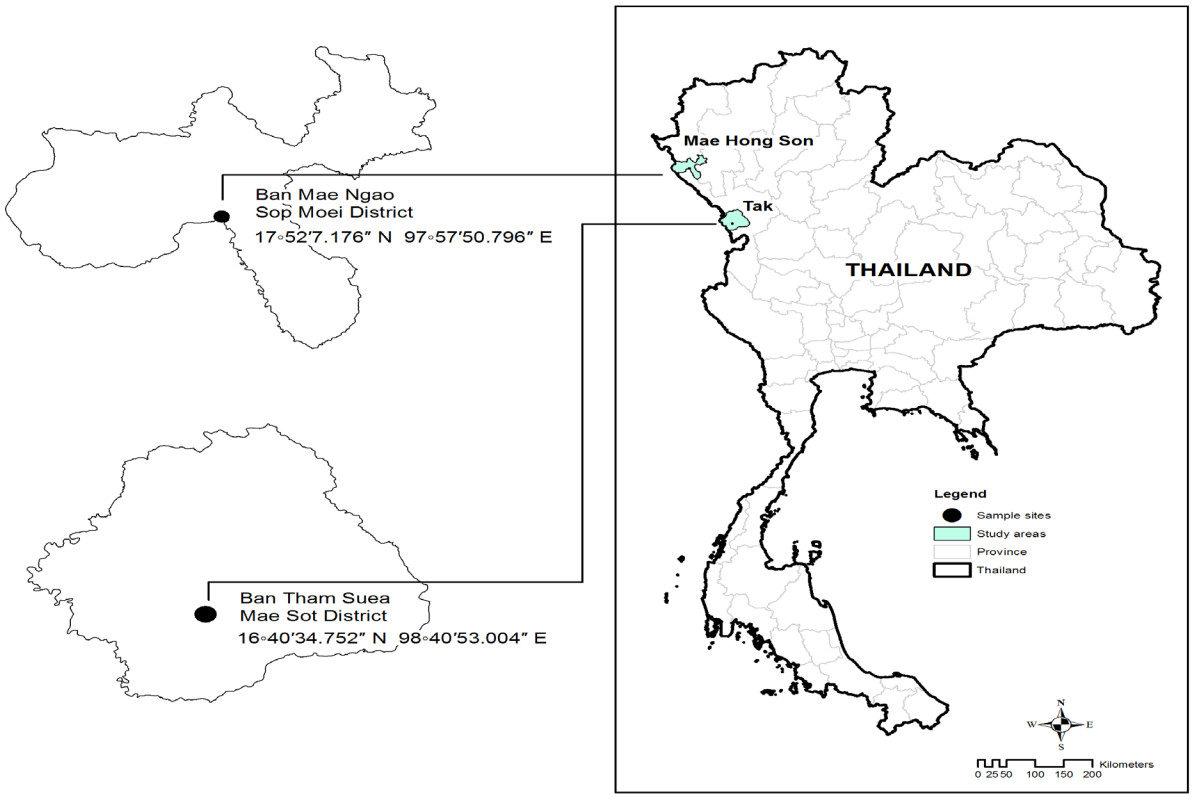


Figure 1. Study site locations in Mae Sot District, Tak Province and Sop Moei District, Mae Hong Son Province.

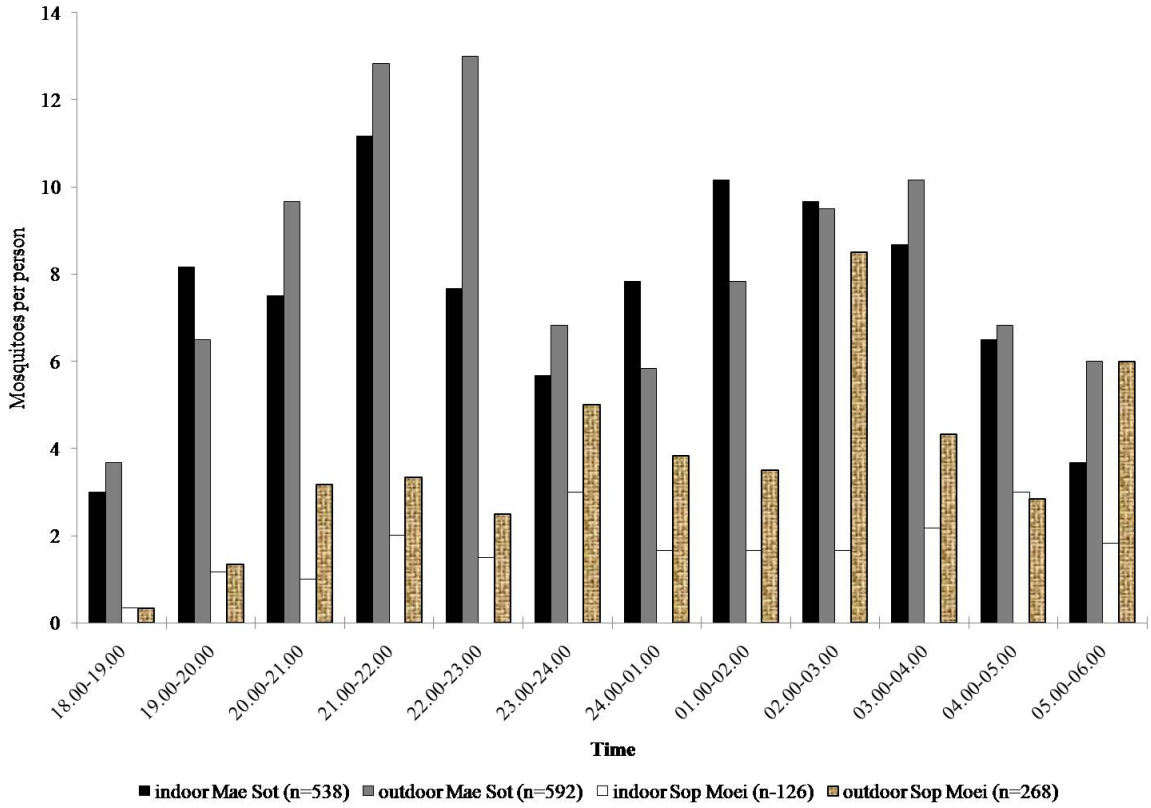


Figure 2. Mean indoor and outdoor human-landing rates by hour for *Anopheles minimus* in Mae Sot and Sop Moei.

Complex and related species, including *Anopheles minimus*, *An. harrisoni*, *An. aconitus, An. varuna* and *An. pampanai* (Sungvornyothin et al. 2006), 2) Dirus Complex, including *Anopheles dirus*, *An. cracens*, *An. scanloni*, *An. baimaii* and *An. nemophilous* (Walton et al. 1999), and 3) Maculatus Group, including *Anopheles maculatus*, *An. sawadwongporni*, *An. pseudowillmori*, *An. dravidicus*, *An. rampae* (former Form K) (Walton et al. 2007, Somboon et al. 2011).

Real Time PCR detection for *Plasmodium* species and *Wuchereria bancrofti* in mosquitoes

Real-time PCR was performed with a Roche LightCycler®480 (Software Version LCS480 1.5.0.39) using TaqMan reagents and hydrolysis probes for the detection of *Plasmodium falciparum*, *P. vivax,* and *P. knowlesi*, following a slightly modified methodology of Divis et al. (2010), and *Wuchereria bancrofti* immature stages using methods of Rao et al. (2006). Two reactions were separately performed including a first round of screening reaction for the detection of all *Plasmodium* species (Primers; Plasmo 1, Plasmo 2 and the *Plasmodium* screening probe) from Rougemont et al. (2004), *Plasmodium vivax* (Primers; VIV-F, VIV-R and probe VIV-PB) from Perandin et al. (2004) and *Wuchereria bancrofti* (Primers; LDR1-F, LDR2-R and probe WB-PB) from Rao et al. (2006). For the second round, a specific reaction was processed for the detection of *P. falciparum* (Primers; FAL-F, FAL-R and FAL probe) from Perandin et al. (2004) and *P. knowlesi* Primers; Plasmo 1 and 2 from Rougemont et al. (2004) and PK probe from Divis et al. (2010).

Data analysis

Numbers of primary malaria vectors collected by different collection methods were analyzed using non-parametric tests. A Wilcoxon test and Pearson's correlation analyses were used to investigate the interaction between the number of mosquitoes and environmental parameters. All data were analyzed with a level of significance set at 0.05% ($p< 0.05$), using a SPSS statistical package (ver. 17.0, SPSS, Chicago, IL).

RESULTS

Species composition and abundance

A total of 7,129 *Anopheles* females was collected during the two-year sampling period of February, 2011 to January, 2013. This sample included 22 species in the subgenus *Cellia* (6,156 specimens), and a single species and three groups in the subgenus *Anopheles* (n=973) (Table 1). Of 5,148 specimens within the two complexes and the Maculatus Group identified by molecular assays, 3,278 were derived from Mae Sot and 1,870 from Sop Moei field sites. The Minimus Complex and related species included *An. minimus*, *An. aconitus* and *An. varuna*; the Dirus Complex was composed of *An. dirus* and *An. baimaii*; and the Maculatus Group was represented by *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus*. Within the subgenus *Cellia*, the most abundant species in Mae Sot and Sop Moei was *An. minimus* accounting for 58.6% and 37.0% of all recorded anophelines, respectively and 49.6% of the total sample from both sites combined (Table 1). *Anopheles maculatus* represented 25.4% and 20.4% of the total anopheline sample by site in Mae Sot and Sop Moei, respectively, and 23.3% of total for both sites. In both sites, *An. dirus* and *An. baimaii* were rarely collected, 0.5% and 0.6%, respectively in Mae Sot and 0.4% for both species in Sop Moei. The other *Anopheles* taxa in the subgenus *Cellia* included *An. annularis* s.l., *An. culicifacies* s.l., *An. jamesii*, *An. jeyporiensis*, *An. karwari, An. kochi* s.l., *An. nivipes*, *An. philippinensis, An. splendidus, An. stephensi*, *An. subpictus, An. tessellatus* and *An. vagus*, all of which were identified by morphological criteria only. In the subgenus *Anopheles*, a species belonging to the *Anopheles hyrcanus* group was the most prevalent with 46.7% and 45.0% collected in Mae Sot and Sop Moei, respectively and representing 45.6% of the total sample in the subgenus, followed by the *Anopheles barbirostris* Group (36.0% in Mae Sot, 21.9% in Sop Moei), for a total of 27% of collection for both sites. In addition, specimens of the *Anopheles umbrosus* group and *Anopheles peditaeniatus* were also found in both study sites.

Mosquito density and feeding behavior

The monthly density of *An. minimus* and two closely related species, *An. aconitus* and *An. varuna,* collected from the two sites during the two years of collection is shown in Table 2. *Anopheles minimus* was found throughout the year with the highest density during the 'summer' period, between February and April, in both sites and years. Comparatively, *An. aconitus,* and *An. varuna* were collected in much smaller densities in Mae Sot and Sop Moei, varying from 0.7% to 1.9% for *An. aconitus*, and from 2.7% to 1.2% for *An. varuna*, respectively (data not shown). Both species were highly zoophilic as all were collected on cattle-baited traps in Mae Sot, and more than 80% were attracted to cattle in Sop Moei. Indoor and outdoor feeding behaviors of *An. minimus* in Mae Sot were quite similar, with peak biting activity occurring between 21:00 and 23:00. In contrast, indoor and outdoor feeding behaviors of *An. minimus* from Sop Moei were markedly different, with the outdoor peak between 02:00 and 04:00 and indoor peak after 05:00 (Figure 2).

The monthly densities of *An. dirus* and *An. baimaii* collected from both study sites are shown in Table 2. Both species were found in similar proportions, with 18 and 21 specimens, respectively, in Mae Sot and 11 specimens for each species in Sop Moei. Nearly twice as many specimens were captured in Mae Sot (n=39) compared to Sop Moei (n=22). In both years, the greatest numbers of individuals were encountered in June. In Mae Sot, *An. dirus* and *An. baimaii* had a preference/attraction to humans with 97% (n=38) collected on human-bait compared to cattle. The indoor:outdoor (9:8) ratios were nearly identical, although the overall numbers were small, while *An. baimaii* was collected in greater number outdoors (n=16) than indoors (n=5) on humans. In Sop Moei, neither species showed any significant anthropophilic behavior. Both species were collected as frequently on humans (n=11) as on cattle (n=11). In Mae Sot, *An. dirus* presented an early peak of biting activity indoors (19:00-20:00) and a later peak outdoors (23:00-midnight)

Table 1. Numbers of *Anopheles* collected by species and percentage divided by subgenus in Mae Sot and Sop Moei from February, 2011 to January, 2013.

Species	Mae Sot (%)	Sop Moei (%)	Total (%)
Anopheles (Cellia)			
An. minimus	2,103 (58.6)	951 (37.0)	3,054 (49.6)
An. aconitus	16 (0.5)	19 (0.7)	35 (0.6)
An. varuna	60 (1.7)	12 (0.5)	72 (1.2)
An. dirus	18 (0.5)	11 (0.4)	29 (0.5)
An. baimaii	21 (0.6)	11 (0.4)	32 (0.5)
An. maculatus	911 (25.4)	525 (20.44)	1,436 (23.3)
An. sawadwongporni	116 (3.2)	274 (10.7)	390 (6.3)
An. pseudowillmori	4 (0.1)	51 (2.0)	55 (0.9)
An. dravidicus	29 (0.8)	16 (0.6)	45 (0.7)
An. annularis s.l.	-	8 (0.3)	8 (0.1)
An. culicifacies s.l.	2 (0.1)	83 (3.2)	85 (1.4)
An. jamesii	78 (2.2)	89 (3.5)	167 (2.7)
An. jeyporiensis	-	2 (0.1)	2 (0.0)
An. karwari	3 (0.1)	11 (0.4)	14 (0.2)
An. kochi s.l.	93 (2.6)	316 (12.3)	409 (6.6)
An. nivipes	9 (0.3)	6 (0.2)	15 (0.2)
An. philippinensis	72 (2.0)	65 (2.5)	137 (2.2)
An. splendidus	2 (0.1)	2 (0.1)	4 (0.1)
An. stephensi	1 (0.0)	1 (0.0)	2(0.0)
An. subpictus	1 (0.0)	9 (0.4)	10 (0.2)
An. tessellatus	-	38 (1.5)	38 (0.6)
An. vagus	47 (1.3)	70 (2.7)	117 (1.9)
Total (s.g. *Cellia*)	3,586 (58.3%)	2,570 (41.7%)	6,156
Anopheles (Anopheles)			
An. barbirostris Group	127 (36.0)	136 (21.9)	263 (27.0)
An. umbrosus Group	42 (11.8)	134 (21.6)	176 (18.1)
An. hyrcanus Group	165 (46.7)	279 (45.0)	444 (45.6)
An. peditaeniatus	19 (5.4)	71 (11.5)	90 (9.2)
Total (s.g. *Anopheles*)	353 (36.3%)	620 (63.7%)	973
Grand total	3,939 (55.3%)	3,190 (44.7%)	7,129

Table 2. Number, distribution and frequency of four primary *Anopheles* malaria vector species collected in Mae Sot and Sop Moei (I= indoor human landing, O=outdoor human landing, C= cattle baited).

	Mae Sot												Sop Moei											
Month	*An. minimus*			*An.dirus*			*An. baimaii*			*An.maculatus*			*An. minimus*			*An.dirus*			*An. baimaii*			*An.maculatus*		
	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C
Year 1																								
Feb-11	134	128	207	-	-	-	-	-	-	1	6	10	8	40	24	-	-	-	-	-	-	-	1	8
Apr-11	121	111	220	1	1	-	-	1	-	3	3	21	21	20	108	-	1	-	-	-	-	-	2	6
Jun-11	65	106	30	6	7	1	2	2	-	9	53	39	6	15	38	1	-	1	1	1	2	2	6	20
Aug-11	-	5	2	-	-	-	-	2	-	3	11	-	-	8	10	-	-	-	-	-	-	-	-	4
Oct-11	23	26	33	-	-	-	-	-	-	26	32	193	2	2	5	-	1	1	-	-	-	2	4	65
Dec-11	30	36	61	-	-	-	-	1	-	20	20	38	-	-	95	-	-	-	-	-	-	3	6	36
Year 2																								
Feb-12	41	31	111	-	-	-	-	-	-	8	8	-	7	49	117	-	-	-	-	-	-	-	-	-
Apr-12	59	79	183	-	-	-	-	-	-	4	4	-	21	20	55	-	-	-	-	-	-	2	-	-
Jun-12	38	37	78	1	-	-	-	5	-	17	20	114	48	87	10	1	1	2	-	4	2	3	50	149
Aug-12	-	3	10	-	-	-	3	1	-	5	25	42	1	9	5	-	-	-	-	-	-	-	2	27
Oct-12	16	6	21	1	-	-	-	4	-	23	45	-	4	10	12	-	-	2	-	-	-	-	2	-
Dec-12	11	24	17	-	-	-	-	-	-	30	78	-	8	8	78	-	-	-	-	-	1	2	2	121
Total	538	592	973	9	8	1	5	16	-	149	305	457	126	268	557	2	3	6	1	5	5	14	75	436

*Summer= February, March, April, May; Wet=May, June, July, August, September, October; Dry= November, December, January.

(Figure 3). In Sop Moei, the numbers were too low to clearly define the precise biting pattern. For *An. baimaii*, outdoor and indoor collections showed nearly identical biting peaks from 20:00 to 22:00 hrs, with a second peak for outdoor collections (04:00-05:00). In Sop Moei, the biting (landing) peak activity was from 01:00 to 02:00 (Figure 4). Neither species was found in great abundance, so that drawing any definitive conclusions about these data should be done with caution.

Four species belonging to the Maculatus Group were identified in Mae Sot and Sop Moei: *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* (Tables 1, 2). Collectively, the group was found throughout the year with the highest density during the rainy period between May and October in both sites. *Anopheles maculatus* was the most abundant and consistent species present in both sites (n=1,436) representing 74.6% of the species group, followed by *An. sawadwongporni* (n=390) representing 20.2%. *Anopheles pseudowillmori* and *An. dravidicus*, were captured in much smaller proportions, 2.9% and 2.3% of total collections, respectively, and having periods (months) of little or no captures recorded. In general, all Maculatus Group members were collected in greater numbers from cow than human, except for *An. sawadwongporni* in Mae Sot and *An. pseudowillmori* in Sop Moei where higher numbers in outdoor HLC were seen (data not shown). The activity of *An. maculatus* in Mae Sot showed a very clear early biting peak between 18:00 and 21:00 for outdoor HLC (Figure 5). The biting activity of *An. sawadwongporni* from outdoor HLC in both villages showed a small peak between 19:00 to 20:00 (data not shown). Like the Dirus Complex, neither species was found in great abundance, so drawing any definitive conclusions regarding these data should be done with caution.

Non-parametric analysis of densities of *Anopheles minimus* and *An. maculatus* in different collection sites between the numbers of mosquitoes collected from indoor/outdoor human-baited collections and environmental data (temperature, relative humidity (RH) and rainfall) found no statistical association except for *An. maculatus* in Sop Moei regards adult densities and variations in mean ambient temperature.

Trophic behavior

The non-parametric Wilcoxon analysis was used to compare the hourly landing rates of the two most abundant mosquito species, *An. minimus* and *An. maculatus,* using different collection methods and study locations. For *An. minimus*, no significant differences (Z=-0.94, p=0.348) were observed in Mae Sot between the indoor (3.76±0.3) and outdoor (4.11±0.41) human landing. However, *An. minimus* showed significantly greater (Z=-2.43, p=0.02) anthropophilic (7.85±0.72) behavior compared to its attraction to cattle (6.76±0.72) (Table 5). In Sop Moei, *An. minimus* was significantly more exophagic (Z=-5.72, $p<0.01$) and zoophilic (Z=-3.99, p<0.01) (Table 5). *An. maculatus* showed similar and highly significant exophagic and zoophilic behavior in both study sites.

Parasite detection

Eight *Anopheles* species (n=1,447) from both collection sites in Mae Sot and Sop Moei were examined for parasite infection rates as follows: *An. minimus* (1,090, 132), *An. aconitus* (16, 10), *An. dirus* (18, 10), *An. baimaii* (14, 19), *An. maculatus* (55, 25), *An. sawadwongporni* (2, 39), *An. pseudowillmori* (2, 39) and *An. dravidicus* (5, 1), respectively. Only one specimen, *An. minimus* from Mae Sot in an outdoor HLC, 00:00-01:00 in April, 2011, was found positive for *P. vivax*.

DISCUSSION

This study is the first detailed description of the diversity of *Anopheles* species based on a molecular identification in the two most malaria endemic areas of Thailand, Mae Sot District in Tak Province and Sop Moei District in Mae Hong Son Province, both located in the northwestern part of the country that borders Myanmar. Results from this study provide accurate species identification as well as seasonal abundance and trophic behavior of *Anopheles minimus* and *Anopheles maculatus*, among the most important malaria vectors in northwestern Thailand.

In this study, six of the most important (primary and secondary) malaria vector species in Thailand were collected. The most abundant species in Mae Sot and Sop Moei was *An. minimus* (42.8%), followed by *An. maculatus* (20.1%) and *An. sawadwongporni* (5.5%). Collectively, these species are responsible to varying degrees for maintaining malaria transmission in both areas which have been listed as 'A1' areas by the Thai Ministry of Public Health, areas where malaria occurs at least 6 months of the year and longer (Chareonviriyaphap et al. 2001, BVBD 2013).

Anopheles minimus represented 43% of the total *Anopheles* mosquitoes recorded from both areas during the two-year collection period (53.4% in Mae Sot and 29.8% of all anophelines in Sop Moei. *Anopheles maculatus* and *An. sawadwongporni* were found in greater numbers compared to *An. pseudowillmori* and *An. dravidicus*, all species within the Maculatus Group. These findings are consistent with previous observations in Mae Sot District (Tum Sua Village) that found *An. minimus* to be the most abundant species (71%), followed by *An. maculatus* Group (28%) and few specimens of the *An. dirus* Complex (Tisgratog et al. 2012). *Anopheles dirus* and *An. baimaii*, closely related and the most important forest-related malaria vectors, were found in very low densities with only a total of 39 specimens collected in Mae Sot and 22 in Sop Moei during a two-year period. These two species were slightly more common during the June collection both years with only a few specimens detected other months. Low collection numbers of the Dirus Complex complicate drawing any definitive conclusions on host-seeking behavior in this study.

In Thailand, the biting activity and behavior of specific malaria vectors, based on reliable molecular identification methods, has been studied in detail in relatively fewer instances compared to earlier studies that based all identification on morphological characters alone. For example, two species

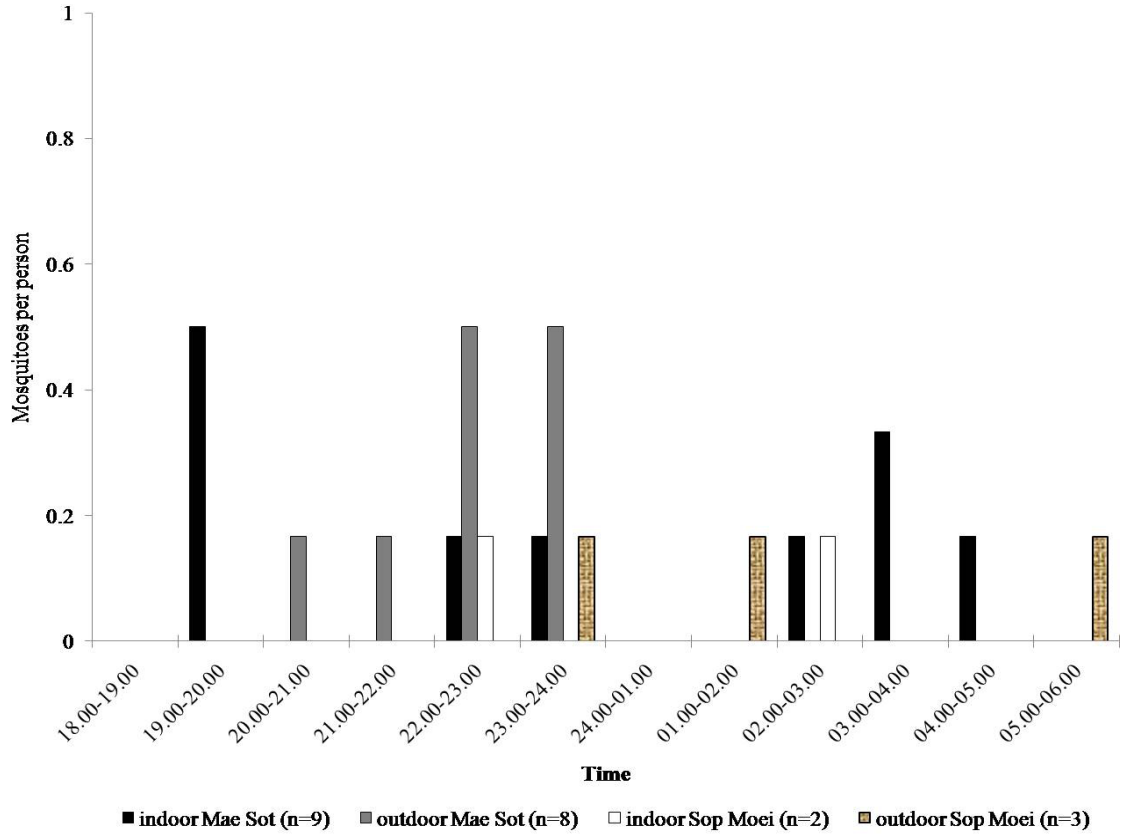


Figure 3. Mean indoor and outdoor human-landing rates by hour for *Anopheles dirus* in Mae Sot and Sop Moei.

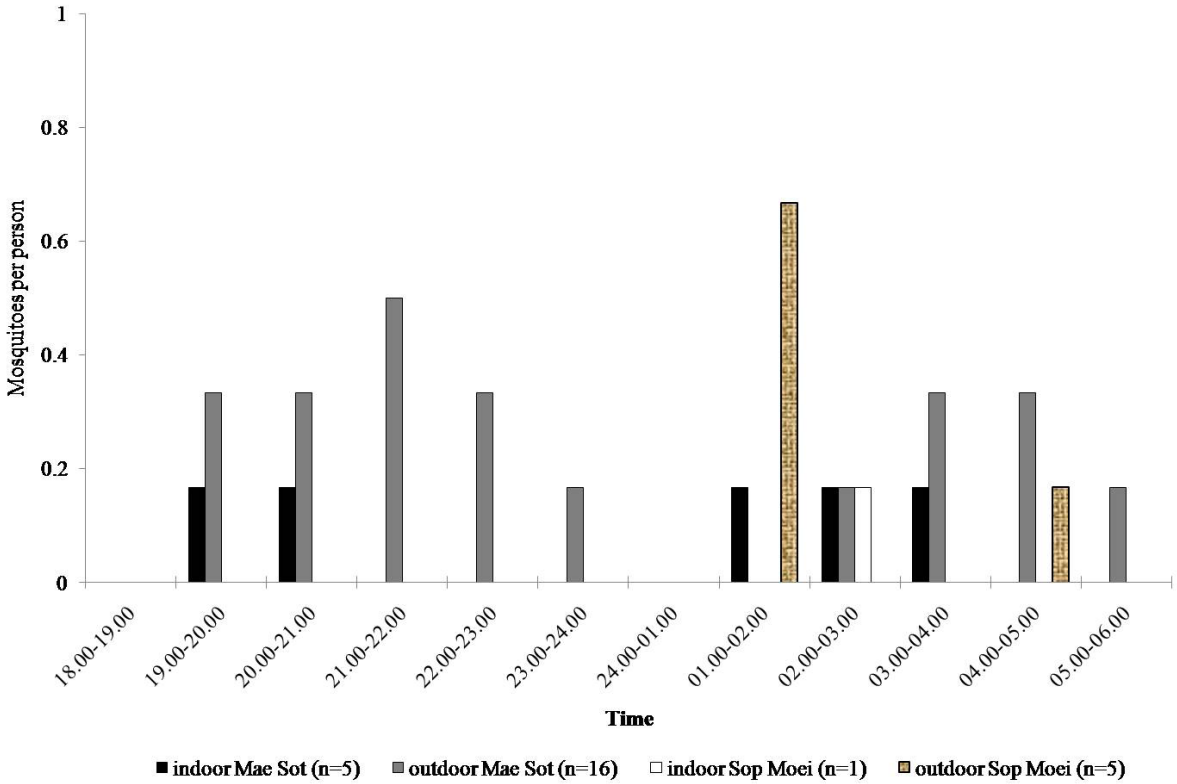


Figure 4. Mean indoor and outdoor human-landing rates by hour for *Anopheles baimaii* by hour in Mae Sot and Sop Moei.

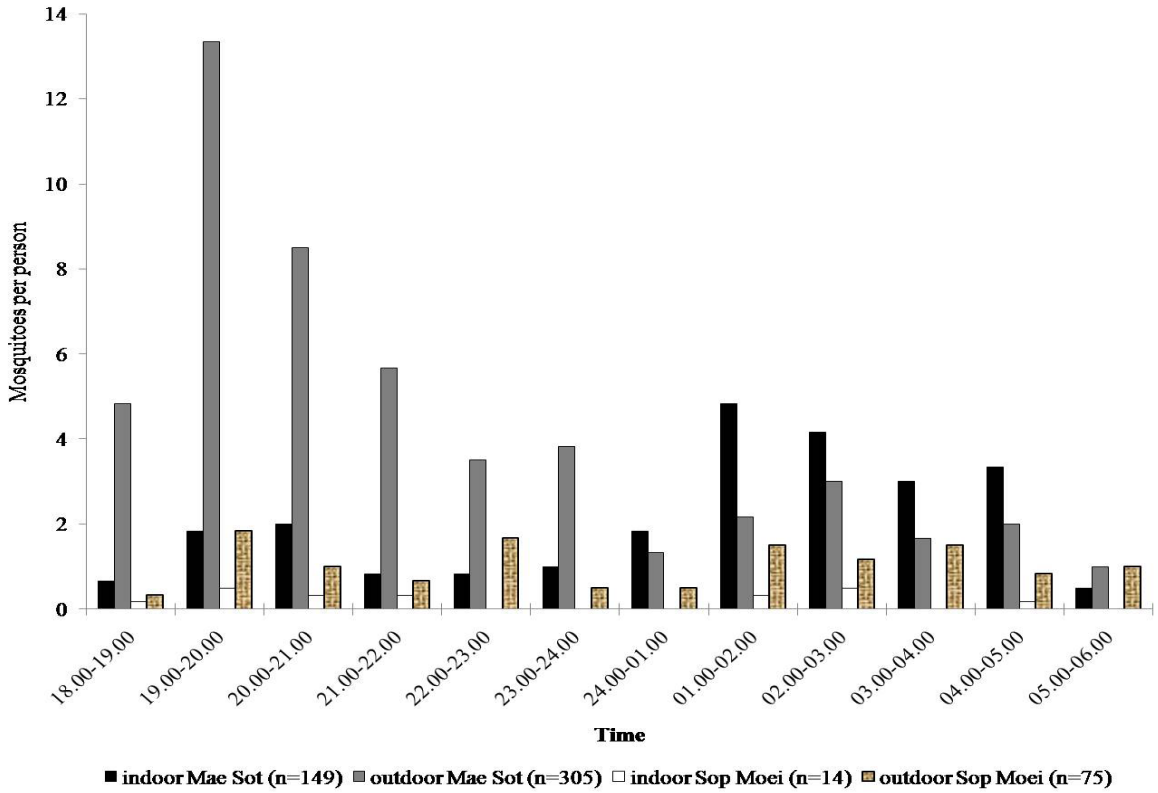


Figure 5. Mean indoor and outdoor human-landing rates by hour for *Anopheles maculatus* in Mae Sot and Sop Moei.

within the Minimus Complex, *An. minimus* and *An. harrisoni* were studied from western Thailand using a molecular identification assay (Sungvornyothin et al. 2006), and more recently, Kongmee et al. (2012) characterized the seasonal abundance, distribution and breeding sites of *An. minimus* and *An. harrisoni* from the same site in Sai Yok District, Kanchanaburi Province in western Thailand. Species diversity, biting activity and trophic behavior of the Minimus Complex were also observed previously in Mae Sot District (Tisgratog et al. 2012) where the two closely related species, *An. minimus and An. aconitus* were collected. In this study, *An. varuna*, another genetically related species to *An. minimus,* was identified for the first time in both locations. Although *An. aconitus* and *An. varuna* are not as efficient vectors compared to *An. minimus*, the presence of these two species, especially *An. aconitus*, in Mae Sot and Sop Moei is of importance as these two species could possibly play a secondary role in malaria transmission (Gould et al. 1967, Scanlon et al. 1968, Junkum et al. 2007).

Tak Province is one of the most malaria-endemic areas of Thailand, accounting for around 23% of total malaria cases in the country in 2012 (5,199 cases) and 2013 (4,977 cases) (BVBD 2013). Six important malaria vectors were identified in the study area using AS-PCR molecular assays. Our findings on the blood-feeding habits of *An. minimus* in Mae Sot showed both zoophilic and anthropophilic behaviors with no strong preference for one host over the other which is similar to previous findings elsewhere in Mae Sot and Kanchanaburi (Sungvornyothin et al. 2006, Tananchai et al. 2012, Tisgratog et al. 2012). In Sop Moei, *An. minimus* demonstrated a stronger zoophilic tendency as more mosquitoes feed on cattle located outside houses. Generally, zoophilic behaviors are considered less conductive for efficient malaria transmission as other animal hosts serve as alternative blood sources, thereby reducing direct contact between vector and human, and thus overall vector capacity. In Pu Teuy, Kanchanaburi Province, *An. minimus* and *An. harrisoni* are present, the latter species being predominant and with generally distinct zoophilic behavior that is believed partly responsible for the typically low levels of malaria transmission in this area (Sungvornyothin et al. 2006). *Anopheles minimus*, *An. dirus* and *An. baimaii,* found in both study areas demonstrated exophagic and zoophilic behaviors which are in agreement with previous observations by Tananchai et al. (2012).

Few studies on species of the Maculatus Group in Thailand have been published (Takai et al. 1987, Upatham et al. 1988, Green et al. 1992, Rongnoparut et al. 1996, 1999, Walton et al. 2007, Muenworn et al. 2009). Studies on this group have been hampered by the lack of reliable tools to identify cryptic species, resulting in misidentification. From our study, four species within this group, *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* were identified molecularly. The most common representative was *An. maculatus*, followed by *An. sawadwongporni*, both malaria vectors in the southern Thailand (Rattanarithikul et al. 1996b, Sinka et al. 2011). Our findings showed that these two important putative vectors were present in study locations in fair proportions, 20.1% and 5.5%, respectively,

compared to all other anophelines identified. The biology of these two malaria vectors, including trophic behavior, infection rate, and parasite susceptibility (vector competence) deserves further study to determine their respective role, if any, in malaria transmission in northwestern Thailand.

Only one mosquito positive for the malaria parasite was detected during the dry season, accounting for a 0.092% (1/1,090) infection rate for *An. minimus*. This mosquito was collected from outdoor HLC from Mae Sot in the early part of the study (April 2011). There was no evidence of malaria infection with *P. falciparum*, *P. malariae, P. knowlesi* or the filarial parasite, *W. bancrofti*. The one positive *An. minimus* was captured during the normal malaria peak in Mae Sot in the late summer months (April-May). The explanation for the low infection rate in mosquitoes tested is unclear, but the probability of finding an infection will have naturally decreased as the overall malaria incidence has been declining year to year in both provinces and where the study took place. For instance, there was a slight decline in reported malaria cases from 2012 to 2013 in Tak Province (0.98 to 0.93 per 1,000 population) and a 38% decline in Mae Hong Son Province (0.52 to 0.32/1000) (BVBD 2013).

It is clear that environmental factors directly influence the distribution and behavior of the malaria vectors (Manguin et al. 2008). For example, the *An. dirus* complex typically occurs in native forest type habitats but also has an ability to adapt to changing environmental conditions that allow it to successfully invade cultivated areas that simulate favored natural forest conditions. Each species within the Dirus Complex is dependent on forest cover (shading) to some degree, and commonly occupies forest-fringe areas where it can interact with human populations to transmit malaria parasites. Habitats include natural dense forest (*An. dirus*), rubber plantations (*An. dirus* and *An. baimaii*) and secondary forest (*An. latens*) (Gingrich et al. 1990, Rosenberg et al. 1990, Suwonkerd et al. 2002). Vanwambeke et al. (2007a,b) found that land-use changes could selectively influence the diversity and likely density of mosquito species, thus having a direct impact on pathogen transmission.

Deforestation, the result of a wide variety of human activities, including land clearing for agricultural development, logging, population resettlement programs, road construction, mining and hydropower development, is one of the most potent factors either promoting or reducing infectious diseases like malaria and dengue in the southeast Asian region, including Thailand (Guerra et al. 2006, Vanwambeke et al. 2007a,b, Yasuoka and Levins 2007). Dramatic changes in environmental conditions are the direct result of modified land use, such as conversion of rice fields to rubber plantations or forest to urbanized zones. The extensive clearing of native and secondary forests has had enormous impacts on local ecosystems, in particular the critical microclimates of mosquitoes by reducing shade cover, changing the humidity regimen, and altering rainfall patterns (Reiter 2001, Overgaard et al. 2002). For example, anopheline species that prefer to use shaded water bodies for oviposition, deforestation can reduce dramatically breeding habitats, thus affecting their propagation (Overgaard et al. 2002). In the future, the inevitable developmental changes that will occur to the landscape of northwestern Thailand will need to be monitored carefully to assess their impacts on potential malaria transmission.

Vector control remains an important component of successful integrated malaria control programs. As demonstrated in this study, a better understanding of the bionomics of a specific vector species, its vector capacity, and epidemiological importance, hinges on the accurate identification of sympatric sibling species in a given area. More investigations are needed in all remaining malaria endemic areas in Thailand so as to develop more cost-effective and targeted vector control strategies based on evidence derived from well-design field studies.

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Influence of Time of Assay on Behavioral Responses of Laboratory and Field Populations *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) to DEET

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ABSTRACT Knowledge on test conditions that may influence behavioral responses of mosquitoes is critical when excito-repellency tests are conducted. The objective of this study was to investigate the effect of test time differences on normal circadian activity and behavioral responses of field and colonized *Aedes aegypti* (L.) (=*Stegomyia aegypti*) and *Culex quinquefasciatus* Say to DEET, one of the most common synthetic repellent active ingredients available. Two field populations of *Ae. aegypti* and *Cx. quinquefasciatus* from Kanchanaburi and Nonthaburi provinces, respectively, and two long-standing laboratory populations, *Ae. aegypti* obtained from the U.S. Department of Agriculture, and *Cx. quinquefasciatus* from the Ministry of Public Health, Thailand, were used. Each population was exposed to DEET during two different periods of time (0900–1500 hours) and (2100–0300 hours). Both field and laboratory *Cx. quinquefasciatus* showed marked differences in spatial repellent escape responses between day and nighttime periods but none in direct contact tests. No significant differences between day and nighttime testing periods were observed with field or laboratory *Ae. aegypti*, except a higher daytime escape response from noncontact DEET treatment. This study indicates that test time may influence the behavioral avoidance responses and is a potential confounder of excito-repellency evaluations.

KEY WORDS DEET, excito-repellency, *Aedes aegypti*, *Culex quinquefasciatus*

Humans living or visiting many tropical and subtropical regions are at risk of contracting diseases transmitted by mosquitoes (WHO 2012). Effective vaccines against many of these diseases, for example, malaria and dengue, are not yet available and are likely years away from reality. Currently, most strategies for the prevention of vector-borne diseases rely on control of the vector and use of personal protection measures using chemicals that either kill or repel the offending insect (Roberts et al. 1997, Grieco et al. 2007, Chareonviriyaphap et al. 2013). In the past, the majority of studies on insecticides have mainly focused on the toxic properties of compounds, whereas behavioral avoidance responses elicited by these same chemicals has been generally neglected.

Aedes aegypti (L.) (=*Stegomyia aegypti*) is predominately an urban mosquito species and an important vector of dengue, yellow fever, and chikungunya viruses. *Culex quinquefasciatus* Say, a common cosmopolitan pest species is an efficient vector of urban lymphatic filariasis, *Wuchereria bancrofti* (Sasa 1976). Both species are difficult to control because of their intimate association with human environments. *Ae. aegypti* is commonly found in and around human dwellings using clean stored water as larval habitats and preferentially feeds on humans during daylight hours, whereas *Cx. quinquefasciatus* is a common urban and rural nuisance mosquito that can breed in a variety of clean and polluted water sources and bites primarily during the night. Vector control against either species is the most effective measure for disease prevention and continues to rely upon various synthetic insecticides applied to control either the larval or adult stage.

A better understanding of the physiological and behavioral responses of mosquitoes to insecticidal and repellent compounds is of operational importance to vector control and disease prevention programs. In general, two forms of behavioral actions have been described following exposure to certain compounds, leading to movement away from the source, contact irritancy (="excitation" in the nonanthropomorphic sense) and noncontact spatial repellency ("avoid-

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ance" in teleological terms; Roberts et al. 1997). Excitation is defined as when an insect leaves an insecticide-treated surface after making physical (tarsal) contact with the chemical, whereas spatial repellency is when an insect moves away from an area treated with insecticide without making direct contact. Spatial repellency involves the detection of airborne volatile components of the chemical (Chareonviriyaphap et al. 1997, Roberts et al. 1997). Collectively, the combined behavioral avoidance response is termed "excito-repellency." The terminology used in this context represents consequences (outcomes), not modes of action. Others have suggested terms like "contact disengagement" and "noncontact disengagement" (irritancy and spatial repellency, respectively) mediated by a "locomotor stimulant" (insecticide, attractant, and repellent) are better suited to the discussion of these various responses (Miller et al. 2009).

Although behavioral responses of mosquitoes to insecticides have been recognized for decades (Kennedy 1947, Muirhead-Thomson 1960), few studies have explored this phenomena in detail. One reason for the poor understanding and under-appreciation for excito-repellency was the lack of an appropriate test system and data analysis to evaluate avoidance responses (Roberts et al. 1997). With the test system and analysis as used in this study, numerous observations on behavioral response of different mosquito species have been documented in Thailand (Chareonviriyaphap et al. 1997, Kongmee et al. 2004, Muenworn et al. 2006, Sathantriphop et al. 2006, Polsomboon et al. 2008, Chareonviriyaphap 2012); however, important information on factors influencing behavioral responses is lacking and insufficiently or poorly understood.

Behavioral responses in adult mosquitoes can be influenced by a number of factors, both physiological and environmental, and should be taken into account during assessments (Kennedy 1947; Busvine 1964; Kaschef 1970; Chareonviriyaphap et al. 1997, 2005; Sungvornyothin et al. 2001; Polsomboon et al. 2008; Boonyuan et al. 2011; Klun et al. 2013). Important biological factors include innate species behavior from field- or laboratory-derived specimens, age, nutritional and physiological conditions (e.g., parity status), body size, and external factors that include ambient temperature and relative humidity, photophase, type and formulation of active ingredients, number of specimens per test, and time of test (Gouck and Smith 1962, Smith et al. 1963, Roberts et al. 1984, Sungvornyothin et al. 2001, Polsomboon et al. 2008, Xue et al. 1995, Chareonviriyaphap et al. 2006). While the behavioral actions of mosquitoes to excito-repellent compounds have been described (Roberts et al. 1997, 2000; Grieco et al. 2007), the wider range of test conditions that might influence the response outcome remain insufficiently explored; for example, the influence of time of testing and normal circadian rhythms. This study investigated if time of testing (day or nighttime hours) significantly affects the normal behavioral responses of either typically day active (*Ae. aegypti*) or night active (*Cx. quinquefasciatus*) species exposed to DEET, one of the most common compounds used in commercial mosquito repellent products (Cox 2005, Bernier et al. 2007).

Materials and Methods

Mosquito Populations. Two mosquito species, consisting of two different populations each, were used in this study.

1. Laboratory (lab) *Ae. aegypti:* Eggs were obtained in December 2012 from the Mosquito and Fly Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, United States Department of Agriculture (USDA). This colony has been maintained in laboratory conditions for >50 yr.
2. Field *Ae. aegypti:* Immature mosquitoes were collected in December 2012 from indoor water containers in Kanchanaburi Province, Sai Yok District, Pu Teuy Village (14° 17′ N, 99° 11′ E). Subsequent colonization was done at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, following the methods described by Kongmee et al. (2004). Only first to third (F1–F3) generation females were used in tests.
3. Laboratory *Cx. quinquefasciatus:* This population was collected from Bangkok, Pom Prab Satru Phai, in 1978 and maintained in continuous at the National Institute of Health, Ministry of Public Health, Thailand.
4. Field *Cx. quinquefasciatus:* Immature stages were collected from Nonthaburi Province Pak Kret District, Tha It Village (13° 54′ N; 100° 29′ E) and reared in plastic trays 25 by 16 by 9 cm^3 and clean fresh water under standard rearing procedures. Adult identification was based on morphological characters Sirivanakarn (1976). Only cohorts of F1–F3 generation females were used in tests.

Impregnated Paper Preparation. DEET (*N, N*-diethyl-m-toluamide or *N, N*- diethyl-3-methylbenzamide) was obtained in December 2012 from the USDA, Beltsville, MD. A 5% concentration of DEET was prepared with absolute ethanol according to the method described by Tisgratog et al. (2011). Filter papers (Whatman), 14.7 in width by 17.5 in length cm^2, were impregnated with the prepared solution and allowed to air dry. Control papers were impregnated with absolute ethanol only. All papers were treated at the rate of 2.75 ml of the insecticide solution per 257.25 cm^2 paper surface area.

Excito-Repellency Test System. Four identical test chambers (23 by 23 by 23 cm^3) were used as previously described (Chareonviriyaphap et al. 2004). The assay system setup consisted of four chambers, each equipped with an exterior receiving box for collecting escaping mosquitoes. Each respective treatment chamber, contact and noncontact, was paired with a matched control chamber. DEET-treated papers were attached to the inner walls of two chambers and "blank" papers inside the two control chambers. Nonblooded, 3–5-d-old female mosquitoes were deprived of all nutrition (sugar) and water for a minimum of

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Table 1. Escape response and mortality of two populations (laboratory and field) of *Ae. aegypti* and *Cx. quinquefasciatus* from exposure chambers conducted at two different time periods

Time	Test populations	Test condition[a]	*Ae. aegypti*				*Cx. qinquefasciatus*			
			No. tested	No. escaped (%)	% Mortality		No. tested	No. escaped (%)	% Mortality	
					Escaped	Remain			Escaped	Remain
Day	Laboratory	Contact	58	19 (33)	0	35	58	42 (72)	0	0
		Control	59	4 (7)	0	0	56	2 (4)	0	0
		Noncontact	54	32 (59)	8	0	54	24 (44)	0	0
		Control	59	1 (2)	0	0	55	2 (4)	0	0
	Field	Contact	58	19 (33)	11	7	59	33 (56)	2	2
		Control	56	1 (2)	0	0	52	3 (6)	0	0
		Noncontact	56	11 (20)	0	2	59	15 (25)	0	9
		Control	58	1 (2)	0	0	55	2 (4)	0	0
Night	Laboratory	Contact	58	23 (40)	0	4	52	31 (60)	10	37
		Control	60	7 (12)	0	0	56	36 (64)	0	0
		Noncontact	60	18 (30)	0	0	58	37 (64)	0	30
		Control	58	1 (2)	0	0	57	34 (60)	0	0
	Field	Contact	58	20 (34)	0	4	60	42 (70)	9	4
		Control	57	11 (19)	0	0	60	31 (52)	0	0
		Noncontact	57	16 (28)	0	0	60	37 (62)	6	6
		Control	55	2 (4)	0	0	60	22 (37)	0	0

[a] Contact irritancy and noncontact spatial repellency.

24 h before test exposure. For each test, 15 female mosquitoes were introduced into each of the four chambers and allowed to acclimate for ≈3 min. Identical test procedures were performed during two different time periods: daylight hours (0900–1500 hours) and nighttime hours (2100–0300 hours) with each test series replicated four times. Observations of escaping mosquitoes were taken at 1-min intervals for the duration of the test (30 min). Immediately afterwards, the number of dead or knockdown mosquitoes was recorded separately inside each chamber and external holding cage. All mosquitoes from control and treatment chambers were held separately in small holding containers, provided with sugar sustenance and water, to record final mortality at 24 h. All tests were performed in the same laboratory setting regard ambient air temperature, percent relative humidity and lightening. Two environmental factors were controlled and recorded in this study including temperature (room temperature under the laboratory condition no matter what day or nighttime ranging 27–30°C) and relative humidity (laboratory condition ranging 60–75%RH). The artificial (LED) illumination was a near constant 68 lumen per watt.

Statistical Analysis. Kaplan–Meier survival analysis was used to analyze and interpret the rate of escape of mosquitoes from each chamber (Chareonviriyaphap et al. 1997). The analysis involved labeling mosquitoes that escaped from the chamber as "dead," while those remaining inside the chamber as "survivors." At the end of the 30-min test, those mosquitoes remaining in the exposure chambers (nonescape) were treated as "censored." A log-rank method (Mantel and Haenzel 1959) was used to compare the patterns of escape behavior of each mosquito population, in both contact and noncontact trials, and between the two experimental time periods of day and night. Statistical significance for all tests was set as 5% ($P < 0.05$).

Results

The percent mortalities and escape responses of laboratory and field-collected *Ae. aegypti* mosquitoes were recorded for each cohort exposed to 5% DEET in paired contact and noncontact trials and respective paired controls (Table 1). Overall, mortality of mosquitoes that escaped and those that remained in the contact chambers was relatively low (0–11%), except for the laboratory population during daytime testing (35%). For noncontact trials, mortality were also low (0–8%). A higher percentage of escaped mosquitoes were observed from contact (33–40%) compared with noncontact (20–30%) trials, except for the laboratory population during noncontact daytime tests (59%). In contact trials, a lower percentage of escape was observed from the treatment tests conducted during the daytime (33% for both laboratory and field test populations) compared with the nighttime period (40 and 34%, respectively). A greater number of escaped mosquitoes from the control chambers were observed during the night time hours (2–19%) as compared with daytime hours (2–7%; Table 1), but differences were not statistically significant between periods of testing ($P = 0.22$).

The behavioral responses of two populations of *Cx. quinquefasciatus* against DEET are presented in Table 1. Mean mortality of escaped and remaining mosquitoes in contact and noncontact trials was relatively low (0–10%), except for mosquitoes remaining in the chambers during the nighttime tests (37% for contact and 30% for noncontact). For laboratory and field populations tested during daytime, a higher percent of escape was observed for contact (72 and 56%, respectively) compared with noncontact (44 and 25%, respectively) trials. A greater percent of escape responses were observed during the night for all tests compared with daytime, except for the contact test with the laboratory population. Control chambers also

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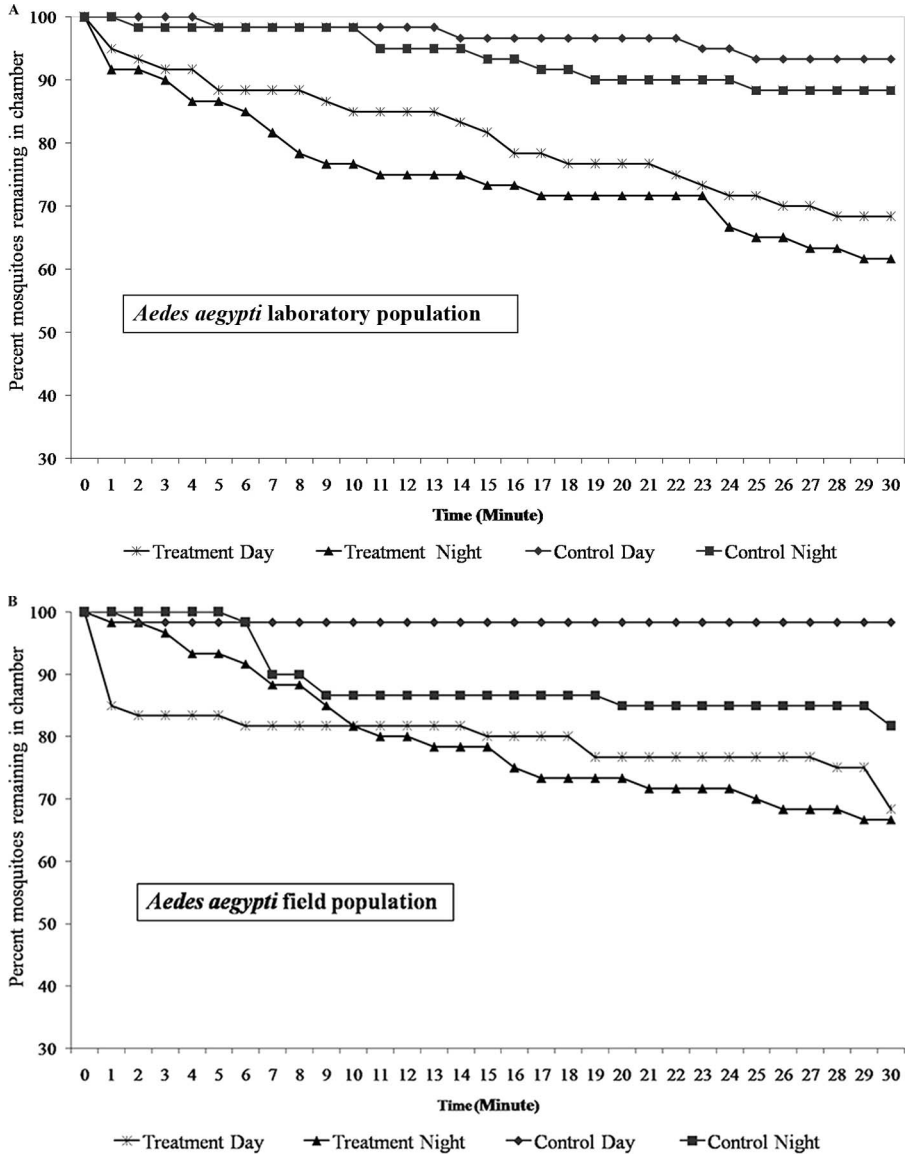


Fig. 1. Escape patterns of *Ae. aegypti* from different chambers in contact trials between day and nighttime trials. (A) Laboratory population. (B) Field population.

showed a very high percent of nighttime escape for both laboratory (64% contact and 60% noncontact) and field populations (52 and 37%, respectively). Conversely, a low percent escape response from the controls was observed in tests conducted during the daytime (4–6%).

Patterns of escape in contact trials and paired controls for each test population conducted during day and night time hours for *Ae. aegypti* and *Cx. quinquefasciatus* are shown in Figs. 1 and 2, respectively. No significant differences based on time of testing were observed for contact escape responses of either population of *Ae. aegypti* or *Cx. quinquefasciatus* from the DEET chambers. However, the escape responses from the control chambers were significantly greater during the night than those tested during daytime (data not shown).

The patterns of escape in noncontact trials and paired controls for each test population conducted during daytime and night time hours are shown in Figs. 3 and 4. There were significant differences in spatial repellency escape responses for both field and laboratory populations of *Cx. quinquefasciatus* for all pairings including controls, with results showing a much stronger escape response during nighttime hours (Table 2). There were no differences in escape responses in noncontact trials between day and night testing for *Ae. aegypti* laboratory control and both treatment and control field populations. The single exception was the laboratory population exposed to vapor phase DEET

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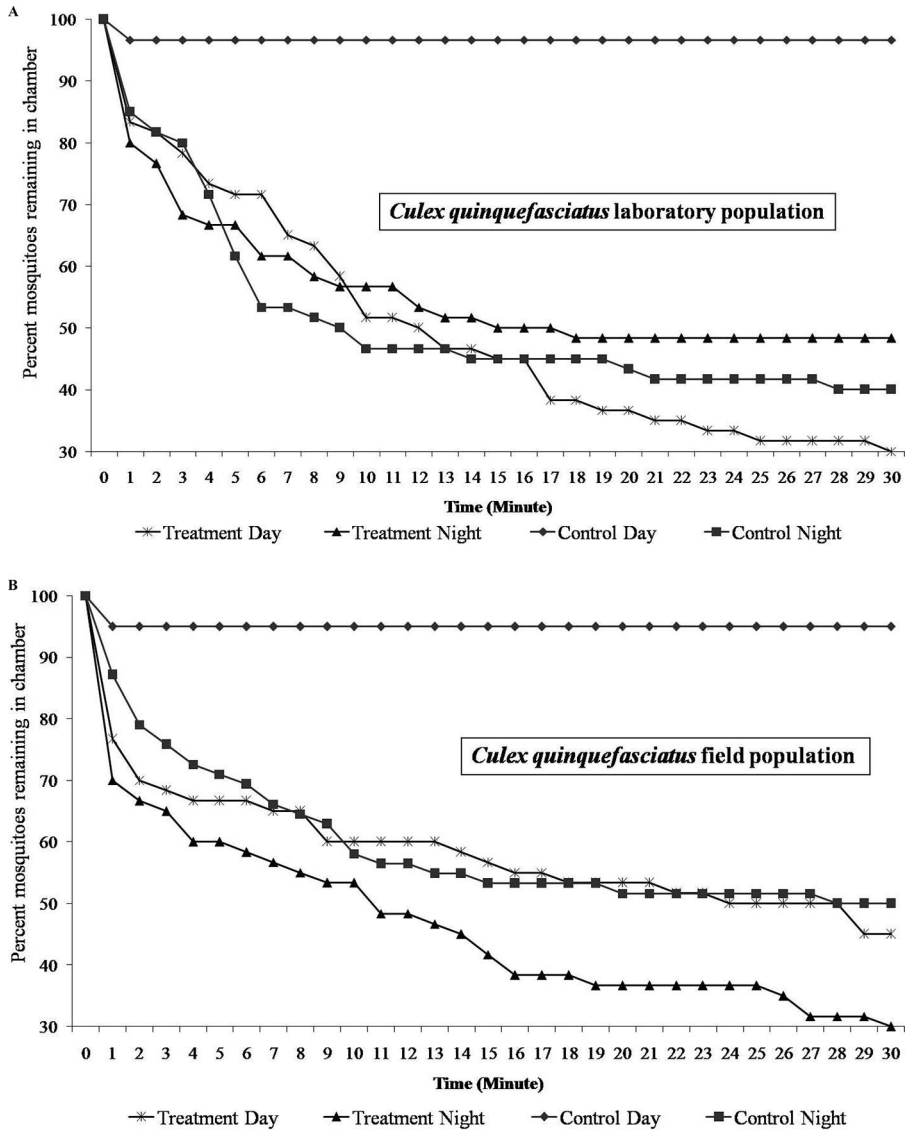


Fig. 2. Escape patterns of *Cx. quinquefasciatus* from different chambers in contact trials between day and nighttime trials. (A) Laboratory population. (B) Field population.

in which a greater and significant escape response was seen during the daytime.

Discussion

Defining the test conditions that may influence the behavioral responses of mosquitoes is critical for the proper interpretation of excito-repellency data. This study investigated the potential effects of altering the time of testing (0900–1500 h vs. 2100–0300 h) on the behavioral responses of field and colonized (lab) *Ae. aegypti* and *Cx. quinquefasciatus* to DEET. The DEET concentration used in this study was deemed sublethal at the 5% concentration. Toxicity was generally low but variable with a higher degree of mortality observed in a few cases depending on the test conditions, mosquito species, population, and time of test. Both populations of *Cx. quinquefasciatus* showed marked differences in escape response between day and nighttime test periods due to repellency, whereas no significant differences were observed between field and laboratory *Ae. aegypti*, except for a greater flight activity and exit from untreated controls during the nighttime hours and escape due to spatial repellency during daylight hours. In this study, with all other experimental factors being equal (e.g., age, physiological and nutritional state, ambient temperature, relative humidity, and lighting), exposure to DEET clearly influenced flight behavior and disrupted normal circadian activity in three of the four mosquito populations tested. The impact of time was seen only with the more subtle spatial repellent responses. The combi-

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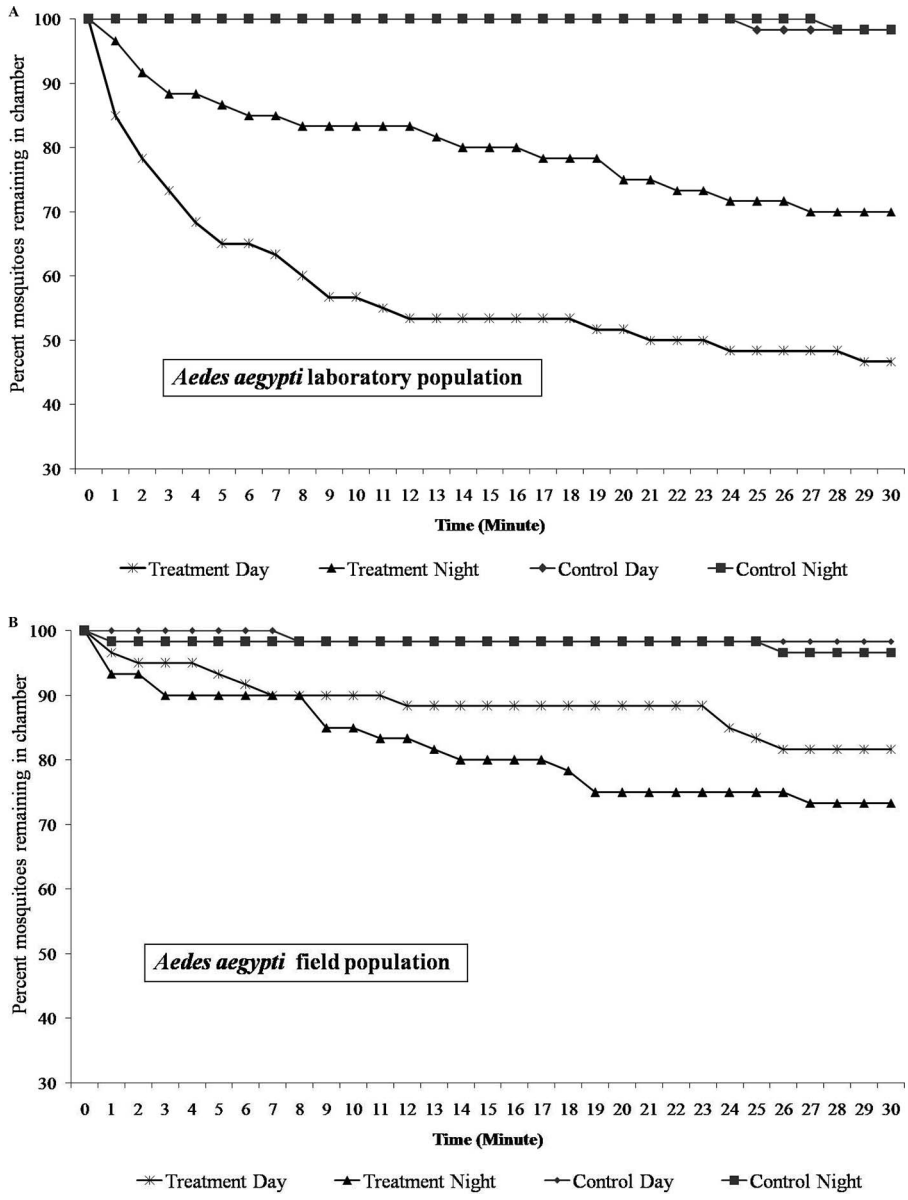


Fig. 3. Escape patterns of *Ae. aegypti* from different chambers in noncontact trials between day and nighttime trials. (A) Laboratory population. (B) Field population.

nation of irritancy and repellency to DEET in the contact tests appeared equally strong regardless of time of test, apparently overwhelming any normal, innate flight activity patterns as a result. This study shows that time of experiment may influence escape response and is a potential confounder of excito-repellency evaluations.

DEET remains the most commonly used active ingredient in commercial topical insect repellents, showing broad effectiveness against many insects and other arthropod species, including mosquitoes (Rutledge et al. 1983, Debboun et al. 2007), yet its mechanism of action and molecular target(s) remain unknown, or at best, unclear (Ditzen et al. 2008, Pickett et al. 2008, Syed and Leal 2008). Although DEET has long been regarded as a repellent (i.e., a substance that causes movement away from a source), more recent studies have concluded it acts as a spatial inhibitor instead (Dogan et al. 1999, Dogan and Rossignol 1999, Kramer et al. 2010). Based on electrophysiological responses, DEET has been shown to inhibit odor-evoked currents and activation mediated by the insect odorant receptor complex (Ditzen et al. 2008), effectively inhibiting perception of host odors and other chemo-attractant cues. Use of DEET may also be operating as an inhibitor of larval habitat chemical cues

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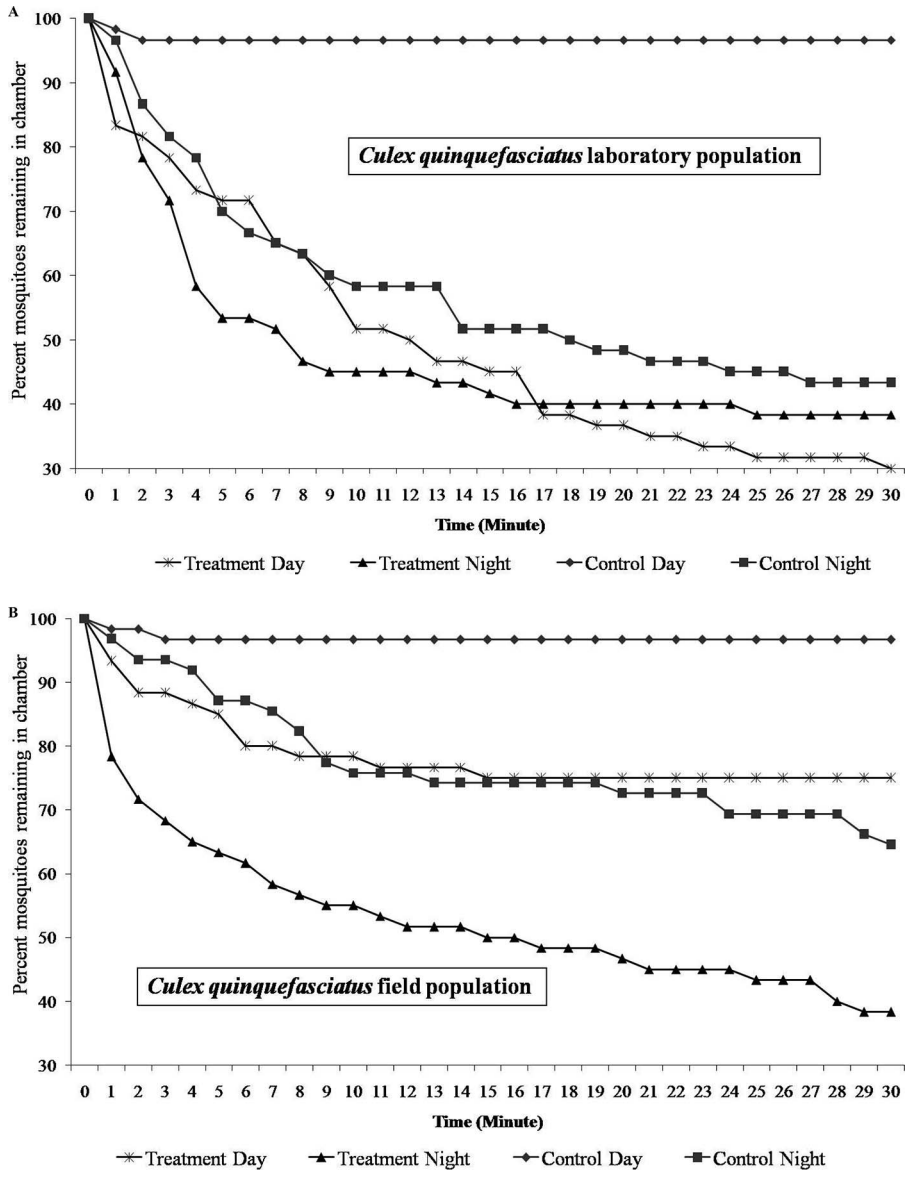


Fig. 4. Escape patterns of *Cx. quinquefasciatus* from different chambers in noncontact trials between day and night time trials. (A) Laboratory population. (B) Field population.

Table 2. The log-rank analysis comparing escaped mosquitoes in each population between day and nighttime trials

Test populations	*P* value (day vs night periods)			
	Contact		Noncontact	
	Treatment	Control	Treatment	Control
Ae. aegypti laboratory	0.42	0.35	**0.0028**	0.99
Ae. aegypti field	0.88	**0.0029**	0.28	0.53
Cx. quinquefasciatus laboratory	0.53	**0.0001**	**0.0040**	**0.0001**
Cx. quinquefasciatus field	0.12	**0.0001**	**0.0001**	**0.0001**

Values in bold text are statistically significant.

(attractants) rather than functioning as a true repellent.

DEET is well-known to exhibit profound behavioral responses in insects (Surgeoner 1995, Cox 2005). Whether this chemical performs as a true repellent or inhibitor (or possibly a combination of both) is a question of mechanism producing the outcome and was not addressed in this study, whereas the final endpoints (escape and mortality) were measured. There has been little published literature describing the two primary mosquito behavioral responses, contact excitation and spatial repellency with DEET. Assuming both contact excitation and spatial repellency are both involved in eliciting escape activity as seen in

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this study, it is not possible to clearly differentiate the actions of excitation and repellency (acting kinetically as well as tactically) in the contact test design. The differences in escape response between the two test designs (contact and noncontact) would presumably help separate and quantify the significance of either excitation or spatial repellency actions alone. The same would apply in factoring out escape responses in paired controls with noncontact tests to arrive at an adjusted percent repellency due to the chemical alone. Nevertheless, neither test design used a known attractant (e.g., host cue) inside the DEET-treated chambers, a requirement to determine if inhibition may be the mode of action. Therefore, we may conclude that those mosquitoes that escaped in the noncontact test designs appear to have been the result of spatial repellency alone. Based on our study findings using the excito-repellency test system, DEET acts as both a contact irritant and spatial repellent.

A variety of factors can influence and regulate mosquito behavior. Numerous intrinsic and extrinsic factors influence escape movement of female mosquitoes from chemical-treated surfaces (Roberts et al. 1984, Sungvornyothin et al. 2001, Chareonviriyaphap et al. 2006). For example, ambient temperature and humidity can affect insect behavioral periodicities as either inhibitory or permissive factors (Muirhead-Thomson 1960). Recently, movement patterns of *Ae. aegypti* females into and out of the field-based experimental huts were shown to be influenced by humidity and ambient air temperatures (Suwannachote et al. 2009). Biting rates of *Ae. aegypti* can vary dramatically depending on mosquito age and time of day in testing of repellent effectiveness of DEET (Gouck and Smith 1962, Smith et al. 1963). For example, interactions between age and time of day suggest that as female mosquitoes age, diel patterns of host biting change (Gouck and Smith 1962). Xue and Barnard (1996) have shown a similar dynamic and change in biting patterns in *Aedes albopictus* (Skuse) based on age and time of testing that impact responses to DEET and interpretation of repellent protection bioassays. Likewise, in our study, time of testing appears to have a differing degree of impact on the behavioral excito-repellency response of the two different mosquito species to DEET.

Ae. aegypti is a diurnally active species and behavioral periodicities generally follow a uni- or bimodal daytime pattern, whereas *Cx. quinquefasciatus* is strongly active during the mid- to late nighttime periods (peak of blood feeding typically from 2200 to 0300 hours; The Armed Forces Pest Management Board [AFPMB] 2009). In this study, *Cx. quinquefasciatus* displayed a significantly greater natural escape response than *Ae. aegypti.* Significant differences in escape responses of *Cx. quinquefasciatus* was found for all controls between day and nighttime trials. Greater escape was observed during the nighttime compared with daytime in concordance with the typical activity patterns of this species. In contrast, *Ae. aegypti* did not show a differing control response in escape between day and night hours except in one case (field population) with greater nighttime escape.

We conclude that time of testing can have considerable bearing on behavioral assay results, especially for *Cx. quinquefasciatus.* To help standardize testing procedures and avoid under- or overestimation of excito-repellency responses and thus prevent systematic error in bioassays, time-of-observation-based testing should be expanded to other important disease vector mosquitoes such as *Anopheles* species, as they too are predominantly night biters, whereas the vast majority of published studies have been conducted during daylight hours. Other biological and external factors that might influence mosquito behavior must be carefully considered when assessing the potential impact of insecticides and repellents for preventing disease transmission.

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Behavioral responses of *Aedes aegypti, Aedes albopictus, Culex quinquefasciatus,* and *Anopheles minimus* against various synthetic and natural repellent compounds

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ABSTRACT: The behavioral responses of colony populations of *Aedes aegypti*, *Aedes albopictus, Culex quinquefasciatus,* and *Anopheles minimus* to four essential oils (citronella, hairy basil, catnip, and vetiver), two standard repellents (DEET and picaridin), and two synthetic pyrethroids (deltamethrin and permethrin) were conducted in the laboratory using an excito-repellency test system. Results revealed that *Cx. quinquefasciatus* and *An. minimus* exhibited much stronger behavioral responses to all test compounds (65-98% escape for contact, 21.4-94.4% escape for non-contact) compared to *Ae. aegypti* (3.7-72.2% escape (contact), 0-31.7% (non-contact)) and *Ae. albopictus* (3.5-94.4% escape (contact), 11.2-63.7% (non-contact)). In brief, essential oil from vetiver elicited the greatest irritant responses in *Cx. quinquefasciatus* (96.6%) and *An. minimus* (96.5%) compared to the other compounds tested. The synthetic pyrethroids caused a stronger contact irritant response (65-97.8% escape) than non-contact repellents (0-50.8% escape for non-contact) across all four mosquito species. Picaridin had the least effect on all mosquito species. Findings from the current study continue to support the screening of essential oils from various plant sources for protective properties against field mosquitoes. ***Journal of Vector Ecology* 39 (2): 328-339. 2014.**

Keyword Index: Aedes aegypti, *Aedes albopictus, Culex quinquefasciatus, Anopheles minimus*, excito-repellency, non-contact repellent, contact irritant.

INTRODUCTION

There are various mosquito-borne diseases transmitted to humans by the bites of infected mosquitoes, including malaria (protozoa), dengue (virus), Japanese encephalitis (virus), chikungunya (virus), and yellow fever (virus). *Aedes aegypti* is the main vector of dengue and yellow fever viruses, whereas *Aedes albopictus* is a secondary dengue vector and a main vector of chikungunya virus (Thavara et al. 2009). *Culex quinquefasciatus* is an abundant nuisance mosquito in urban areas. It has been reported that *Cx. quinquefasciatus* in Thailand is a potential vector of Japanese encephalitis virus (JEV) (Nitatpattana et al. 2005). Moreover, *Cx. quinquefasciatus* in Thailand was first reported to be susceptible to the nocturnal periodic strain of Myanmar *Wuchereria bancrofti* that causes human lymphatic filariasis (Triteeraprapab et al. 2000). *Anopheles minimus* is now the most important malaria vector in Thailand and occurs in hilly, forested areas. Malaria is a serious and sometimes fatal disease that is routinely reported along the Thai-Cambodia and Thai-Myanmar borders as well as the southern part of Thailand (Chareonviriyaphap et al. 2013, Suwonkerd et al. 2013).

Among the many strategies that have been used for mosquito control are insecticides and repellents. Synthetic pyrethroids are widely used for controlling adult mosquitoes, especially deltamethrin and permethrin, as recommended by the World Health Organization. The Ministry of Public Health of Thailand has used these two pyrethroid insecticides for space spraying applied via fogging or misting machines and mosquito net treatments (Chareonviriyaphap et al. 1999, Jirakanjanakit et al. 2007). *Previous* studies have found that both deltamethrin and permethrin elicit strong contact irritant response in mosquito vectors (Chareonviriyaphap et al. 2004, 2012, 2013, Kongmee et al. 2004, Mongkalangoon et al. 2009).

The term "excito-repellency" is used to describe mosquito behavior that is triggered by the combination of either irritancy or repellency. Irritancy results from direct tarsal contact with an insecticide that can cause a mosquito to leave treated surfaces before acquiring a lethal dose, therefore repeated contact is required before mortality occurs (Roberts et al. 2000). On the other hand, repellency refers to the stimulation by a chemical that orients mosquito movement away from the treated surfaces without making tarsal contact (Roberts et al. 2000). These forms of behavioral responses can be quantitatively assessed by using an excito-repellency test system (Roberts et al. 1997).

For personal protection, DEET (N,N-Diethyl-meta-toluamide) is the most common active compound used as insect repellents with a strong effect against mosquitoes (Fradin and Day 2002, Klun et al. 2006). However, DEET has been shown to have toxic effects on humans, especially when misapplied at very high concentrations and used often or over a long period of time (Robbins and Cherniack 1986, Qui et al.

1998). Plant essential oils have become popular to use as safe mosquito repellents, such as citronella oil that is approved by the U.S. Environmental Protection Agency (EPA) (Fradin 1998). Besides citronella oil, numerous essential oils extracted from plants have been available for insect repellent activity for protection against mosquitoes. The objective of this research was to investigate the behavioral responses of four mosquito species to three different types of test compounds - essential oils, standard repellents, and synthetic chemicals.

MATERIALS AND METHODS

Test mosquitoes

Laboratory populations of *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus* and *An. minimus* were used in this study. The *Ae. aegypti* insecticide susceptible test population (United States Department of Agriculture laboratory [USDA]) was obtained as eggs from the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. The USDA population of *Ae. aegypti* has been reared in the laboratory for more than 40 years. *Aedes albopictus* has been maintained in the entomological laboratory at Kasetsart University for six years. A colony of *Cx. quinquefasciatus* were obtained from the National Institute of Health (NIH), Ministry of Public Health, Nonthaburi, Thailand, where the colony has been established since 1978. The *Anopheles minimus* colony has been maintained in the Department of Entomology, Kasetsart University for >15 years. The colony originated from the Malaria Division, Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand, in 1998.

All four species of mosquitoes were reared in the insectary of the Department of Entomology, Faculty of Agriculture, Kasetsart University. All larvae and adults were held under laboratory conditions of 25 $\pm$ 5° *C* and 80 $\pm$ 10% *RH* with a 12:12 *L:D* photoperiod. Larvae were fed with fish food twice a day. Adults were reared in a screened cage and provided 10% sugar solution as food. Female mosquitoes aged three to five days old were starved for 24 h before testing.

Test compounds

Pyrethroid insecticides. Deltamethrin ([Cyano-[3-(phenoxy)phenyl]methyl] 3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate) (98% purity) was obtained from BASF (Lot No: HDDLTK034). Permethrin (3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) (97.6% purity) was provided by Sherwood Chemicals Public Company Limited (Lot No: ABJFPAP118).

Repellents. DEET and picaridin were selected as representative synthetic repellents. DEET (N, N-diethyl-3-methylbenzamide) (97% purity) was obtained from the USDA, Beltsville, MD (Lot No: 0326/2009). Picaridin (2-(2-hydrozyethyl-1-piperidincarboxylic acid 1-methylpropyl ester) (98.4% purity) was obtained from Bayer Thai Company Limited (Lot No: CHCAEN0020).

Plant essential oils. Citronella, *Cymbopogon nardus* (Lot No: NO5410008-1/1910), hairy basil, *Ocimum americanum* (Lot No:5308093/0408), catnip, *Nepeta cataria* (Lot No: 01022011) and vetiver, *Vetiveria zizanoides* (Lot No: 5506713/2706) were selected from previous studies as *effective in repelling mosquitoes* (Tawatsin et al. 2001, Trongtokit et al. 2005, Phasomkusolsil and Soonwera 2010). Citronella, hairy basil and vetiver oils were supplied in 100% purity from Thai-China Flavours and Fragrances Industry Company Limited. Catnip was received from the Chemicals Affecting Insect Behavior Laboratory, United States Department of Agriculture, Beltsville, MD.

Paper treatment

Deltamethrin and permethrin were dissolved in a mixture of acetone and silicone (Dow Corning 556) to obtain doses of 25 mg/m^2 and 500 mg/m^2, respectively, the highest doses of insecticidal-treated nets (ITNs). DEET, picaridin, and four essential oils were diluted in ethanol to a concentration of 2.5%, based on optimal concentrations identified from previous mosquito behavioral studies (Polsomboon et al. 2008, Suwansirisilp et al. 2012). With a micropipette, 2.8 ml of the solution was dropped onto 14.7 × 17.5 cm^2 Whatman No.1 filter paper. Impregnated papers were allowed to air dry 1 h before testing (Licciardi et al. 2006). Control papers were treated with only ethanol or solvent mixture for insecticide tests.

Behavioral tests

The excito-repellency test assay used in this study has previously been described (Mongkalangoon et al. 2009, Suwansirisilp et al. 2012). Briefly, a set of four test chambers was used to evaluate both non-contact repellent and contact irritant behaviors. Each test chamber was connected with a receiving box for collecting mosquitoes exiting from the test chamber. A matched control was performed for each chemical treatment evaluation. Fifteen unfed female mosquitoes of three to five days old were released into each of four test chambers and mosquitoes were allowed to adjust themselves to environmental conditions inside the test chamber for 3 min before opening the exit door. The number of escaping mosquitoes was recorded every minute for 30 min during exposure to test repellent compounds. At the end of the exposure period, escaped and non-escaped mosquitoes were transferred to individual containers and provided 10% sugar solution. Knockdown was observed after 30 min and mortality after 24 h in both treatments and controls for both escaped and non-escaped cohorts. Each repellent compound was tested in four replicates between 08:00 am and 16:30.

Statistical analysis

Kaplan-Meier survival analysis was used to evaluate escaping mosquitoes from each test chamber of the excito-repellency test system (Roberts et al. 1997). The time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of assay populations to escape was calculated for each product assessed and the log-rank test used to compare the escape responses of test populations. Observed percentage escape was corrected with Abbott's formula. (Finney 1964).

RESULTS

The escape patterns during 30-min exposures for the four mosquito species are given in Figures 1 and 2. Results in contact trials indicate responses of *Ae. albopictus* to the synthetic pyrethroids (deltamethrin and permethrin) were significantly greater than DEET, picaridin, or essential oils ($P<0.05$). With deltamethrin, *Ae. aegypti* produced significantly greater responses than others ($P<0.05$), except hairy basil ($P=0.0892$). For permethrin, the repelling effect was not significantly different from that of all essential oils, *P>0.05* (Table 1). All compounds were found to elicit escape responses in both *Cx. quinquefasciatus* from 69.2% to 96.6% and *An. minimus* from 65.4% to 97.8%, except picaridin where minimal escape occurred (3.4% and 12.2%, respectively). Moreover, two test populations, *Cx. quinquefasciatus* and *An. minimus* showed high escape responses to three essential oils, citronella, hairy basil, and catnip in contact and non-contact trials (72.4% to 94.4%), whereas vetiver was associated with extremely high escape responses of both these species from the contact chambers (>95%). *Mortality rates were low overall, e*xcept in *An. minimus* when exposed to catnip where 76.7% and 89.9% mortality was reported in contact and non-contact, respectively.

Escape time in minutes (ET) 25%, 50%, and 75% during the 30-min exposure assay is shown in Table 2. *Aedes aegypti* showed the fastest response to permethrin with an ET_{25} value of 1 min and an ET_{50} value of 4 min in contact trials but no response was found in the non-contact trials. *Aedes albopictus* had low ET_{25} values in both contact and non-contact trials (≤1 min) against deltamethrin, DEET, catnip, and vetiver. For ET_{50}, this species displayed the values of <1 min only in contact trials for deltamethrin and permethrin and ET_{75} values of 2 min and 4 min appeared, respectively, for deltamethrin and permethrin in contact treatment chambers. *Culex quinquefasciatus* displayed ET_{25} values of <1 min in contact trials against all four essential oils tested and ≤1 min in non-contact trials for citronella, hairy basil, and catnip. *Anopheles minimus* showed fast escape responses of ≤1 min at ET_{25} to DEET, hairy basil, and catnip. Only catnip was found on *An. minimus* to have an ET_{25}, ET_{50} and ET_{75} for both contact and non-contact trials ≤4 min. *Anopheles minimus* had a delayed escape response to permethrin, DEET, and citronella with ET_{50} values of 6, 6, and 7 min, respectively.

A comparison of escape responses among the mosquito test populations between contact and non-contact trials is shown in Table 3. All species exhibited significant differences in response patterns between trials for deltamethrin and permethrin ($P<0.0001$). *Aedes aegypti* showed slightly different patterns among DEET (P=0.0460), hairy basil (P=0.0166), and catnip (P=0.0201), but there were no significant differences for picaridin, citronella, and vetiver (P>0.05). A significant difference was found between DEET and vetiver (P<0.0001) trials using *Culex quinquefasciatus.* Escape responses were also significantly different against citronella and vetiver trials using *An. minimus.*

Statistical comparisons between any two species exposed to deltamethrin, permethrin, DEET, picaridin, citronella, hairy basil, catnip, and vetiver in either contact or non-contact trials are shown in Table 4. For deltamethrin, there were significant differences in escape responses when *Ae. albopictus* was compared to the other species in both test conditions ($P<0.0001$). With permethrin, significance in escape responses were found when *Ae. aegypti* was compared to *Ae. albopictus* and *An. minimus* ($P<0.0001$) . In addition, the significant differences in escape responses between two species were most found in citronella, hairy basil, catnip and vetiver ($P<0.05$) (Table 4).

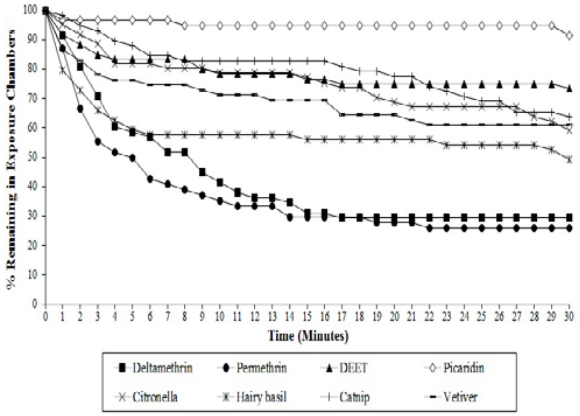
Statistical comparisons between pyrethroids and essential oils against four mosquito species are shown in Table 5. Significant differences in escape responses between pyrethroids and essential oils were most observed in *An. minimus* and *Ae. albopictus* (P<0.05). In contact trials, statistical significances in escape responses of all species were observed when catnip and vetiver were compared to deltamethrin (P<0.05). In non-contact trials, there were significant differences in escape responses when citronella and hairy basil were compared to deltamethrin for all species ($P<0.0001$).

DISCUSSION

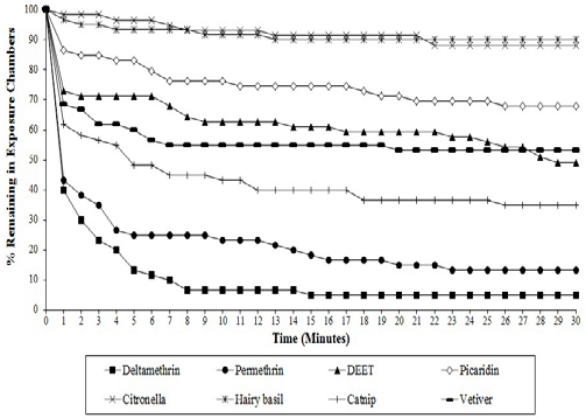
Insecticides commonly used to control the vectors of human diseases are within the pyrethroid chemical group, mainly deltamethrin and permethrin, but several papers have reported pyrethroid resistance in *Ae. aegypti*, *Ae. albopictus,* and *An. minimus*, and in the nuisance-biting *Cx. quinquefasciatus* within Thailand (Ponlawat et al. 2005, Paeporn et al. 2006, Jirakanjanakit et al. 2007, Chareonviriyaphap et al. 2013). Thus, topical repellents could be used in combination with traditional indoor residual spraying (IRS) and bednet strategies for preventing mosquito bites. Plant-based mosquito repellents are more popular for self-protection because their natural scents are perceived as being safer to humans. Many plants have been studied for their efficacy in repelling mosquitoes. For example, Phasomkusolsil and Soonwera (2010) reported that citronella essential oil (*Cymbopogon nardus*) and phlai (*Zingiber cassumunar*), a famous Thai herbal medicine belonging to the same family as ginger and sweet basil (*Ocimum basilicum*), provided protection against *Cx. quinquefasciatus*, *An. minimus, and Ae. aegypti* when applied to the skin. Misni et al. (2008) also found there was no significant difference in efficacy between *Piper aduncum* essential oil (Malaysian plant) and DEET when tested against *Ae. aegypti*. Also, Tawatsin et al. (2001) reported turmeric, citronella grass, and hairy basil combined with 5% vanillin provided improved protection time against *Ae. aegypti*, *An. dirus,* and *Cx. quinquefasciatus*.

The current study measured irritant contact and repellent non-contact characteristics of eight compounds: citronella, hairy basil, catnip, and vetiver (plant essential oils), DEET and picaridin (synthetic repellents), and two synthetic pyrethroids (deltamethrin and permethrin), using the excito-repellency test system. The results show that both pyrethroid insecticides were effective in eliciting an irritant rather than a repellent response against test vector populations, similar to

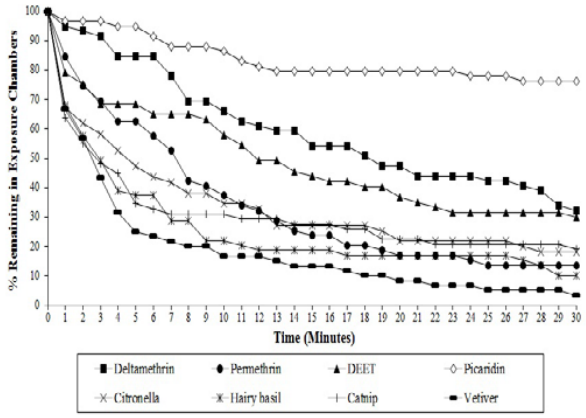
Aedes aegypti



Aedes albopictus



Culex quinquefasciatus



Anopheles minimus

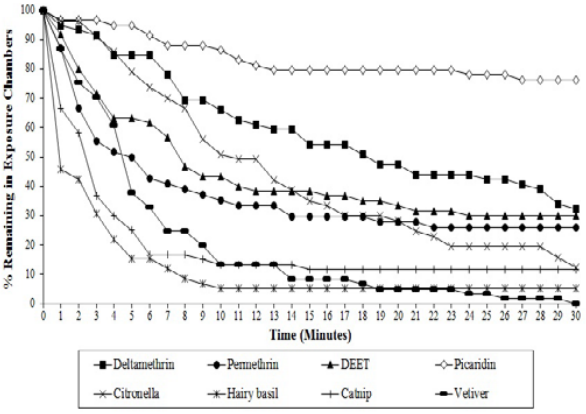
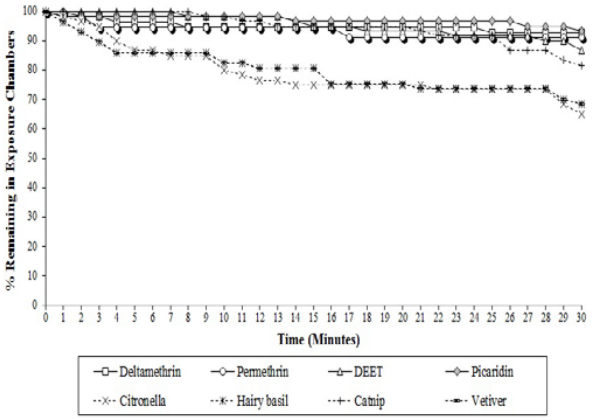
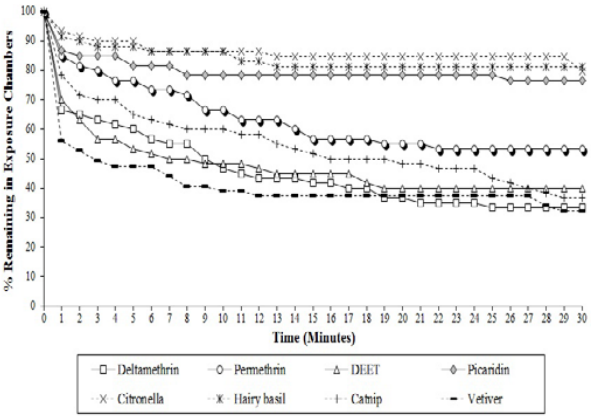


Figure 1. Kaplan-Meier survival curves indicating the percent of four mosquito species remaining inside a treated chamber during 30-min exposure to eight test compounds in contact behavioral assays.

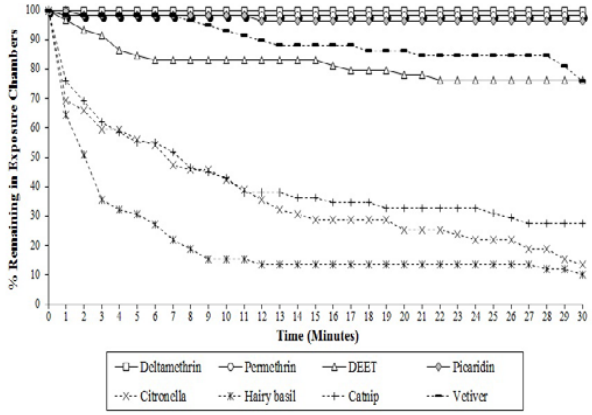
Aedes aegypti



Aedes albopictus



Culex quinquefasciatus



Anopheles minimus

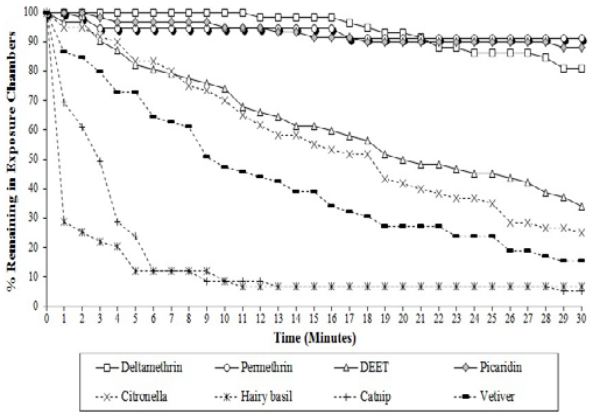


Figure 2. Kaplan-Meier survival curves indicating the percent of four mosquito species remaining inside a treated chamber during 30-min exposure to eight test compounds in non-contact behavioral assays.

Table 1. Knockdown, mortality, and escape response of four mosquito species exposed to deltamethrin (0.025 g/m^2), permethrin (0.5 g/m^2), DEET, picaridin, citronella, hairy basil, catnip, and vetiver each at 2.5% (2.72 g/m^2).

Compound[1]	*Aedes aegypti*					*Aedes albopictus*					*Culex quinquefasciatus*					*Anopheles minimus*				
	% Esc[2]	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)	
		Es[2]	NEs[3]	Es	NEs		Es	NEs	Es	NEs		Es	NEs	Es	NEs		Es	NEs	Es	NEs
Contact																				
Deltamethrin	67.9	46.6	22.4	46.6	27.6	94.4	36.7	5.0	13.3	5.0	72.4	0	0	0	0	65.4	5.1	5.1	15.3	10.2
Deltamethrin-C	8.6	0	0	0	0	10.0	0	0	0	0	3.3	0	0	0	0	10.0	0	0	0	0
Permethrin	72.2	27.8	24.1	24.1	24.1	85.2	25.0	8.3	33.3	10.0	85.9	0	1.7	0	0	97.8	0	3.4	6.9	0
Permethrin-C	7.0	0	0	0	0	10.0	0	0	0	0	3.4	0	0	0	0	10.0	0	0	0	0
DEET	21.4	0	0	0	0	47.3	0	1.7	0	1.7	69.2	0	0	0	0	80.7	0	0	0	0
DEET-C	6.7	0	0	0	0	6.7	0	0	0	0	3.3	0	0	0	0	6.7	0	0	0	0
Picaridin	3.7	0	0	0	0	28.6	0	0	0	0	3.4	0	0	0	0	12.2	0	1.7	0	1.7
Picaridin-C	5.0	0	0	0	0	5.0	0	0	0	1.7	3.4	0	0	0	0	5.0	0	0	0	0
Citronella	37.9	0	0	0	0	5.6	0	76.3	0	0	80.8	0	0	0	0	86.3	0	0	0	0
Citronella-C	0	0	0	0	0	6.7	0	0	0	0	5.0	0	0	0	0	6.7	0	0	0	0
Hairy basil	47.3	0	0	0	0	3.5	0	88.3	0	1.7	89.3	0	0	0	0	90.8	0	6.9	0	3.4
Hairy basil-C	6.7	0	0	0	1.7	6.7	0	0	0	0	5.1	0	0	0	0	6.7	0	0	0	0
Catnip	32.8	5.2	32.8	0	0	61.1	0	8.3	0	1.7	80.7	0	8.6	0	5.2	87.9	0	10.0	76.7	0
Catnip-C	5.1	0	0	0	0	10.0	0	0	0	0	1.7	0	0	0	0	10.0	0	0	3.4	0
Vetiver	37.8	0	0	0	0	42.9	0	0	0	1.7	96.6	0	0	0	0	96.5	0	0	0	0
Vetiver-C	1.7	0	0	0	0	6.7	0	0	0	1.7	1.7	0	0	0	0	6.7	0	0	0	0

Continued.

Table 1. Continued.

Compound[1]	*Aedes aegypti*					*Aedes albopictus*					*Culex quinquefasciatus*					*Anopheles minimus*				
	% Esc[2]	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)		% Esc	% KD		%Mort (24h)	
		Es[2]	NEs[3]	Es	NEs		Es	NEs	Es	NEs		Es	NEs	Es	NEs		Es	NEs	Es	NEs
Non-contact																				
Deltamethrin	3.7	0	10.7	0	12.5	62.3	0	0	0	0	0	0	0	0	0	16.2	0	0	3.4	3.4
Deltamethrin-C	3.5	0	0	3.5	0	3.3	0	0	0	0	0	0	0	0	0	3.3	0	0	0	0
Permethrin	0	0	0	0	3.6	40.8	0	0	0	0	0	0	0	0	0	50.8	0	1.7	1.7	3.3
Permethrin-C	8.9	0	0	0	1.8	15.3	0	0	0	0	1.7	0	0	0	0	15.3	0	0	0	1.7
DEET	8.7	0	0	0	0	57.9	0	0	0	1.7	21.1	0	0	0	0	77.7	0	0	0	0
DEET-C	5.0	0	0	0	0	10.3	0	0	0	0	3.3	0	0	0	0	10.3	0	0	0	0
Picaridin	0	0	0	0	0	14.8	0	0	0	0	1.8	0	0	0	0	2.1	0	0	1.7	0
Picaridin-C	6.7	0	0	0	0	10.0	0	0	0	0	1.7	0	0	0	0	10.0	0	0	0	0
Citronella	30.4	0	0	0	0	14.6	0	52.5	0	1.7	85.9	0	1.7	0	1.7	73.2	0	0	0	0
Citronella-C	3.4	0	0	0	0	6.6	0	0	0	0	3.3	0	0	0	0	6.6	0	0	0	0
Hairy basil	30.4	0	17.5	1.8	1.8	11.2	0	72.9	0	5.1	89.6	0	0	0	0	78.1	1.8	17.5	1.8	3.5
Hairy basil-C	1.7	0	0	0	1.7	11.7	0	0	0	1.7	1.7	0	0	0	0	11.7	0	0	0	0
Catnip	18.6	1.7	35.0	0	0	60.0	0	6.7	0	0	72.4	0	3.4	0	0	94.4	18.6	5.1	89.8	0
Catnip-C	1.7	0	0	0	0	8.3	0	0	1.7	0	0	0	0	0	0	8.3	0	0	5.0	0
Vetiver	31.7	0	0	0	0	63.7	0	0	3.3	0	24.1	0	0	0	0	66.0	0	0	3.3	4.9
Vetiver-C	0	0	0	0	0	3.4	0	0	1.7	1.7	0	0	0	0	0	3.4	0	0	0	0

Fifteen sugar-unfed female mosquitoes aged three to five days old were used in each of the four replications for the behavioral test. [1]C = control test without insecticide/repellent/essential oil. [2]Esc or Es = escaped mosquitoes. [3]NEs = not escaped mosquitoes. All treatment escapes were adjusted for control escapes by Abbott's formula (Finney 1964).

Table 2. Time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of four mosquitoes test populations to escape from test chambers treated with deltamethrin, permethrin, DEET, picaridin, citronella, hairy basil, catnip, and vetiver. Fifteen sugar-unfed female mosquitoes aged three to five days old were used in each of the four replications for the behavioral test.

Compound	*Aedes aegypti*			*Aedes albopictus*			*Culex quinquefasciatus*			*Anopheles minimus*		
	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact												
Deltamethrin	2	8	-	<1	<1	2	4	9	-	7	18	-
Permethrin	1	4	-	<1	<1	4	2	7	14	2	6	12
DEET	17	-	-	<1	28	-	2	12	-	1	6	21
Picaridin	-	-	-	10	-	-	-	-	-	-	-	-
Citronella	15	-	-	-	-	-	<1	4	19	5	7	22
Hairy basil	1	29	-	-	-	-	<1	3	8	<1	2	4
Catnip	21	-	-	<1	15	-	<1	3	18	<1	2	4
Vetiver	6	-	-	<1	-	-	<1	2	5	3	5	11
Non-contact												
Deltamethrin	-	-	-	<1	24	-	-	-	-	-	-	-
Permethrin	-	-	-	5	-	-	-	-	-	10	22	-
DEET	-	-	-	<1	6	-	-	-	-	5	16	27
Picaridin	-	-	-	-	-	-	-	-	-	-	-	-
Citronella	26	-	-	-	-	-	<1	6	22	8	10	30
Hairy basil	15	-	-	-	-	-	<1	2	6	<1	1	16
Catnip	-	-	-	1	4	-	1	7	-	<1	2	4
Vetiver	9	-	-	<1	2	-	-	-	-	3	19	-

Table 3. Comparison of escape responses between paired contact and non-contact trials for four mosquito species exposed to deltamethrin, permethrin, DEET, picaridin, citronella, hairy basil, catnip, and vetiver using the log rank test. Fifteen sugar-unfed female mosquitoes aged three to five days old were used in each of the four replications for the behavioral test.

Compound	*Aedes aegypti* (*P*)	*Aedes albopictus* (*P*)	*Culex quinquefasciatus* (*P*)	*Anopheles minimus* (*P*)
Deltamethrin	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
Permethrin	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
DEET	0.05*	0.22	< 0.0001*	0.06
Picaridin	0.71	0.31	0.44	0.08
Citronella	0.42	0.20	0.97	0.04*
Hairy basil	0.02*	0.12	0.46	0.20
Catnip	0.02*	0.45	0.13	0.74
Vetiver	0.38	0.04*	< 0.0001*	< 0.0001*

*Indicates significant differences ($P<0.05$) between contact and non-contact trials.

Table 4. Comparison of escape responses between two species from the four mosquito species exposed to deltamethrin, permethrin, DEET, picaridin, citronella, hairy basil, catnip, and vetiver using the log rank test.

Compound	Mosquito species	Contact (*P*)	Non-contact (*P*)
Deltamethrin	*Ae. aegypti* and *Ae. albopictus*	< 0.0001*	< 0.0001*
	Ae. aegypti and *Cx. quinquefasciatus*	0.71	0.04*
	Ae. aegypti and *An. minimus*	0.11	0.07
	Ae. albopictus and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *An. minimus*	< 0.0001*	< 0.0001*
	Cx. quinquefasciatus and *An. minimus*	0.15	0.00*
Permethrin	*Ae. aegypti* and *Ae. albopictus*	< 0.0001*	< 0.0001*
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	0.08
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	0.07	< 0.0001*
	Ae. albopictus and *An. minimus*	0.59	0.52
	Cx. quinquefasciatus and *An. minimus*	0.12	< 0.0001*
DEET	*Ae. aegypti* and *Ae. albopictus*	0.01*	< 0.0001*
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	0.11
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	0.05	< 0.0001*
	Ae. albopictus and *An. minimus*	0.05	0.52
	Cx. quinquefasciatus and *An. minimus*	0.76	< 0.0001*
Picaridin	*Ae. aegypti* and *Ae. albopictus*	0.00*	0.01*
	Ae. aegypti and *Cx. quinquefasciatus*	0.72	0.45
	Ae. aegypti and *An. minimus*	0.02*	0.32
	Ae. albopictus and *Cx. quinquefasciatus*	0.0004*	0.00*
	Ae. albopictus and *An. minimus*	0.26	0.08
	Cx. quinquefasciatus and *An. minimus*	0.01*	0.10
Citronella	*Ae. aegypti* and *Ae. albopictus*	0.00*	0.09
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *An. minimus*	< 0.0001*	< 0.0001*
	Cx. quinquefasciatus and *An. minimus*	0.26	0.01*
Hairy basil	*Ae. aegypti* and *Ae. albopictus*	< 0.0001*	0.15
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *An. minimus*	< 0.0001*	< 0.0001*
	Cx. quinquefasciatus and *An. minimus*	0.03*	0.04*
Catnip	*Ae. aegypti* and *Ae. albopictus*	0.0001*	< 0.0001*
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	0.08	0.19
	Ae. albopictus and *An. minimus*	0.00*	< 0.0001*
	Cx. quinquefasciatus and *An. minimus*	0.20	< 0.0001*
Vetiver	*Ae. aegypti* and *Ae. albopictus*	0.27	< 0.0001*
	Ae. aegypti and *Cx. quinquefasciatus*	< 0.0001*	0.23
	Ae. aegypti and *An. minimus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *Cx. quinquefasciatus*	< 0.0001*	< 0.0001*
	Ae. albopictus and *An. minimus*	< 0.0001*	0.33
	Cx. quinquefasciatus and *An. minimus*	0.37	< 0.0001*

*Indicates significant differences ($P<0.05$) between two species.

Table 5. Comparison of contact irritant and non-contact repellent of pyrethroids and synthetic repellents or essential oils against the four mosquito species by using the log rank test.

Species	Compound	Contact		Non-contact	
		Deltamethrin	Permethrin	Deltamethrin	Permethrin
Aedes aegypti	DEET	< 0.0001*	0.0118*	0.30	0.50
	Picaridin	< 0.0001*	< 0.0001*	0.90	0.64
	Citronella	0.00*	0.24	0.00*	0.00*
	Hairy basil	0.09	0.59	0.00*	0.00*
	Catnip	< 0.0001*	0.07	0.08	0.16
	Vetiver	0.00*	0.30	0.00*	0.00*
Aedes albopictus	DEET	< 0.0001*	< 0.0001*	0.63	0.05
	Picaridin	< 0.0001*	< 0.0001*	< 0.0001*	0.01*
	Citronella	< 0.0001*	< 0.0001*	< 0.0001*	0.00*
	Hairy basil	< 0.0001*	< 0.0001*	< 0.0001*	0.00*
	Catnip	< 0.0001*	0.00*	0.46	0.10
	Vetiver	< 0.0001*	< 0.0001*	0.77	0.01*
Culex quinquefasciatus	DEET	0.73	0.03*	< 0.0001*	0.00*
	Picaridin	< 0.0001*	< 0.0001*	0.15	0.54
	Citronella	0.07	1.00	< 0.0001*	< 0.0001*
	Hairy basil	0.00*	0.12	< 0.0001*	< 0.0001*
	Catnip	0.02*	0.60	< 0.0001*	< 0.0001*
	Vetiver	< 0.0001*	0.00*	< 0.0001*	0.00*
Anopheles minimus	DEET	0.17	0.00*	< 0.0001	0.42
	Picaridin	< 0.0001*	< 0.0001*	0.34	< 0.0001*
	Citronella	0.01*	0.00*	< 0.0001*	0.07
	Hairy basil	< 0.0001*	0.00*	< 0.0001*	< 0.0001*
	Catnip	< 0.0001*	0.14	< 0.0001*	< 0.0001*
	Vetiver	< 0.0001*	0.12	< 0.0001*	0.0001*

*Indicates significant differences (P<0.05) between pyrethroids and repellents/essential oils.

previous findings reported by Chareonviriyaphap et al. (2004), Kongmee et al. (2004), and Dusfour et al. (2009). In these papers, pyrethroids had an irritant effect when mosquitoes came into contact with the treated surface. Additionally, our results indicated that pyrethroids, particularly deltamethrin, induced a knockdown effect and mortality against *Ae. aegypti* and *Ae. albopictus* in contact trials. In general, synthetic pyrethroids have a knockdown and killing effect through direct contact (Elliott et al. 1965).

Most essential oils were effective against four mosquito vector species, especially *Cx. quinquefasciatus* and *An. minimus*, but some of them were much weaker than DEET and/or deltamethrin and permethrin when tested against *Ae. aegypti* and *Ae. albopictus*. Previous studies using the same excito-repellency test system found that *Cx. quinquefasciatus* had a strong behavioral escape response to clove, citronella, and cinnamon, whereas *Ae. aegypti* was less responsive and some were knocked down with clove (Suwansirisilp et al. 2012). Phasomkusolsil et al. (2010) found that *An. minimus* and *Cx. quinquefasciatus* were more sensitive to several different oils than were *Ae. aegypti.* Polsomboon et al. (2008) reported that *Ae. aegypti* showed increased escape rates in the contact chamber with 5% catnip, but *An. harrisoni* had a greater irritancy escape response to 2.5% catnip. Thus, the irritant/repellent efficacy of essential oils depends upon the active ingredient, concentration, and mosquito species tested. Moreover, some plants are toxic to mosquitoes. Deletre et al. (2013) reported that 1% of cinnamon, citronella, and thyme essential oils exhibited a toxic effect to *An. gambiae*, and Phasomkusolsil and Soonwera (2011b) found that 10% lemongrass produced 100% mortality for *Ae. aegypti, Cx. quinquefasciatus* and *An. dirus.* In our study, hairy basil and citronella achieved over 50% knockdown of *Ae. albopictus*. Similarly, Boonyuan et al. (2013), using the excito-repellency test system, demonstrated that 10% hairy basil gave almost 90% mortality for *Ae. aegypti* in a contact assay and 69% mortality for a non-contact assay, while 10% citronella showed very high knockdown in *Ae. aegypti*. Therefore, hairy basil and citronella are potential candidates for development as natural insecticides.

Behavioral responses against 2.5% DEET in the present study indicate that this compound elicited both a moderate repellent action against *Ae. albopictus* and *An. minimus* and an irritant effect against *Ae. albopictus*, *Cx. quinquefasciatus* and *An. minimus*. Tisgratog et al. (2011) showed the result of DEET at 5% compared to bifenthrin with a field dose of 25 mg/m^2, that *An. minimus* had a greater escape response to DEET than bifenthrin, and also showed that DEET was both

a contact irritant and spatial repellent.

Overall, this study showed synthetic pyrethroids to be the most effective against all four mosquito species tested. In addition, each mosquito species responded in different escape patterns to the various test compounds. Understanding how alternative natural products function to prevent mosquito human contact is vital in optimizing tools for personal protection. Insecticide resistance phenotypes in many important vector species will continue to pressure the scientific community to develop new and improved vector control strategies. Combination approaches are a good way to ensure the beneficial qualities of the various tools implemented (Gratz 1993, Yap et al. 1994, Lee et al. 2010). Essential oils are one such area for exploration. As a result of the current study, citronella, hairy basil, catnip, and vetiver essential oils could serve as potential mosquito repellent products against *Cx. quinquefasciatus* and *An. minimus*. Further testing with higher concentrations of all essential oils should be performed to find an effective repellent dose against *Ae. aegypti*. According to Phasomkusolsil and Soonwera (2011a), increasing concentrations of essential oils can increase their repellent activity. However, development of a natural repellent formulation with long-lasting protection is necessary to be comparable to the effectiveness of the standard topical repellent DEET. The irritant and/or repellent effects of test compounds described here must be validated with tests on field mosquitoes for confirmation.

Acknowledgments

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Biting patterns and host preference of *Anopheles epiroticus* in Chang Island, Trat Province, eastern Thailand

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ABSTRACT: A study of species diversity of *Anopheles* mosquitoes, biting patterns, and seasonal abundance of important mosquito vectors was conducted in two villages of Chang Island, Trat Province, in eastern Thailand, one located along the coast and the other in the low hills of the central interior of the island. Of 5,399 captured female anophelines, 70.25% belong to the subgenus *Cellia* and remaining specimens to the subgenus *Anopheles*. Five important putative malaria vectors were molecularly identified, including *Anopheles epiroticus, Anopheles dirus, Anopheles sawadwongporni, Anopheles maculatus,* and *Anopheles minimus. Anopheles epiroticus* was the most commonly collected species in the coastal site, whereas *An. dirus* was found to be most abundant in the forest-hill site. From both locations, a greater number of mosquitoes was collected during the dry season compared to the wet. *Anopheles epiroticus* showed greater exophagic and zoophilic behavior with the highest blood feeding densities occurring between 18:00 and 19:00. In contrast, *An. dirus* demonstrated an activity peak between midnight and 01:00. We conclude that *An. epiroticus* and *An. dirus*, in coastal and inland areas, respectively, appear to be the most epidemiologically important malaria vectors on Chang Island. As no studies of vector competency specific to Chang Island have been conducted, our conclusions that these two species play a primary role in malaria transmission are based on evidence from other localities in Thailand and mainland Southeast Asia. This information serves as a basis for designing improved vector control programs that target specific species, and if integrated with other interventions could result in the elimination of malaria transmission on the island. ***Journal of Vector Ecology* 39 (2): 361-371. 2014.**

Keyword Index: *Anopheles*, species diversity, malaria, Chang Island, Thailand.

INTRODUCTION

In Thailand, malaria is a significant cause of morbidity and remains prevalent and entrenched in the more remote forested and hilly areas, especially along the international borders with Cambodia and Myanmar where efficient malaria vectors are common and access to health care distant (Kongmee et al. 2012). Of the approximately 73 *Anopheles* species recognized in Thailand, member species in the Leucosphyrus Group, Maculatus Group and Minimus Subgroup, include five of the primary malaria vectors (Rattanarithikul et al. 2006). Nine species of mosquitoes have been incriminated as malaria vectors in Thailand (Green et al. 1991, Rattanarithikul et al. 2006, Suwonkerd et al. 2013), including *Anopheles dirus* (Baimai et al. 1988, Rosenburg et al. 1990), *An. baimaii* (Baimai et al. 1988, Green et al. 1991), *An. minimus* (Ratanatham et al. 1988, Rattanarithikul et al. 1996), *An. pseudowillmori*, *An. maculatus* (Cheong et al. 1968), *An. aconitus* (Maheswary et al. 1992), *An. sawadwongporni,* and *An. campestris* (Somboon et al. 1998, Coleman et al. 2002), all of which are associated with hilly forest environments and forest-fringe areas. The last malaria vector, *An. epiroticus,* occurs along mainland coastal areas and islands where this species predominantly utilizes brackish water habitats (Sumruayphol et al. 2010).

Anopheles sundaicus s.l. is regarded as the principal vector of malaria along many coastal areas in Southeast Asia (Adak et al. 2005, Alam et al. 2006, Dusfour et al. 2007a). The species complex is widely distributed from northeastern India, eastwards to southern Vietnam (south of the 11th parallel), and southwards to the Andaman and Nicobar Islands (India), Malaysia (peninsular and northern Borneo), and Indonesia (Java, Sumatra, Sulawesi, and Lesser Sunda islands) (Linton et al. 2001, Dusfour et al. 2004a). At least four sibling species are recognized in the complex, *An. epiroticus* (formerly *An. sundaicus* species A), *An. sundaicus* s.s., *An. sundaicus* species E, and *An. sundaicus* species D (Dusfour et al. 2007b, Alam et al. 2006). In Thailand, only *An. epiroticus* is regarded as present and is found along the coastal regions and islands of the eastern and southern regions (Scanlon et al. 1968, Sukowati et al. 1996, 1999, Linton et al. 2005, Rattanarithikul et al. 2006) and has been incriminated as a secondary malaria vector (Gould et al. 1966, Harinasuta et al. 1974, Chowanadisai et al. 1989).

Chang Island is located in the Gulf of Thailand, (Ko Chang District, Trat Province), eastern Thailand, 30 km east

of the mainland (Figure 1). Although Chang Island is a major tourist destination, it is also a malaria endemic area that poses a health risk to visitors and local inhabitants alike. The island is roughly divided into either forested foothills or coastline in which malaria remains a significant health threat and concern. From 2006 to 2013 a total of 201 malaria cases were recorded from the island. During this period, the number of infections peaked in 2006 with 61 cases including *Plasmodium falciparum* and 52 *P. vivax* infections (Bureau of Vector Borne Diseases 2013). While malaria is endemic, information is lacking not only on the diversity of mosquitoes present, but also medical importance (vector status), basic biology, and behavioral aspects of each. Vector incrimination and species bionomics is critical for defining the vectorial capacity of each species, understanding the spatial and temporal disease transmission risk, and for designing appropriate vector prevention and control strategies against target species.

Recently, molecular-based assays have been developed to reliably identify individual species within the different *Anopheles* complexes (Manguin et al. 2008). An allele-specific polymerase chain reaction assay using cytochrome oxidase I (COI) and cytochrome-*b* (Cyt-b) has been developed for distinguishing three allopatric species of the Sundaicus Complex that includes *An. epiroticus* (Thailand, Vietnam, Cambodia, peninsular Malaysia), *An. sundaicus* s.s (Borneo Malaysia, Kalimantan Indonesia), and *An. sundaicus* E (Sumatra, Java, Lesser Sunda Islands, Indonesia) (Dusfour et al. 2007a). Additionally, *An. epiroticus* can be identified using the internal transcribed spacer 2 (ITS2), domain 3 (D3), and COI (Sumruayphol et al. 2010). Accurate identification of sibling species of important vectors contributes to more accurate studies of vector species and effective control of target species (Sinka et al. 2011, Kongmee et al. 2012).

As there is very limited bionomic information about anophelines on the island, this study was designed to describe the trophic behavioral patterns (biting cycle and feeding preferences) and seasonal abundance of *An. epiroticus* and other species present on Chang Island.

MATERIALS AND METHODS

Study sites

Chang Island (Ko Chang) is located approximately 350 km from Bangkok in Trat Province, eastern Thailand, near the border of Cambodia. Chang Island is the second largest island in the Gulf of Thailand, covering an area of approximately 217 km^2 with a human population of nearly 8,000. Most of the island consists of forested foothills and coastal zones mostly covered by tropical rainforest.

Anopheles mosquitoes were collected from two different sites on the island, including a coastal site at Khlong Yuan Village (12° 02'N, 102° 23'E) and an inland forest site at Khlong Jao Lueam waterfall (12° 06'N, 102° 18'E) (Figure 1). Coastal Khlong Yuan Village is 500 m from the sea at an elevation of approximately 39 m above sea level and is surrounded by fruit orchards and rubber plantations along with natural mangrove forests. Interior Khlong Jao Lueam is near a waterfall surrounded by deep forests and high hills. Khlong Jao Lueam site is approximately 2.5 km from the sea and approximately 71 m above sea level.

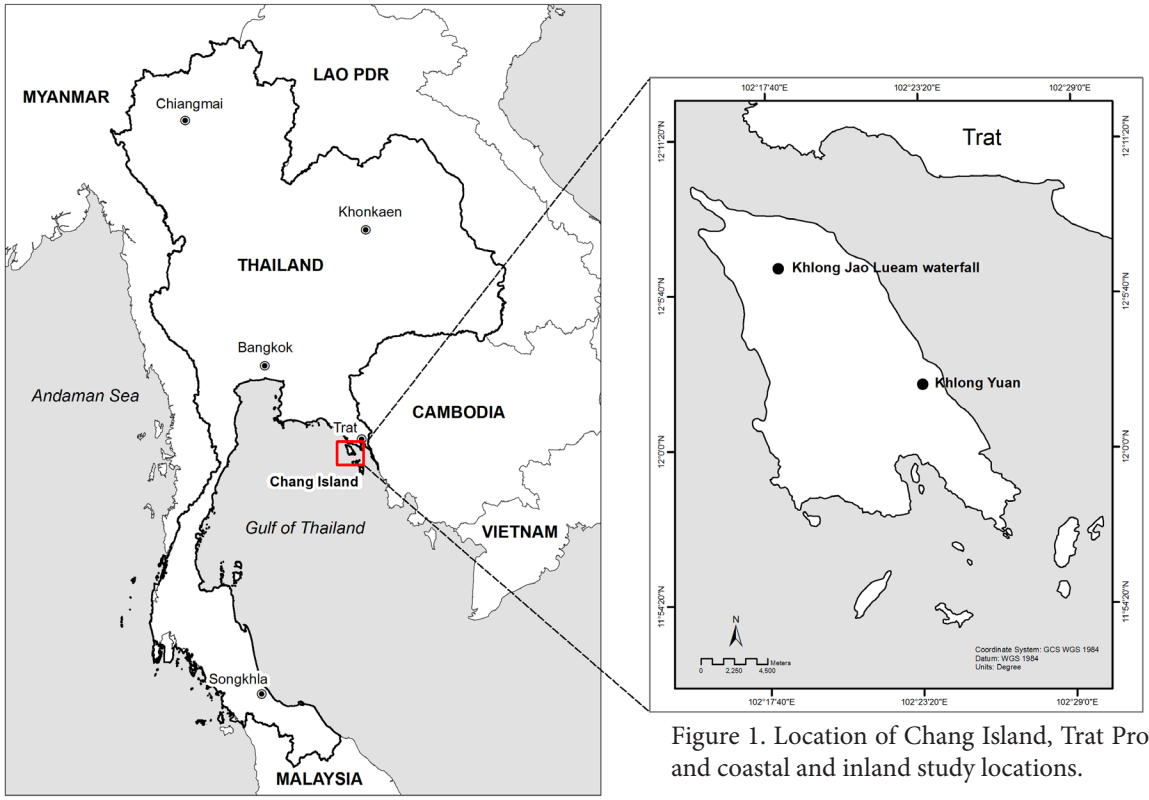


Figure 1. Location of Chang Island, Trat Province, Thailand, and coastal and inland study locations.

Collection methods

Adult mosquitoes were collected once every two months during three consecutive nights for a period of two years (2011 to 2012). Mosquito collections were carried out using up to three methods: human-landing indoor (HLI), human-landing outdoor (HLO), and buffalo-bait collections (BBC) when applicable. Human-landing collections (HLC) were performed in a local (inhabited) house in each site. Each site consisted of two mosquito collection teams (four persons each), with each team divided into two groups of two collectors each for indoor and outdoor activities, respectively. Mosquitoes were collected from 18:00 to 06:00 with each team dividing collection time from the first half of the night (18:00 to 24:00), followed by the second team beginning at midnight to 06:00. Every hour the team members rotated between indoor and outdoor collection positions to mitigate collector bias. Outdoor collectors sat within 50 m of the study house. HLC were done for 45 min each hour (e.g., 18:00-18:45). Each collector captured mosquitoes landing on exposed legs (knee to foot) using a flashlight and a mouth aspirator. Approval for the HLC activity was provided by the Ethics Review Committee for Research Involving Human Subjects, Health Science Group of Faculties, Colleges and Institutes, Chulalongkorn University, Thailand (Approval COA No. 167/2013).

The buffalo-baited capture (BBC) method was only performed in Khlong Yuan Village as the animal represented the only buffalo (*Bubalus bubalis*) on the entire island, The method placed a tethered animal inside a suspended net 20 m^2 in area (2 m height) in a designated holding area to attract host-seeking mosquitoes. Netting was set up from ground level with a small gap (0.3 m) to allow the mosquitoes access. Mosquitoes that entered were allowed to rest or blood feed and subsequently rest on the inside of the netting before collection using a mouth aspirator. The BBC was performed 15 min each hour from 18:00-06:00. *Anopheles* females were held in plastic cups, labeled by hour and site of collection, and covered with netting and a cotton pad saturated with 10% sugar solution. Mosquitoes were transferred to another location for morphological identification and sorting. Ambient air temperature and relative humidity were recorded from indoor and outdoor locations each hour. At the site of the buffalo-baited trap, a manual thermo-hygrometer (BARICO GmbH, Villingen-Schwenningen, Germany) was used to collect the hourly air temperature and percent relative humidity. Rainfall data was obtained from the Trat Province meteorological station located approximately 110 km from the study sites.

Morphological identification

Anopheles females were initially identified and sorted in the field using a standard morphological identification key (Rattanarithikul et al. 2006). Afterward, all specimens were preserved in liquid nitrogen and returned to Kasetsart University, Bangkok, for molecular identification and further analysis.

Molecular identification

DNA from specimens initially identified as *An. sundaicus* s.l., *An. minimus* s.l., *An. dirus* s.l., and *An. maculatus* s.l. were extracted from each adult mosquito following the methods of Linton et al. (2001) and Manguin et al. (2002). Molecular identification of members of the *An. sundaicus* complex used the AS-PCR assay developed by Dusfour et al. (2007a) using species-specific primers for *An. sundaicus* s.s, *An. epiroticus,* and *An. sundaicus* E. For the Dirus Complex, the AS-PCR method of Walton et al. (1999) was performed using specific primers for *An. dirus*, *An. cracens*, *An. scanloni*, *An. baimaii,* and *An. nemophilous.* For members of *An. minimus* complex, the AS-PCR assay of Garros et al. (2004) was conducted using the specific primers for *An. minimus* and *An. harrisoni*. For the *An. maculatus* group, molecular identifications were performed using the AS-PCR assay of Walton et al. (2007) using specific primers for *An. maculatus* and *An. sawadwongporni*.

Data analysis

Seasons, collection times, locations, and specific collection methods were used in the analysis. Seasonal periods were separated to include wet (May to October) and dry (November to April) seasons, collection time periods were group classified as early evening (18:00–21:00), late night (21:00–24:00), predawn (24:00–03:00), and dawn (03:00–06:00), and collection types and locations were designated as HLI, HLO, and BBC. The nocturnal biting behavior of *An. epiroticus* was tabulated by averaging the number of *Anopheles* landing per hour per human, separated by indoor and outdoor positions and by averaging the number of mosquitoes captured in the buffalo trap for 15 min each hour. Comparisons of human-landing data were analyzed by non-parametric Kruskal-Wallis, Wilcoxon, and Mann-Whitney tests. The level of significance was set at 0.05% (P valve < 0.05), followed by correlation coefficient (r) analysis taking into account specimens captured and environmental variables. All data were analyzed using the SPSS statistical package (SPSS version 17.0, Chicago, IL).

RESULTS

In the two-year period, combined study sites had a total of 5,399 anophelines captured, comprising 14 *Anopheles* taxa separated within two subgenera, *Cellia* and *Anopheles* (Table 1), representing nine (n=3,793, 70.25%) and five (n= 1,606), respectively. From all collections, 97.5% (5,264) were captured from the coastal site of Klong Yuan and only 2.5% (135) were collected from the inland forested location. However, buffalo-baited trapping only occurred in Khlong Yuan and represented 83.2% of all anophelines collected. Excluding the BBC data, when comparing only HLC data, Khlong Yuan still produced the majority (85.1%) of captured anophelines compared to Khlong Jao Lueam. Within the subgenus *Cellia,* 3,444 specimens (90.8%) belonged to the Sundaicus Complex, 140 (3.7%) within the Dirus Complex, 43 (1.13%) to the Maculatus Group, and nine (0.24%) to the Minimus Complex. Many members within these four taxonomic assemblages are

regarded as potential malaria vectors in Thailand (Saeung 2012). Species that are typically regarded as non-malaria vectors within the subgenus included *Anopheles jamesii*, *An. kochi*, *An. karwari*, *An. vagus,* and *An. philippinensis* (Table 1). Additionally, five taxa within the subgenus *Anopheles* were identified, including *An. umbrosus*, *An. barbirostris* group, *An. aitkenii* group, *An. hyrcanus* group, and *An. peditaeniatus* (Table 1). Only members in the *Barbirostris* and *Hyrcanus* groups have been found infected with either *P. falciparum* and/or *P. vivax* parasites (Rattanarithikul et al. 1996).

Only specimens from the four putative malaria vector species complexes or groups in the subgenus *Cellia* were subjected to definitive species identification using the appropriate multiplex AS-PCR assay. Five important species were identified, including *An. dirus* (former *An. dirus* species A) (3.9% of samples assayed), *An. minimus* (former *An. minimus* species A) (0.25%), *An. sawadwongporni* (0.8%), *An. maculatus* (0.4%), and *An. epiroticus* (former *An. sundaicus* species A) (94.7%). The initial morphological identification recorded 14 species or assemblages, whereas follow-up PCR allowed the identification of *An. sawadwongporni* (Maculatus Group), resulting in a total of 15 species collected on Chang Island. The multiplex assay was able to identify nearly 90% of all assayed specimens initially identified as members of the Sundaicus Complex, whereas the remaining 10% were not identifiable by PCR for reasons that remain unclear (Figure 2). However, following the most current revision of the Sundaicus Complex in Southeast Asia, *An. epiroticus* is the only species believed present in Thailand (Suwonkerd et al. 2013).

In Klong Yuan, *An. epiroticus* was the most abundant (90.8%) of the 15 *Anopheles* species identified and when compared to only the other four key potential malaria vectors present, it contributed to 98.4% of the collection (Tables 1 and 2). *Anopheles epiroticus* was not collected from Khlong Jao Lueam. At this latter site, only two species were identified among 135 *Anopheles* captured, the majority being *An. dirus* (94.1%) followed by *An. minimus* (5.9%) (Table 2).

The majority of *An. epiroticus* from Klong Yuan were captured in the buffalo trap (78.4%) compared to HLC. The distribution of this species in the HLC found 65.5% outdoors compared to timed-matched indoor collections (Table 3). With 21.6% of *An. epiroticus* collected on humans either indoors or outdoors, this species demonstrated some degree of anthropophily but appears attracted to both humans and buffalo (alternative animal blood sources). The *An. epiroticus* feeding patterns by hour and collection method are shown in Figures 3A and 3B. The indoor human biting activity showed small peaks from 01:00-02:00 and 03:00-05:00. The outdoor human biting activity presented two small peaks from 21:00-22:00 and 01:00-02:00. None of these small increases in biting density are considered marked and it appears this species is active throughout the evening, both indoors and out (Figure 3A). In contrast, the mosquito activity patterns associated with buffalo bait showed the greatest activity at the beginning of the collection period (18:00 to 19:00), and declining progressively thereafter until the following morning (Figure 3B).

A greater number of *An. epiroticus* were collected during the dry season (November to April) with a notable peak

Table 1. Total numbers of *Anopheles* mosquitoes collected at Chang Island, Trat Province, from January, 2011 to September, 2012 based on initial morphological species identification.

Anopheles species	Khlong Yuan		Khlong Jao Lueam	Total
	Human bait	Buffalo bait	Human bait	
Anopheles (Cellia)				
An. dirus s.l.	5	8	127	140
An. minimus s.l.	1	0	8	9
An. maculatus s.l.	0	43	0	43
An. sundaicus s.l.	743	2,701	0	3,444
An. jamesii	0	25	0	25
An. kochi	6	90	0	96
An. karwari	1	32	0	33
An. vagus	0	1	0	1
An. philippinensis	0	2	0	2
Anopheles (Anopheles)				
An. umbrosus	14	382	0	396
An. barbirostris group	0	12	0	12
An. aitkenii group	0	175	0	175
An. hyrcanus group	0	997	0	997
An. peditaeniatus	0	26	0	26
Total	770	4,494	135	5,399

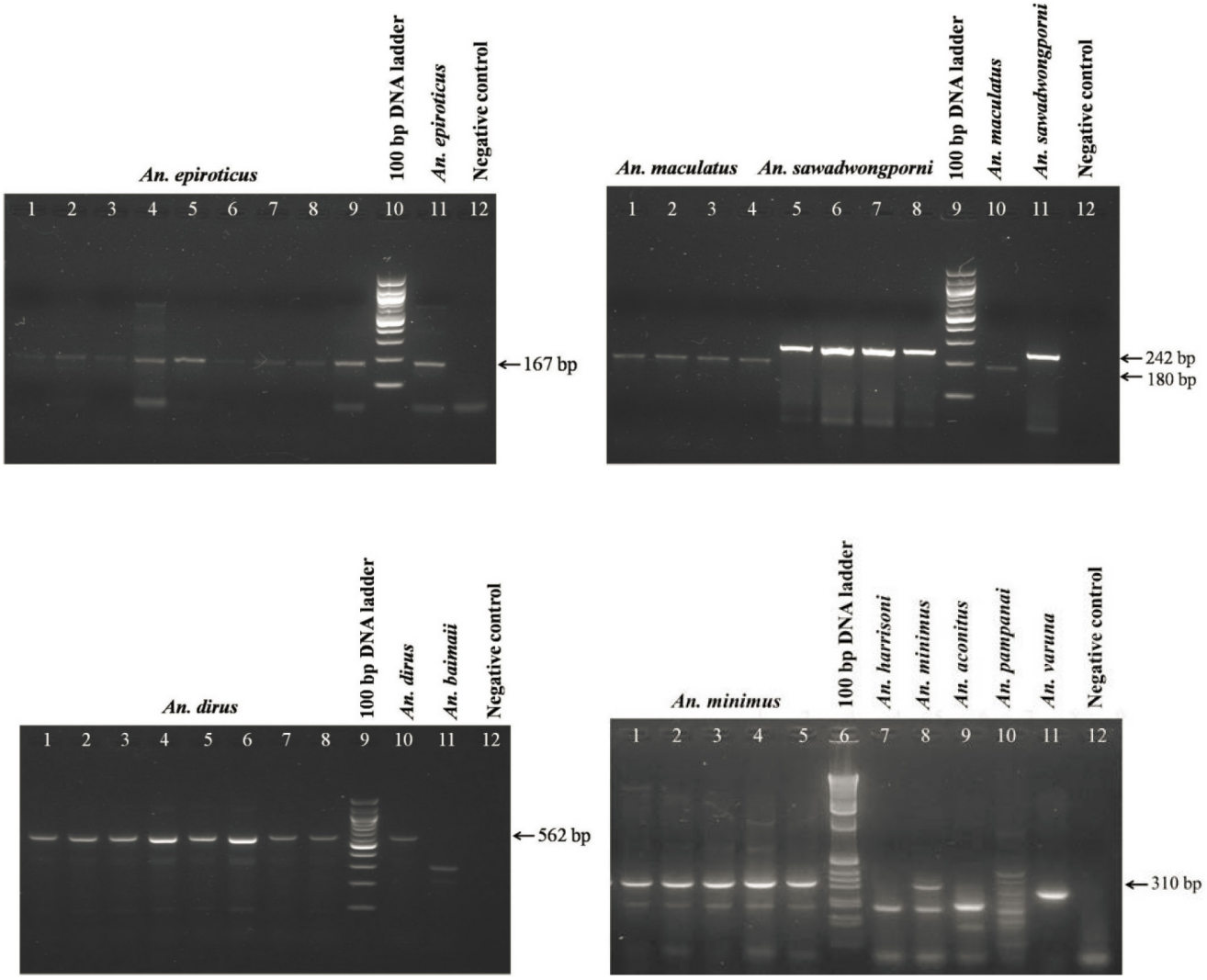


Figure 2. Amplified fragments using allele-specific PCR assay for identifying *An. epiroticus* *An. maculatus*, *An. sawadwongporni*, *An. dirus,* and *An. minimus* mosquitoes with appropriate control sample DNA.

in the early dry season from November to January (Figure 4). In contrast, *An. epiroticus* was found in relatively low adult densities during the wet season (May to October). In the inland forest site, both *An. dirus* and *An. minimus* were collected in higher abundance during the dry season (peak January-March).

Comparisons of HLC data were analyzed by non-parametric Kruskal-Wallis, Wilcoxon, and Mann-Whitney tests. A strong significant difference in the number of *An. epiroticus* was found between seasons (Z= -4.696, $P = 0.000$), and between indoor, outdoor human landing, and buffalo-baited collection methods (F = 5.319, df=2, $P = 0.010$). The Wilcoxon pairwise comparison between indoor vs outdoor collections (Z = -2.803, $P = 0.005$), between indoor vs buffalo (Z = -2.936, $P = 0.003$), and between outdoor vs buffalo (Z = -2.994, $P = 0.003$) were significantly different from one another. There was no significant difference in the number of *An. epiroticus* collected between the four quarterly evening time intervals (χ^2=0.04, *df*=3, *P*=0.998 indoor; χ^2=0.91, *df*=3, *P*=0.823 outdoor and χ^2=0.579, *df*=3, *P*=0.903 buffalo). Data from all collection methods were pooled to determine the correlation between mosquito abundance and measured environmental variables (Table 3 and Figures 3A, 3B). Results indicated that *An. epiroticus* densities were strongly correlated with rainfall patterns ($r = -0.667$; $P = 0.009$) and relative humidity ($r = -0.640$; $P = 0.012$) but were not associated with relative minimum or maximum ambient air temperatures ($P > 0.05$).

DISCUSSION

In this study, five important malaria vectors in Thailand were molecularly identified as occurring on Chang Island, including *An. dirus*, *An. minimus, An. maculatus, An. sawadwongporni,* and *An. epiroticus*. *Anopheles epiroticus* was only found at Khlong Yuan, a coastal site. In contrast, *An. dirus*, arguably the most efficient malaria vector in Thailand, was identified in Khlong Jao Lueam, an inland site near forested hills. Khlong Yuan contained all five key vector species and showed much higher anopheline species diversity (15 species in all) compared to only two species captured in Khlong Jao Lueam, of which *An. dirus* represented 94% of the catch. Khlong Yuan contains several potential breeding habitats for *An. epiroticus*, a species that typically requires/prefers sunlit, mostly brackish, water habitats containing floating algae (Sinka et al. 2011). The most favorable habitats are abandoned or poorly maintained shrimp/fish ponds or inland seawater canals, but immature stages will also inhabit coastal ponds, swamps, mangrove, and rock pools (Manguin et al. 2008). In our study, we attempted sampling of all available potential aquatic habitats in and around each study site every two months during the same time as adult collections; however, larval collections were generally unproductive and not particularly informative. We were unable to identify any productive larval habitats of *An. epiroticus* in and around Khlong Yuan. The inability to detect immature stages of this species is puzzling and something that was entirely unexpected given its relatively high abundance in adult collections. A careful, systematic search for all possible larval habitats to include greater geographical coverage along the coastline of Chang Island is required.

The behavior of *An. sundaicus* sibling species appears to differ depending on the locality (Dusfour et al. 2004a, Linton et al. 2005). The biting activity of *An. sundaicus* complex typically occurs between 20:00 to 03:00 depending on the locality (Sinka et al. 2011). In Thailand, Gould et al. (1966) observed that *An. sundaicus* s.l. (= *An. epiroticus*) had a greater outdoor biting frequency and feeding preference on cows, indicating more pronounced exophagy and zoophily. In contrast, the trophic behavior of *An. sundaicus* s.l. in Cambodia varied from exophagy to endophagy (Dusfour et al. 2004a). In Rayong Province, Thailand, Sumruayphol et al. (2010) observed *An. epiroticus* blood feeding predominantly between 18:00-24:00 with a peak of biting activity around midnight with a maximum of 6.6 bites/person/h. In Chang Island, we found the buffalo to be a potent attractant for *An. epiroticus* relative to humans, indicating stronger zoophilic behavior. Buffalo-baited collections showed the highest activity during the beginning of the 12-h collection period between 18:00 and 19:00. Although we recognize that the movement of *An. epiroticus* to bait and blood feeding could have taken place before 18:00, it would have unlikely occurred in high numbers outside forested or well-shaded environments. The biting patterns of *An. epiroticus* in indoor and outdoor human landing collections were very similar, and only small peaks of activity were observed between 01:00-02:00 indoors and between 20:00-21:00 and 01:00-02:00 in outdoor captures. The limited number of specimens of *An. dirus* (140), *An. minimus* (9), *An. maculatus* (15), and *An. sawadwongporni* (28) collected in this study did not allow for accurate interpretations of host-seeking patterns or definitive conclusions regarding seasonality and species abundance.

The seasonal abundance of *An. epiroticus* in this study appeared to be influenced by several factors, most notably precipitation patterns. The population densities of *An. epiroticus* showed the greatest abundance during the dry season (November to April) for both the first and second years of observations. *An. epiroticus* was found active every collection period throughout the two years with the highest densities in November. In Indonesia, high densities of *An. sundaicus* s.l. were also associated with the dry season as lagoons and brackish water impoundments became more suitable habitats as water flow was impeded from entering the sea, creating large stagnant bodies of water and abundant floating algal mats (Sundararaman et al. 1957). However, in Rayong Province, *An. epiroticus* was found active throughout the year with the highest densities in the rainy season (September). We also noted a significant negative association with adult densities and with higher mean ambient relative humidity.

Thailand faces recurring threats of emerging and re-emerging arthropod-borne diseases, especially malaria (Chareonviriyaphap et al. 2013). Malaria remains most prevalent along the less developed international borders of eastern Myanmar, northern Malaysia, and western Cambodia, as well as several coastal zones where the blackish water

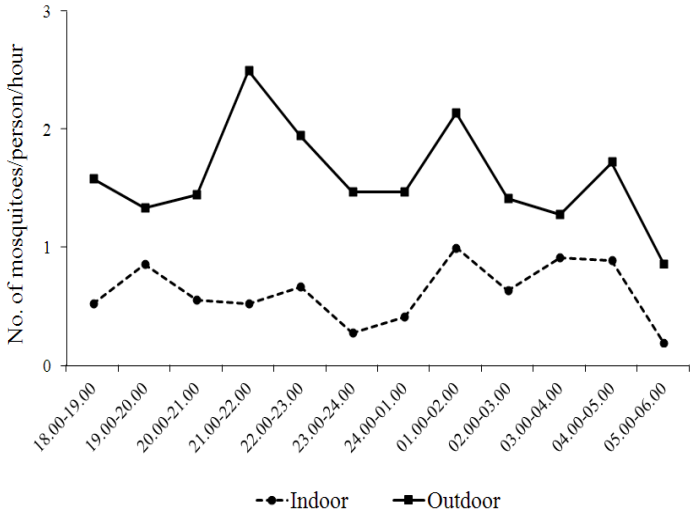


Figure 3A. Mean hourly densities of *Anopheles epiroticus* by human (indoor and outdoor) landing collections in Khlong Yuan.

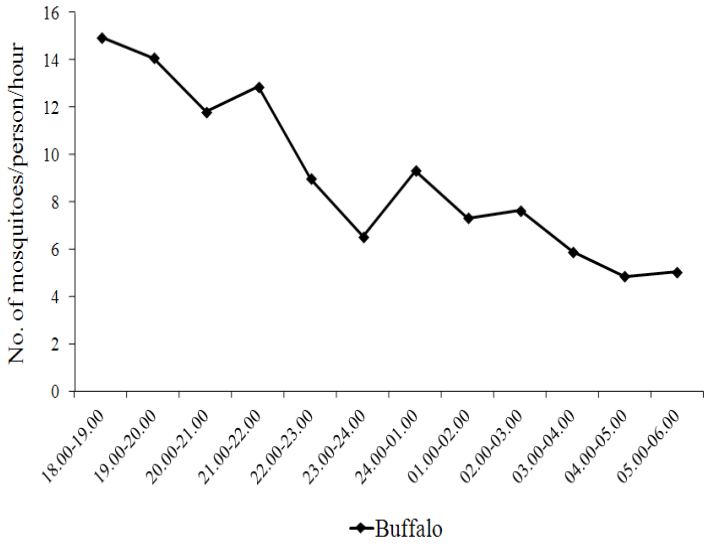


Figure 3B. Mean hourly densities of *Anopheles epiroticus* using a buffalo-baited trap in Khlong Yuan.

mosquito, *An. epiroticus*, exists. Chang Island is the second largest island of Thailand and is regarded as one of the most attractive and popular tourist sites in the country. However, this island is still a malaria endemic area with around 50-100 cases annually since the beginning of the 1990s. However, in 2013, only five malaria cases (all *P. vivax*) were detected and presumably transmitted on the island. The malaria risk areas are generally located in two relatively undeveloped areas on the island, including a forest fringe in the interior hills and a coastal area where *An. dirus* and *An. epiroticus* are present, respectively. The Chang National Park on the eastern side of the island is primarily an inland forest with creeks, rivers, and waterfalls which provide many potential habitats for malaria vectors.

Although malaria vectors have been identified on Chang Island, no information about their biology and behavior (population dynamics, biting and host preference, and seasonal abundance) have been described from the island. Moreover, until this study, there had been no attempt to identify the species present based on molecular methods. A critical component to understanding the local epidemiology is a precise identification of the vector species in various locales. The vectorial capacity of different sibling species can often vary in behavior, resulting in different abilities to transmit malaria. Such information is important to help identify the respective roles of each vector species in disease transmission and to implement appropriate prevention and control strategies.

Anopheles epiroticus belongs to the Sundaicus Complex in the Pyretophorus Series, a grouping of important malaria

Table 2. Numbers of select *Anopheles* species (as suspected malaria vectors) based on molecular analysis collected at Chang Island, Trat Province, between January, 2011 and September, 2012.

Anopheles	Khlong Yuan			Khlong Jao Lueam		Total
	Buffalo	Indoor	Outdoor	Indoor	Outdoor	
An. dirus	8	0	5	58	69	140
An. minimus	0	0	1	8	0	9
An. sawadwongporni	28	0	0	0	0	28
An. maculatus	15	0	0	0	0	15
*An. epiroticus**	2,701	256	487	0	0	3,444
Total	2,752	256	493	66	69	3,636

*Approximately 10% of samples failed to generate a PCR-based confirmation, however, based on initial morphological identification, all specimens were deemed *An. epiroticus*.

Table 3. Total of monthly captures of *An. epiroticus* from three collection methods in Chang Island, Trat Province.

Month	In	Out	Buffalo	Total	Mean		
					Temp. (°C)	RH (%)	Rainfall (mm)
Year 1							
Jan 11	40	52	310	402	26.8	65	0.0
Mar 11	29	33	395	457	27.0	78	235.6
May 11	18	27	45	90	28.4	83	353.5
Jul 11	1	0	2	3	27.1	86	895.8
Sep 11	0	0	3	3	26.6	88	1446.7
Nov 11	77	179	583	839	28.2	73	9.5
Year 2							
Jan 12	76	119	716	911	27.3	76	141.1
Mar 12	8	28	32	68	28.1	80	136.5
May 12	1	3	10	14	27.6	86	622.9
Jul 12	0	1	0	1	27.0	87	857.4
Sep 12	0	2	3	5	26.3	89	1311.0
Nov 12	6	43	602	651	27.7	84	392.8
Total	256	487	2,701	3,444			

RH = percent relative humidity.

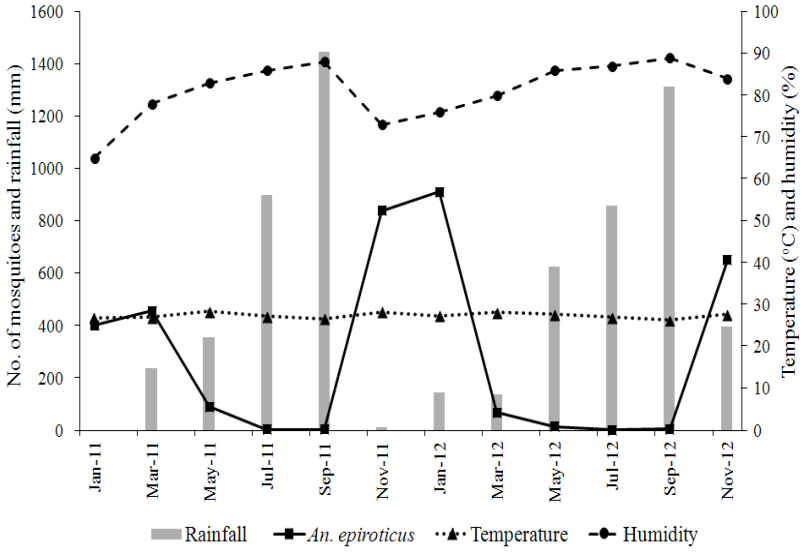


Figure 4. Seasonal abundance of *An. epiroticus* in relation to monthly precipitation and percent relative humidity on Chang Island, Trat Province.

vectors in Asia and Africa (Harbach 2013). Members of this species complex have long been recognized as malaria vectors in coastal areas and on islands in Southeast Asia (Sukowati et al. 1999, Dusfour et al. 2004b, Linton et al. 2005); however, their relative importance as either major or secondary malaria vectors varies by locality and epidemiological factors influencing transmission (Schaefer and Kirnowardayo 1983, Dusfour et al. 2007a). Of the four known allopatric species in the complex, only *An. epiroticus* has been identified in Thailand and distributed in eastern and southern coastal areas (Rattanarithikul et al. 2006, Dusfour et al. 2004b, Linton et al. 2005, Dusfour et al. 2007b). With the development of PCR assays that identify individual sibling species, a growing number of studies have successfully described the trophic behavior, biting activity, and seasonal abundance of several mainland malaria vector species in Thailand (Chareonviriyaphap et al. 2003, Sungvornyothin et al. 2006, Muenworn et al. 2008, Tananchai et al. 2012, Tisgratog et al. 2012, Kongmee et al. 2012), whereas similar investigations on coastal and island species, like *An. epiroticus*, has been limited.

Although *An. epiroticus* is typically regarded as a secondary vector of malaria (Harinasuta et al. 1974), its potential as an efficient vector remains a prime concern near tourist areas and local coastal settlements on Chang Island. The information gathered in this study indicates two primary vectors on the island: *An. epiroticus* along the coastal zone and *An. dirus* in the interior parts. Although malaria parasites were not tested in any of the anophelines captured on humans or in buffalo-baited traps, these two species were the predominant vectors in their respective localities throughout much of the study period, albeit adult densities varied by season. Therefore, from an epidemiological perspective, all available evidence implicates both anophelines as the most likely vector candidates. As only two sites were observed, this does not preclude other potential vector species playing a greater role in transmission on the island. Further investigations in other localities will better define and map vector distribution in relation to malaria transmission. Moreover, a far better understanding of the full range and preferred *An. epiroticus* larval habitats is also required and a pre-requisite to any meaningful attempt to control this species and transmission of malaria.

Recent malaria statistics reveal that malaria transmission has been lower than in the past, especially since 2006 when a high of 113 cases was reported. Between 2007 and 2013, an average of less than 13 cases of malaria per year occurred. For this reason, it may be feasible to attempt an island-wide integrated campaign to eliminate malaria entirely from the island by identifying all residual foci and treating all human reservoirs of malaria. For those areas with evidence of recent or high risk for renewed transmission, a time-limited vector control strategy could also complement the elimination effort when appropriate. As importation of malaria into Chang Island remains a threat, any successful elimination effort will still require a robust surveillance system to be in place to quickly identify cases and prevent secondary transmission. To advance the most appropriate vector control on Chang Island, additional investigations are needed on vector biology and transmission potential of local anopheline populations.

Acknowledgments

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Resistance to Synthetic Pyrethroids in *Aedes aegypti* (Diptera: Culicidae) in Thailand

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ABSTRACT

The insecticide susceptibility level of mosquito populations is one of the major factors influencing the success of vector control. In this study, *Aedes aegypti* from seven localities with a current dengue outbreak in Thailand were subjected to synthetic pyrethroid insecticide susceptibility assays. The results revealed that *Ae. aegypti* from all localities were strongly resistant to bifenthrin, permethrin and deltamethrin. High resistance to lambda-cyhalothrin was detected from all localities with the exception of *Ae. aegypti* from Bangkok and Uttaradit which demonstrated incipient resistance. However, *Ae. aegypti* from Bangkok, Phra Nakhon Si Ayutthaya, Sakon Nakhon and Chumphon showed incipient resistance to alpha-cypermethrin whereas *Ae. aegypti* collected from Uttaradit, Mukdahan and Phatthalung were susceptible. In addition, *Ae. aegypti* from Bangkok, Phra Nakhon Si Ayutthaya and Uttaradit demonstrated incipient resistance to cypermethrin while susceptibility of *Ae. aegypti* from Mukdahan, Sakon Nakhon, Phatthalung and Chumphon to cypermethrin was observed. It was concluded that field-collected *Ae. aegypti* from all localities had developed resistance to the synthetic pyrethroids, with the majority of these being to bifenthrin, permethrin and deltamethrin.

Keywords: *Aedes aegypti*, synthetic pyrethroid, resistance, Thailand

INTRODUCTION

The incidence of dengue fever and dengue hemorrhagic fever (DF/DHF) has increased dramatically around the world in recent decades, especially in tropical and subtropical regions; about 2.5 billion people—two fifths of the world's population—are now at risk from DF/DHF and it is estimated that there are 50 million dengue infections worldwide every year (World Health Organization, 2012, 2014). The incidence of DF/DHF is still consistently high with 153,765 reported cases in Thailand (Ministry of Public Health, 2013). The disease is transmitted by *Aedes aegypti*, a primary vector of DF and DHF (Gubler, 1997).

Aedes aegypti, a day-biting mosquito, is highly anthropophilic and often rests and feeds in or near human dwellings (Christophers, 1960). This mosquito has been found to be highly adapted to all man-made and natural environments. The key to preventing dengue transmission relies mainly on vector control, the most effective method for reducing disease transmission (Pant, 1979; Reiter and Gubler, 1997). During the past decade, several synthetic pyrethroids—namely permethrin, deltamethrin, lambda-cyhalothrin and etofenprox—were

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introduced in the malaria and dengue control program, particularly with the impregnation of bed nets (permethrin) and indoor/outdoor sprays (deltamethrin) (Chareonviriyaphap *et al*., 1999). At present, pyrethroids are the main insecticides used in controlling vector-borne diseases throughout the country. However, the long-term continuous use of insecticides has led in some cases to high levels of chemical resistance by certain pests and disease vectors (Chareonviriyaphap *et al*., 1999). This is considered to be a major factor in the development of resistance in mosquitoes (Chareonviriyaphap *et al*., 1999; Sathantriphop *et al*., 2006; Chuaycharoensuk *et al*., 2011). Previous studies have shown the occurrence of insecticide resistance in several populations and species of mosquitoes, including *Ae. aegypti* (Chareonviriyaphap *et al*., 1999; Ponlawat *et al*., 2005; Jirakanjanakit *et al*., 2007; Chuaycharoensuk *et al*., 2011).

According to World Health Organization (WHO) guidelines for insecticide susceptible tests on mosquito populations (World Health Organization, 1998, 2006), the insecticide discriminating dose is important for determining the susceptibility status in mosquito populations. However, few diagnostic doses are currently available for insecticide susceptible assays on *Ae. aegypti* mosquitoes. In 2007, diagnostic doses of two synthetic pyrethroids (permethrin and deltamethrin) were established for *Ae. aegypti* (Jirakanjanakit *et al*., 2007). In recent years, diagnostic doses of six synthetic pyrethroids, consisting of alpha-cypermethrin, bifenthrin, cypermethrin, deltamethrin, lambda-cyhalothrin and permethrin, were obtained for monitoring the insecticide susceptibility of *Ae. aegypti* (Juntarajumnong *et al*., 2012). Therefore, the aim of this study was to determine the insecticide susceptibility levels to six synthetic pyrethroids diagnostic doses recently available for *Ae. aegypti* populations collected from seven outbreak localities in Thailand.

MATERIALS AND METHODS

Study sites

Aedes aegypti larvae and pupae were collected from containers located in and around houses in seven collection sites within Thailand, namely—Bangkok (Khet Prawet), Phra Nakhon Si Ayutthaya (Ban Phraek district), Uttaradit (Tron district), Mukdahan (Mueang district), Sakon Nakhon (Mueang district), Phatthalung (Mueang district) and Chumphon (Mueang district) as shown in Figure 1. The samples were collected between 2011 and 2012 and the geographical coordinates of the locations are provided in Table 1. Larvae and pupae collected from the study sites were then reared in the Department of Entomology, Faculty of Agriculture, Kasetsart University for morphological identification and colonization. The standard insecticide susceptible laboratory strain of *Ae. aegypti* was obtained from the United States Department of Agriculture (USDA), Gainesville, Florida, USA.

Table 1 Location and global positioning system (GPS) coordinates of *Ae. aegypti* collection sites.

Province	District	GPS coordinates	
Bangkok	Prawet	13°40′9.5″N	100°41′27.6″ E
Phra Nakhon Si Ayutthaya	Ban Phraek	14°38′35.9″ N	100°34′27.3″ E
Uttaradit	Tron	17°29′3.1″ N	100°6′23″ E
Mukdahan	Mueang	16°32′30.25″ N	104°43′1.1″ E
Sakon Nakhon	Mueang	17°15′13.3″ N	104°11′7.9″ E
Phatthalung	Mueang	7°36′52.7″ N	100°4′52.5″ E
Chumphon	Mueang	10°30′34.4″ N	99°6.5′25″ E



Figure 1 *Aedes aegypti* collection sites in various parts of Thailand.

Mosquito rearing

All strains of *Ae. aegypti* were reared and kept separately to ensure no accidental cross-breeding (hybridization) between populations. All developmental stages were reared in a temperature-controlled space at 25 ± 5 °C and 80 ± 10% relative humidity using a 12h:12h light:dark photoperiod according to the method of Kongmee *et al.* (2004). Immature stages were reared in plastic pans with identical physical and nutritional conditions

throughout the study. Pupae were transferred into cups with tap water and placed in screened cages (30 cm × 30 cm × 30 cm). Adult males and females were provided cotton pads soaked with 10% sugar solution. The resultant progeny and adults from the F_1 to F_3 generations were utilized for testing.

Insecticides

Six pyrethroid insecticides were used in this study: 1) alpha-cypermethrin (Sherwood Chemicals Public Company Limited, Bangkok, Thailand, purity 97.05%); 2) deltamethrin (Sherwood Chemicals Public Company Limited, purity 98.46%); 3) permethrin (Sherwood Chemicals Public Company Limited, purity 97.6%); 4) bifenthrin (Sherwood Chemicals Public Company Limited, purity 95.12%); 5) cypermethrin (T.J.C. Chemical Company Limited, Bangkok, Thailand, purity 92%); and 6) lambda-cyhalothrin (Syngenta Company, Bangkok, Thailand, purity 91.8%). Diagnostic concentrations for all test compounds are shown in Table 2 (Juntarajumnong *et al.*, 2012).

Insecticide-treated paper

Test papers (Whatman® No. 1 size 12 cm × 15 cm) were impregnated with diagnostic doses of synthetic pyrethroids, following the WHO standard protocol (World Health Organization, 1998). These synthetic pyrethroids (bifenthrin, permethrin, cypermethrin, alpha-cypermethrin, deltamethrin and lambda-cyhalothrin) were used for determining the susceptibility tests. All treated papers were treated at the rate of 2 mL of insecticide solution per paper. Control papers were impregnated with only carrier diluents (acetone and silicone oil).

Insecticide susceptibility test

Three to five day-old, non-blood fed adult females of *Ae. aegypti* were used for the susceptibility tests. Test procedures were obtained from the WHO, including analysis and interpretation (World Health Organization, 1981a, b). Treated papers were prepared at the Department of Entomology, Faculty of Agriculture, Kasetsart University, according to WHO guidelines (World Health Organization, 1998). Each test was replicated four times. Twenty-five mosquitoes were carefully introduced into each holding tube lined with clean (untreated) paper for 1 hr to observe the health of the mosquitoes before insecticide exposure. Dead and moribund mosquitoes were removed before beginning the insecticide exposure. Mosquitoes from each holding tube were exposed for 1 hr to either insecticide-impregnated or control papers prepared in serial dilutions (determined from base-line findings). Knockdown mosquitoes were recorded after 1 hr. All mosquitoes were then carefully returned to separate clean holding tubes and provided with 10% sugar solution. Mortality was recorded at 24 hr post-exposure.

Data analysis

The mortality of *Ae. aegypti* at 24 hr was averaged for each test series. Interpretation and analysis of resistance/susceptibility status was determined according to WHO criteria (World Health Organization, 1998, 2006). If the percentage of mosquito mortality was between 98 and 100%, it was interpreted as completely susceptible. Mosquitoes were defined as incipiently resistant if the mortality rate was between 80 and 97% and were considered resistant if the mosquito mortality was less than 80%. If mortality of the control was between 5% and 20%, the test results were corrected using Abbott's formula (Abbott, 1925).

RESULTS

Diagnostic concentrations for six synthetic pyrethroids were established in previous work (Table 2). In this study, these "diagnostic doses" were used to determine the insecticide susceptibility level for eight populations of

Table 2 Diagnostic concentration of six synthetic pyrethroids based on dose/mortality relationships tested against *Ae. aegypti*, susceptible strain (US Department of Agriculture) (Juntarajumnong *et al*., 2012).

Insecticide	No. tested	LC_{50} (%)	95% FL	LC_{99} (%)	95% FL	Diagnostic concentration (%)	*P* > Chi square
α-Cypermethrin	302	0.0009	0.0004-0.0013	0.043	0.0220-0.1449	0.0863	0.9419
Bifenthrin	282	0.0185	0.0171-0.0202	0.047	0.0396-0.0599	0.0938	0.3082
Cypermethrin	311	0.0052	0.0031-0.0072	0.111	0.0662-0.2760	0.2212	0.1333
Deltamethrin	297	0.0007	0.0006-0.0007	0.002	0.0020-0.0034	0.0049	0.8955
λ-Cyhalothrin	299	0.0012	0.0010-0.0014	0.006	0.0043-0.0087	0.0116	0.8633
Permethrin	275	0.0379	0.0345-0.0407	0.073	0.0632-0.0922	0.1466	0.9718

LC = Lethal concentration; FL = Fiducial limits at 95% confidence level; LC_{50} = Lethal concentration required to kill 50% of the tested population; LC_{99} = Lethal concentration required to kill 99% of the tested population; Diagnostic concentration/ discriminating dose calculation = 2 × LC_{99}.

Ae. aegypti—one laboratory and seven field populations. The results of susceptibility tests using the single diagnostic dose of 0.147% permethrin, 0.094% bifenthrin, 0.005% deltamethrin, 0.221% cypermethrin, 0.086% alpha-cypermethrin and 0.012% lambda-cyhalothrin for different populations of *Ae. aegypti* are shown in Table 3. The ability of mosquitoes to survive the diagnostic dose after 24 hr is indicative of resistance in the population as defined by the percent mortality in mosquito test populations. The results from the current study indicated that the control population (USDA) was completely susceptible to all test compounds as evidenced by 100% mortality (Table 3). Based on the diagnostic doses established by Juntarajumnong *et al.* (2012), the USDA *Ae. aegypti* control population was found to be completely susceptible to all six synthetic pyrethroids along with 100% knockdown after 60 min. All seven populations of *Ae. aegypti* were highly resistant to deltamethrin (0–37% mortality), followed by bifenthrin (4–39% mortality) and permethrin (2–55% mortality). In addition, high resistance to lambda-cyhalothrin (0–57% mortality) was detected from all localities with the exception of *Ae.aegypti* from Bangkok and Uttaradit which demonstrated incipient resistance (93–96% mortality). Moreover, the two populations from Bangkok and Phra Nakhon Si Ayutthaya were found to have evidence of incipient resistance to alpha-cypermethrin and cypermethrin (91–97% mortality) whereas the populations from Mukdahan and Phattalung were susceptible to both insecticides indicated by 98–100% mortality. The populations from Sakon Nakhon and Chumphon demonstrated incipient resistance to alpha-cypermethrin (90–95% mortality); however, the population from Uttaradit was susceptible. The populations from Sakon Nakhon and Chumphon demonstrated susceptibility to cypermethrin (98–100% mortality) but the population from Uttaradit was incipient resistant.

In general, higher levels of physiological tolerance/resistance to bifenthrin, permethrin and deltamethrin were seen in northeastern populations (Mukdahan and Sakon Nakhon) and one southern population (Chumphon) compared to other geographical regions in Thailand. The strongest resistance to permethrin and lambda-cyhalothrin was seen in the population from Sakon Nakhon (2% mortality for permethrin and 0% mortality for lambda-cyhalothrin) whereas the highest resistance to deltamethrin was observed in the population from Mukdahan (0% mortality), followed closely by Chumphon (2% mortality) and Sakon Nakhon (6% mortality). Interestingly, the population

Table 3 Percent knockdown (KD) following 60 min exposure and mortality rate at 24 hr of *Ae. aegypti* to diagnostic concentrations of six pyrethroid insecticides.

Area	Bifenthrin		Permethrin		Deltamethrin		λ-Cyhalothrin		α-Cypermethrin		Cypermethrin	
	% KD	% Mortality ± SE	% KD	% Mortality ± SE	% KD	% Mortality ± SE	% KD	% Mortality ± SE	% KD	% Mortality ± SE	% KD	% Mortality ± SE
USDA (susceptible strain)	100	100	100	100	100	100	100	100	100	100	100	100
Bangkok (Prawet)	0	6	5	6 ± 1.18	2	14 ± 1.02	16	93 ± 2.51	91	97 ± 2.51	96	97 ± 1.93
Phra Nakhon Si Ayutthaya (Ban Phraek)	0	4	9	23 ± 6.15	2	16 ± 5.01	1	26 ± 7.39	89	91 ± 3.53	89	97 ± 1.99
Uttaradit (Tron)	3	18 ± 6.00	4	10 ± 4.49	7	14 ± 7.75	17	96 ± 2.36	98	99 ± 1.00	82	89 ± 5.59
Mukdahan (Mueang)	1	8 ± 2.83	7	26 ± 2.58	0	0	16	22 ± 8.87	94	98 ± 1.15	97	99 ± 1.00
Sakon Nakhon (Mueang)	0	9 ± 3.15	0	2 ± 1.11	1	6 ± 2.00	0	0	86	90 ± 2.06	94	98 ± 1.85
Phatthalung (Mueang)	20	39 ± 3.32	30	55 ± 9.04	25	37 ± 0.91	24	57 ± 5.68	99	100	100	98 ± 1.85
Chumphon (Mueang)	1	5 ± 1.88	1	4 ± 1.57	1	2 ± 2.00	1	7 ± 3.28	85	95 ± 3.00	92	100 ± 1.00

collected from Chumphon demonstrated very high resistance to four synthetic pyrethroids (5% mortality for bifenthrin, 4% mortality for permethrin, 2% mortality for deltamethrin and 7% mortality for lambda-cyhalothrin) and showed incipient resistance to alpha-cypermethrin (95% mortality) and no resistance to cypermethrin (100% mortality). In contrast, the population from Phatthalung showed higher resistance to bifenthrin (39% mortality) and deltamethrin (37% mortality) than from permethrin (55% mortality) and lambda-cyhalothrin (57% mortality) as shown in Table 3.

In this study, the population from Bangkok, Central Thailand was strongly resistant to bifenthrin (6% mortality), permethrin (6% mortality) and deltamethrin (14% mortality). However, it showed incipient resistance to the other three synthetic pyrethroids (93% for lambda-cyhalothrin, 97% for alpha-cypermethrin and 97% for cypermethrin). In addition, the population from Phra Nakhon Si Ayutthaya in central Thailand showed high resistance to bifenthrin (4% mortality), followed by deltamethrin (16 % mortality) and permethrin (23% mortality). In brief, strong resistance to bifenthrin, permethrin and deltamethrin was observed in all field test populations whereas alpha-cypermethrin and cypermethrin demonstrated incipient or no resistance.

DISCUSSION

Aedes aegypti (L.) is the primary vector of dengue viruses in Southeast Asia, a region which represents the epicenter of disease transmission (Gubler, 1998). This vector is primarily a day-biting mosquito and is more prevalent near and inside dwellings. In addition, *Ae. aegypti* is considered a secondary vector of the chikungunya virus, a disease that has recently emerged in a more frequent epidemic form in Asia, Indian Ocean countries and southern Europe (Charrel *et al.*, 2007). *Ae. aegypti* also prefers to rest indoors in undisturbed places, complicating control of this vector (Reiter and Gubler, 1997). Despite some research progress, an effective and commercially acceptable dengue vaccine is not yet available; thus, the prevention and control of disease transmission relies almost entirely on vector control strategies using synthetic insecticides (Roberts and Andre, 1994; Reiter *et al.*, 1995; Chareonviriyaphap *et al.*, 2004).

Synthetic compounds, including organophosphates, carbamates and pyrethroids have long been used with varying levels of success in national control programs to control dengue vectors (Reiter and Gubler, 1997). Since 1994, the Ministry of Public Health in Thailand has recommended the use of deltamethrin for emergency vector control and adulticide during dengue outbreaks and this latter chemical remains the only compound used in dengue control programs (Chareonviriyaphap *et al.*, 1999; Kongmee *et al.*, 2004). Recent studies have reported that there has been an increased deltamethrin resistance in several field populations of *Ae. aegypti* in Thailand (Jirakanjanakit *et al.*, 2007; Thanispong *et al.*, 2008; Chuaycharoensuk *et al.*, 2011). The increased incidence of resistance is raising awareness of the need for alternative insecticides or newer, more innovative methods of controlling mosquito vectors. Alpha-cypermethrin, another synthetic pyrethroid, is being used in Thai homes for protection against indoor biting mosquitoes and other arthropod pests.

Numerous synthetic pyrethroids, such as permethrin, resmethrin, cypermethrin, cyfluthrin, lambda-cyhalothrin and bifenthrin-based formulations (for example, aerosols, coils and gels), are commercially available to the general public (Paeporn *et al.*, 1996; Chareonviriyaphap *et al.*, 1999; Jirakanchanakit *et al.*, 2007). The selection for resistance to pyrethroids by mosquitoes is largely attributed to frequent exposure to sub-lethal concentrations of commonly applied chemicals and has a direct bearing on the effective management and

prevention of vector-borne diseases (Hemingway and Ranson, 2000). Many studies have reported mosquito resistance to synthetic pyrethroids in Thailand (Chareonviriyaphap *et al.*, 1999; Prapanthadara *et al.*, 2002; Somboon *et al.*, 2003; Paeporn *et al.*, 2004; Ponlawat *et al.*, 2005; Yaicharoen *et al.*, 2005; Sathantriphop *et al.*, 2006; Jirakanjanakit *et al.*, 2007; Thanispong *et al.*, 2008; Chuaycharoensuk *et al.*, 2011). However, the majority of published reports on pyrethroid resistance in *Ae. aegypti* have been restricted in their geographical scope with the susceptibility level of the insecticides needing updating frequently (Chadwick *et al.*, 1977; Chareonviriyaphap *et al.*, 1999; Paeporn *et al.*, 2004; Yaicharoen *et al.*, 2005; Sathantriphop *et al.*, 2006; Jirakanjanakit *et al.*, 2007; Thanispong *et al.*, 2008; Chuaycharoensuk *et al.*, 2011). Without a better understanding of the temporal effects and outcomes of the insecticides used to determine mosquito susceptibility, sustainable and successful vector control activities will never be positive.

As noted, WHO has established only two diagnostic doses for synthetic pyrethroids—permethrin and lambda-cyhalothrin (World Health Organization, 1998, 2006). Therefore, estimation of the resistance status of *Ae. aegypti* to pyrethroid compounds is incomplete. Recently, Juntarajumnong *et al.* (2012) developed diagnostic doses for six synthetic pyrethroids from the standard susceptible strain of *Ae. aegypti* from the USDA. These compounds currently represent the predominant chemical classes utilized for space spray applications and treated materials (for example, window curtains) in Thailand (Juntarajumnong *et al.*, 2012). In the current study, diagnostic doses were used for these compounds to investigate mosquito resistance. This study was consistent with the results from previous studies (Juntarajumnong *et al.*, 2012). It was found that there was a clear development of mosquito resistance to synthetic pyrethroids in the collected areas. In addition, incipient resistant was found to alpha-cypermethrin in some populations of *Ae. aegypti* which were similar to those reported by Thanispong *et al.*, 2008. In the current study, five populations of *Ae. aegypti* demonstrated moderate to high resistance to lambda-cyhalothrin which had not been detected in any previous studies (Chuaycharoensuk *et al.*, 2011).

In summary, *Ae. aegypti* from several localities were resistant to bifenthrin, permethrin, deltamethrin and lambda-cyhalothrin as indicated by the low percentage mortality. Monitoring insecticide resistance should be carried out more frequently and should be increased in geographical coverage to include as many known vectors as possible. Further steps should include the identification of biochemical mechanisms responsible for resistance and should be an integral part of insecticide evaluation programs for effective integrated vector management practices.

CONCLUSION

Aedes aegypti were collected from seven localities in Thailand where there had been a dengue outbreak between 2011 and 2012. Susceptibility or resistance of mosquitoes to the synthetic pyrethroids, bifenthrin, permethrin, deltamethrin, lambda-cyhalothrin, alpha-cypermethrin and cypermethrin were determined using the WHO susceptibility test and the diagnostic doses determined by Juntarajumnong *et al.*, 2012. The results from the susceptibility tests revealed that *Ae. aegypti* from all localities were extremely resistant to bifenthrin, permethrin and deltamethrin. *Ae. aegypti* from most localities were also resistant to lambda-cyhalothrin with the exception of *Ae. aegypti* from Bangkok and Uttaradit which were incipient resistant. *Ae. aegypti* from Bangkok, Phra Nakhon Si Ayutthaya, Sakon Nakhon and Chumphon had incipient resistance to alpha-cypermethrin whereas *Ae. aegypti* collected from Uttaradit, Mukdahan and Phatthalung were still susceptible. Mosquitoes collected from all areas were found to be susceptible to cypermethrin except for Bangkok, Phra Nakhon Si Ayutthaya and Uttaradit which showed incipient

resistance.

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Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing

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The capacity of mosquitoes to resist insecticides threatens the control of diseases such as dengue and malaria. Until alternative control tools are implemented, characterizing resistance mechanisms is crucial for managing resistance in natural populations. Insecticide biodegradation by detoxification enzymes is a common resistance mechanism; however, the genomic changes underlying this mechanism have rarely been identified, precluding individual resistance genotyping. In particular, the role of copy number variations (CNVs) and polymorphisms of detoxification enzymes have never been investigated at the genome level, although they can represent robust markers of metabolic resistance. In this context, we combined target enrichment with high-throughput sequencing for conducting the first comprehensive screening of gene amplifications and polymorphisms associated with insecticide resistance in mosquitoes. More than 760 candidate genes were captured and deep sequenced in several populations of the dengue mosquito *Ae. aegypti* displaying distinct genetic backgrounds and contrasted resistance levels to the insecticide deltamethrin. CNV analysis identified 41 gene amplifications associated with resistance, most affecting cytochrome P450s overtranscribed in resistant populations. Polymorphism analysis detected more than 30,000 variants and strong selection footprints in specific genomic regions. Combining Bayesian and allele frequency filtering approaches identified 55 nonsynonymous variants strongly associated with resistance. Both CNVs and polymorphisms were conserved within regions but differed across continents, confirming that genomic changes underlying metabolic resistance to insecticides are not universal. By identifying novel DNA markers of insecticide resistance, this study opens the way for tracking down metabolic changes developed by mosquitoes to resist insecticides within and among populations.

[Supplemental material is available for this article.]

Mosquitoes are vectors of numerous human diseases, representing a major threat for public health worldwide (Lounibos 2002). Dengue and Chikungunya viruses are both transmitted by the mosquito *Aedes aegypti* and represent a burden in more than 100 countries putting more than 2.5 billion people at risk (WHO 2009, 2014). Since the Second World War, chemical insecticides have been massively used for controlling vector populations and reducing disease transmission, but their efficacy is now threatened by resistance mechanisms developed by mosquitoes. Insecticide resistance is widespread in *Ae. aegypti* and affects most insecticides used for vector control (Ranson et al. 2010). Resistance to pyrethroid insecticides, the primary insecticide family used against adult mosquitoes, is particularly worrying in the context of the re-emergence of dengue and other arboviruses worldwide (Bhatt et al. 2013). Although attempts are made to develop new insecticides or alternative mosquito control strategies (Scholte et al. 2004; Lacey 2007; Hoffmann et al. 2011; Walker et al. 2011; Harris et al. 2012), their large-scale implementation in tropical

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regions will require at least a decade. Until this, characterizing molecular mechanisms underlying resistance is crucial for tracking down resistance alleles and improving resistance management strategies (Corbel et al. 2013).

Resistance to pyrethroids can be the consequence of various mechanisms, such as nonsynonymous mutations affecting the voltage-gated sodium channel targeted by these insecticides, i.e., knockdown resistance (*kdr*) mutations, a lower insecticide penetration, its sequestration, or its biodegradation (metabolic resistance) (Hemingway et al. 2004; Li et al. 2007). *Kdr* mutations and metabolic resistance are known as the two primary resistance mechanisms in mosquitoes. Several *kdr* mutations have been identified in *Ae. aegypti*, and the association between the V1016G/I and the F1534C mutations and pyrethroid resistance has been confirmed (Brengues et al. 2003; Saavedra-Rodriguez et al. 2007; Yanola et al. 2011). Monitoring the frequency of these mutations in field populations is possible through simple DNA-based diagnostic assays and provides key data for resistance management strategies.

Metabolic resistance is far less understood, although this type of resistance is frequent and often accounts for a significant part of the resistance phenotype (Li et al. 2007; Nkya et al. 2013). Such a resistance mechanism is caused by an increased activity of detoxification enzymes. These detoxification enzymes include cytochrome P450 monooxygenases (P450s or *CYPs* for genes), carboxy/cholinesterases (CCEs), glutathione S-transferases (GSTs), and UDP-glycosyl-transferases (UDPGTs), although other families can be involved (Hemingway et al. 2004; David et al. 2013). Their high diversity (roughly 300 genes in *Ae. aegypti*) and the complexity of insecticide biodegradation pathways make the identification of those conferring resistance challenging. Theoretically, metabolic resistance can be the consequence of an increased expression of one or multiple detoxification enzymes capable of metabolizing the insecticide and/or the selection of variants showing a higher insecticide metabolism rate due to conformational modifications. Because overexpression is frequently associated with overtranscription, most candidate genes were identified based on their differential transcription in resistant populations compared to susceptible counterparts using transcriptomics (for review, see David et al. 2013). Although subjected to inherent difficulties associated with gene expression studies (e.g., RNA handling and degradation, uncontrolled variation across time, tissues, and populations), these approaches identified several detoxification enzymes overtranscribed in resistant populations, some of them being later validated as pyrethroid metabolizers (for review, see Vontas et al. 2012; David et al. 2013). Although these approaches proved their value for identifying metabolic resistance genes, they failed to pinpoint the genomic changes associated to their overexpression. In addition, because a limited quantity of mRNA can be extracted from a single mosquito, these studies were conducted at the population level without estimating the frequency of resistant alleles within populations. A constitutive overtranscription can be the consequence of an up-regulation controlled by *cis*/*trans* genomic regulatory elements or an increased gene copy number (Li et al. 2007). Although gene amplifications have been associated with the overtranscription of a few pyrethroid resistance genes in mosquitoes (Wondji et al. 2009; Itokawa et al. 2010, 2011; Bariami et al. 2012), no comprehensive screening of gene amplifications linked to insecticide resistance has ever been conducted. Considering the central role of copy number variation (CNV) in adaptations associated with a "gene dosage" effect (for review, see Kondrashov 2012) and the richness of the *Ae. aegypti* genome in transposable elements known to favor duplication events (Nene et al. 2007), conducting a comprehensive screening of gene amplifications associated with insecticide resistance may allow identifying reliable DNA markers of metabolic resistance. Besides, most genes linked to metabolic resistance were identified based on their differential expression while the importance of nonsynonymous changes affecting detoxification enzymes have clearly been neglected by molecular screenings. As for gene amplifications, these polymorphisms are of high interest for monitoring the dynamics of insecticide resistance mechanisms among and within mosquito populations.

In this context, the present study aimed at combining the target enrichment technology with high-throughput sequencing for identifying gene amplifications and polymorphisms linked to pyrethroid resistance in the dengue vector, *Ae. aegypti*. More than 760 genes, including those encoding for detoxification enzymes, cuticle proteins, ATP-binding cassette (ABC) transporters, neurotransmitter receptors, and voltage-gated channels, were captured and deep sequenced in several susceptible and pyrethroid-resistant populations from three geographical regions (Asia, South America, and laboratory populations). After phenotyping populations for resistance to the pyrethroid deltamethrin and segregating most resistant individuals, gene amplifications and polymorphisms associated with deltamethrin resistance were identified by deep targeted sequencing of large pools of individuals. Gene amplifications were validated on individual mosquitoes, and their impact on transcription levels were validated. Polymorphism data were first used to identify genomic regions under selection through a hierarchical Bayesian approach taking into account population structure. Then, nonsynonymous variants strongly linked to deltamethrin resistance were identified by combining those identified by the Bayesian approach and those displaying allele frequencies mostly associated with resistance. Quantitative PCR assays were used for cross validating polymorphism data and studying the association between gene amplifications and known target-site mutations. Results are discussed in regard to known insecticide resistance mechanisms and the identification of novel DNA markers of insecticide resistance.

Results

Deltamethrin resistance levels

Bioassays on adult females revealed a broad range of resistance to deltamethrin across populations (Table 1). Exposing mosquitoes for 1 h to various doses of insecticide showed that the lethal dose killing 50% of individuals (LD_{50}) varied up to 750-fold between susceptible populations and the most resistant populations from French Guiana. Resistant populations from Thailand showed an intermediate resistance level (about 250-fold), and the laboratory selected population showed a slight resistance (approximately fivefold). These different resistance levels were confirmed by WHO diagnostic assays, in which the time necessary to knock down 50% of individuals (KDT_{50}) varied from 11 min for susceptible populations to >8 h for the most resistant ones.

Sequencing metrics and coverage

The use of target enrichment technology followed by multiplexed Illumina sequencing allowed obtaining an average of 4.35 million 75-bp reads per sample (Supplemental Table 1). Filtering them according to read pairing, sequence quality, and mapping quality

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Table 1. Deltamethrin resistance levels

Population	Group	Resistance level	KDT^a_{50}	KDT_{50}95% CI	LD^b_{50}	R+/R− segregation	
						LT_{25} (min)[c]	LT_{75} (min)[c]
Livp S	Genome	Susceptible	11.5	10.8–12.1	0.001	—	—
Bora S	Laboratory	Susceptible	12.1	11.6–12.7	0.001	—	—
Delta R	Laboratory	Slightly resistant	14.2	13.5–15.0	0.005	30	80
Patg S	Thailand	Susceptible	17.0	15.1–19.2	0.001	—	—
Phet R	Thailand	Resistant	98.2	91.4–106.7	0.25	40	120
Nakh R	Thailand	Resistant	92.9	87.7–99.2	0.25	40	120
NwOr S	S. America	Susceptible	11.9	11.2–12.6	0.001	—	—
Cayn R	S. America	Highly resistant	>500	—	0.75	30	180
StGe R	S. America	Highly resistant	>500	—	0.75	30	180

[a]KDT_{50}: time (min) necessary to knock down 50% of individuals. KDT_{50} was determined using papers impregnated with 0.05 g/100 mL deltamethrin following WHO insecticide testing recommendations.
[b]Dose of insecticide (g/100 mL) used for paper impregnation leading to 50% mortality after 1 h exposure.
[c]R−/R+ segragation of resistant populations consisted in separating individuals dead at LT_{25} from those surviving at LT_{75} using impregnated paper corresponding to the LD_{50}.

allowed successful mapping of 68% of reads on the *Ae. aegypti* genome. The majority of mapped reads consisted in perfect matches (46.6%), whereas reads matching with one and two or more mismatches represented 12.6% and 9.2% of sequenced reads, respectively. The mean coverage was homogenous across samples with a total mean coverage of 83× (Supplemental Fig. 1).

Gene amplifications linked to deltamethrin resistance

Of the 763 targeted candidate genes, 727 showed a mean coverage greater than 90 reads per kilobase per million reads (RPKM) and were considered for further analysis. Among them, 41 genes showed an increased coverage >1.5-fold in any resistant population compared to the mean coverage of all susceptible ones, together with an increased coverage >1.0-fold in LT_{75} survivors (R+ phenotype) compared to individuals dead at LT_{25} (R− phenotype). These genes were considered amplified in association with deltamethrin resistance (Supplemental Table 2). These genes mostly encoded detoxification enzymes with a significant enrichment in P450s (Fig. 1). Most gene amplifications occurred in gene clusters located within a few genomic supercontigs spread across all chromosomes (Fig. 2). Gene amplification profiles were highly conserved within each region but showed marked differences between continents. Thai resistant populations (Nakh R and Phet R) showed a strong amplification of four *CCEs* located in supercontig 1.142 and another one in supercontig 1.1678. Two *CYP6s* located in supercontig 1.702 and two sulfotransferases in supercontig 1.975 were also amplified in Thai resistant populations. In contrast, resistant populations from South America (Cayn R and StGe R) showed a marked amplification of four *CYP6s* located in supercontig 1.1327. Resistant populations from both regions also shared the amplifications of several *CYP9Js* in supercontigs 1.1188 and 1.221. As expected, the less resistant laboratory population (Delta R) exhibited fewer gene amplifications associated with resistance. The amplification profile of the Delta R population shared particular gene amplifications with field resistant populations. This included a chloride channel also detected in Thailand together with two *CYP4Js* and a glycosyltransferase also detected in South America.

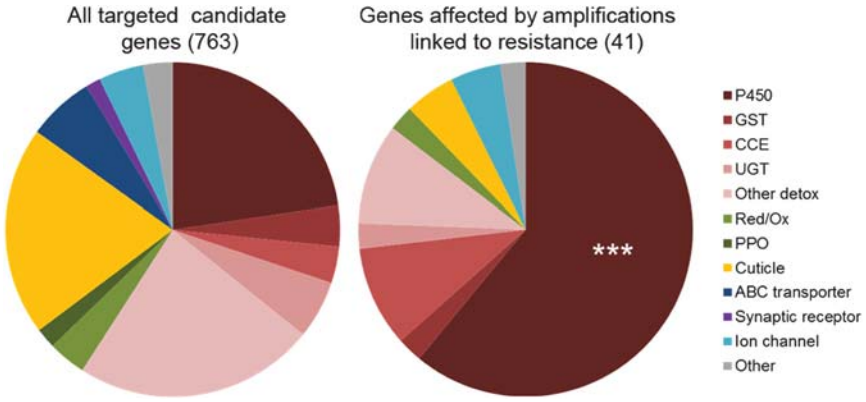


Figure 1. Gene families affected by gene amplifications associated with deltamethrin resistance. (*Left*) Frequency of each gene family among the 763 captured genes. (*Right*) Frequency of each gene family among the 41 genes affected by genic amplifications associated with deltamethrin resistance. Proportions of each gene family between all captured genes and genes affected by genic amplifications were compared using a one-sided Fisher's exact test: (***) $P < 0.001$.

Validation of gene amplifications on individual mosquitoes

Nine gene amplifications identified by deep sequencing belonging to two P450 clusters and one CCE cluster heavily impacted by gene amplifications were cross-validated by qPCR on individual mosquitoes. The presence of gene amplifications was confirmed for all targeted genes. A good correlation was observed between CNVs obtained by deep sequencing and qPCR (Supplemental Fig. 2). Individual qPCR data reveal high copy number polymorphism within resistant populations (Supplemental Fig. 3). Estimating the frequency of gene amplifications within each population revealed that amplifications are frequent in resistant populations and sometimes also present at low frequencies in susceptible populations (Supplemental Fig. 4). Comparing the amplification profile of each gene across all individuals revealed *CYP6* and *CCE* clusters are likely affected by single amplification events (Supplemental Fig. 5). In contrast, different copy numbers were frequently observed for genes belonging to the *CYP9J*

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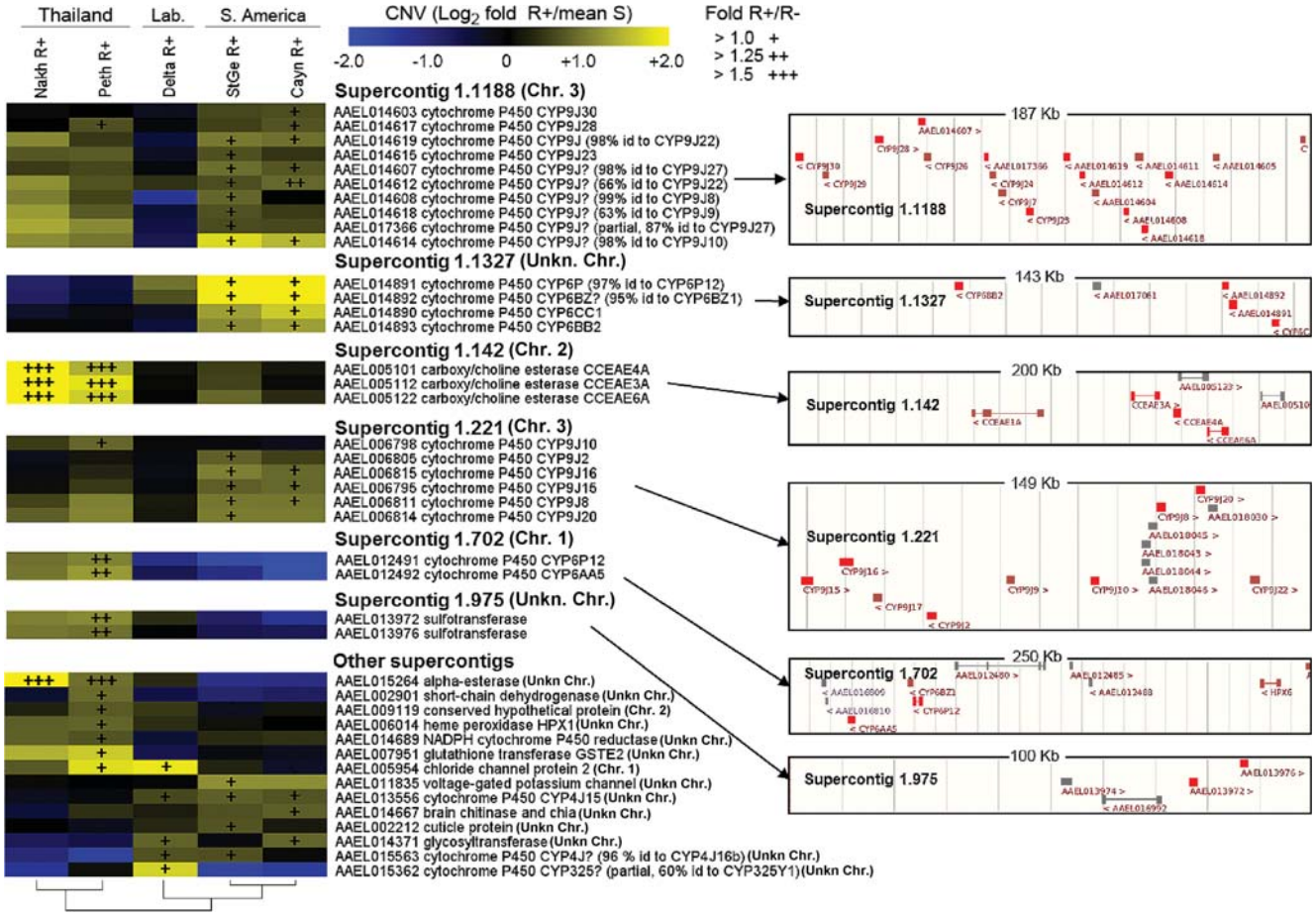


Figure 2. Gene amplifications associated with deltamethrin resistance. (*Left*) CNV profiles of genes affected by gene amplifications associated with deltamethrin resistance. Color scale shows (R+)/(meanS) CNV for each resistant population, and overimposed "+" marks show (R+)/(R−) CNV. (*Right*) Location of gene amplifications on genomic supercontigs. Amplified genes are shown in red. Nonamplified genes are shown in maroon. Genes not included in the capture design are shown in gray. Chromosomal locations are shown as described in Timoshevskiy et al. (2014) and Juneja et al. (2014).

cluster on supercontig 1.1188, suggesting that distinct duplication events affect this large P450 cluster.

Impact of gene amplifications on transcription levels

Comparing gene amplification levels obtained by DNA-sequencing with transcription levels obtained by RT-qPCR for the nine candidate genes across all resistant populations confirmed that gene amplifications are often associated with increased transcription (Supplemental Fig. 6). High and significant positive correlations were observed for most genes except for two *CYP9Js* in supercontig 1.1118, where positive correlations were not significant.

Association of gene amplifications with target-site mutations

Among the nine gene amplifications genotyped by qPCR, only one was found associated with *kdr* mutations (Supplemental Table 3). This association occurred in the Nakh population from Thailand, where a positive correlation between the presence of *CYP9J28* amplification and the presence of the V1016G mutation was found ($r = 0.61$; $P = 0.025$). As a consequence of the total segregation of V1016G and F1534C mutations in Thailand, the F1534C mutation was negatively correlated to *CYP9J28* amplification in this population ($r = -058$; $P = 0.036$).

Polymorphisms identified by deep targeted sequencing

A total of 41,469 polymorphisms supported by an important coverage were called against the reference genome. Among them, 30,400 were polymorphic among populations. A principal component analysis (PCA) based on allele frequency variation across all populations confirmed the overall population structure and the different genetic background of Thai, South-American, and laboratory (French Polynesia) populations (Supplemental Fig. 7). The reliability of polymorphism data was confirmed by comparing the frequencies of V1016I, V1016G, and F1534C *kdr* mutations estimated by deep targeted sequencing and those obtained by qPCR on individual mosquitoes (Supplemental Fig. 8). The presence of distinct *kdr* mutations at position 1016 in Thailand and South America was confirmed, and the F1534C mutation was also detected in most populations.

Polymorphisms associated with deltamethrin resistance

Searching for selection footprints using a Bayesian approach taking into account population structure (Foll et al. 2014) identified 156 outlier loci significantly associated with deltamethrin resistance. These outlier loci included 31 nonsynonymous variants and affected multiple supercontigs spread over all chromosomes.

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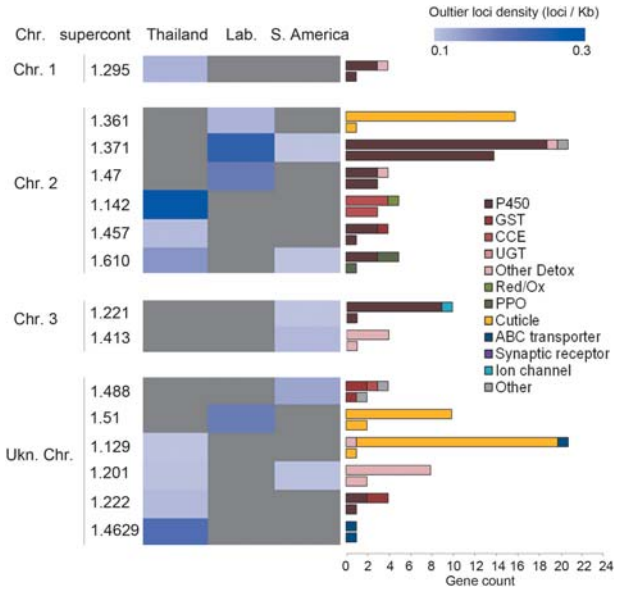


Figure 3. Supercontigs showing selection footprint. For each region, loci displaying a significant BayeScan3 F_{sc} *Q*-value <0.05 and both f(R+)-f (S) and f(R+)-f(R−) allele frequency variation occurring in the same direction were considered as outliers. For each supercontig, outlier loci densities were obtained by dividing the number of outlier loci by the total length of all captured genes (outlier loci per kb of captured region). Outlier loci densities are shown as a blue color scale. Gray stands for an absence of outlier loci. For each supercontig, horizontal bars show the number of captured genes from each gene family (*upper* bar) compared to those affected by outlier loci (*lower* bar). Supercontigs are clustered according to their putative chromosomal location as described in Timoshevskiy et al. (2014) and Juneja et al. (2014).

Selection footprints varied between geographical regions with outlier loci densities ranging from 0 to 4.1 per kb of captured sequence (Fig. 3). In Thailand, supercontigs 1.142 and 1.4629 showed high outlier loci densities with three CCE and one ABC transporter being affected. Six other supercontigs also displayed selection footprints impacting two P450s, two other detox enzymes, one PPO, and one cuticle protein. High outlier loci densities were identified in laboratory populations in supercontigs 1.371, 1.47, and 1.51 affecting 17 P450s and two cuticle proteins. Selection footprints were less pronounced in South America but showed overlap with laboratory and Thai populations.

Of the 30,400 polymorphic loci, 3054 loci showed an allele frequency variation between R+ phenotypes and their respective susceptible population ≥40% together with an allele frequency variation between R+ and R− phenotypes ≥5%. These loci included 465 nonsynonymous variants (Supplemental Table 4). Comparing the Bayesian and the frequency filtering approaches revealed that the Bayesian approach was overall more stringent (Supplemental Fig. 9). However, the stringency of the Bayesian approach was high in South America, medium in Thailand, and low in laboratory populations following the decreasing diversity of these groups. The frequency filtering approach showed an opposite stringency with more variants identified in South America than Thailand and even less in laboratory populations. Frequency filtering identified 16 nonsynonymous variants shared by distinct geographic regions, whereas the Bayesian approach only identified variants being specific to each region.

Combining the best candidates identified by each approach identified 55 nonsynonymous variants strongly associated with deltamethrin resistance (Fig. 4; Supplemental Table 4). These variants affected 39 distinct genes mainly belonging to P450s, UGTs, CCEs, and cuticle proteins. As for gene amplifications, allele frequency patterns were conserved within regions but often differed among continents. Most nonsynonymous variants affecting P450s were identified from South American or laboratory populations. These P450s mostly belong to CYP6, CYP9, and CYP12 families. Conversely, most variants affecting CCEs were identified from Thai populations. Two were both affected by gene amplifications and nonsynonymous variants with *CCEAE4A* bearing height distinct nonsynonymous variants associated with resistance. Several UGTs were also affected by nonsynonymous variants, with three showing an increased frequency across multiple regions. ABC transporters and cuticle proteins were most frequently affected in South America. Several other enzymes potentially involved in insecticide detoxification were also affected by nonsynonymous variants, including two aldehyde oxidases, one alcohol dehydrogenase, and one sulfotransferase. Finally, the nicotinic acetylcholine receptor (nAchR) AAEL004935 targeted by neonicotinoid insecticides and the voltage-gated sodium channel (VSGC) AAEL006019 targeted by pyrethroid insecticides were also affected. Variants detected in the VGSC corresponded to the S989P and F1534C *kdr* mutations previously described in this species. None of the V1016I/G *kdr* mutations previously associated with pyrethroid resistance was retained as best candidates. Neither of them was retained by the Bayesian approach, and the V1016G mutation only passed the allele frequency filtering in Thailand (Supplemental Table 4).

Discussion

Deep targeted sequencing for studying insecticide resistance

Screening for genomic changes associated with insecticide resistance remained challenging until the sequencing of mosquito genomes (Holt et al. 2002; Nene et al. 2007; Arensburger et al. 2010). Afterward, the development of mosquito DNA microarrays allowed screening for resistance genes based on their differential transcription, leading to the identification of detoxification genes conferring resistance (David et al. 2005, 2013; Strode et al. 2008; Vontas et al. 2010; Edi et al. 2014). However, these screenings focused on differential transcription while genomic changes such as CNVs and polymorphisms were neglected. Then, the development of high-throughput DNA sequencing allowed screening for genomic variants across whole genomes. However, such an approach required a huge amount of sequences to reach the minimum genome coverage necessary for accurately quantifying CNV and allele frequencies, making it unaffordable when several samples have to be compared. Although RNA-seq is more affordable and can generate gene expression and variant data concomitantly, this approach is not suitable for identifying CNVs and does not provide reliable polymorphism data from poorly expressed genes. In addition, allele frequencies inferred from RNA-seq are to be taken with caution because they can be altered by allele-specific expression events (Gaur et al. 2013).

Recently, the development of target enrichment techniques allowed conducting DNA-seq focused on genomic regions of interest, hence, drastically increasing the sequencing depth while maintaining costs at a reasonable level (Altmüller et al. 2014). By targeting more than 760 genes from all protein families potentially involved in insecticide resistance in the dengue mosquito, our

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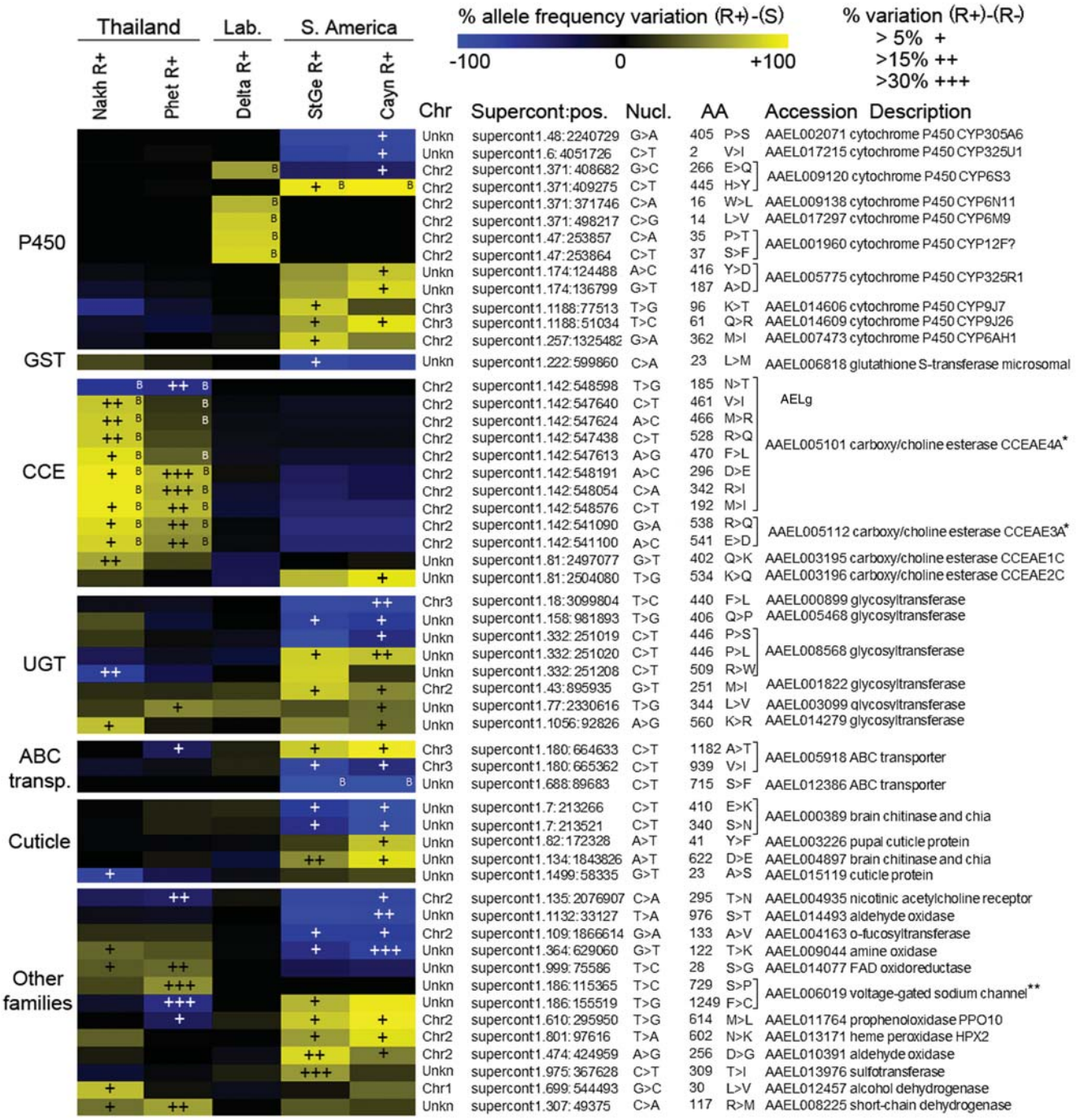


Figure 4. Best nonsynonymous polymorphisms associated with deltamethrin resistance. Only the 55 best differential nonsynonymous variants identified from frequency-based filtering and the Bayesian approach are shown (see Methods). For each region, allele frequency variation between each resistant population (R+ phenotypes) and their susceptible counterpart (S) are shown as a blue-yellow color scale. Blue indicates an enrichment in the reference allele, whereas yellow indicates an enrichment in the variant allele. Variants identified by the Bayesian approach are indicated by "B" marks. For variants passing frequency-based filtering or BayeScan3 filtering, allele frequency variation between R+ and R− phenotypes are shown as overimposed "+" marks. Variants are grouped by gene families and are described by the following annotations: chromosomal location (according to Juneja et al. 2014; Timoshevskiy et al. 2014), supercontig position, nucleotide change, amino acid position, amino acid change, gene accession number, and gene description. (*) Genes also found affected by CNVs linked to deltamethrin resistance. (**) The sodium channel S729P and F1249C variants correspond to the S989P and F1534C *kdr* mutations described in the literature due to changes in AAEL006019-RD transcript annotation.

study screened for both CNVs and polymorphisms across several samples. Our experimental design allowed reaching a very deep coverage (>80×) across multiple resistant and susceptible populations originating from different geographical areas, providing the first comprehensive screening of CNVs and polymorphisms associated with insecticide resistance. Targeted DNA-seq data were

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consistent with qPCR data obtained from individual mosquitoes validating the robustness of the approach. Altogether, this study demonstrates that target enrichment coupled with high-throughput sequencing is a powerful tool for pinpointing genomic changes associated with adaptive traits from which candidate gene families are known.

Gene amplifications associated with pyrethroid resistance

Only a few CNVs were previously associated with insecticide resistance, and no comprehensive screening has ever been conducted in mosquitoes (for review, see Bass and Field 2011). The present study identified 41 genes affected by gene amplifications linked to deltamethrin resistance. Most were previously found significantly overtranscribed in pyrethroid resistant *Ae. aegypti* populations according to data publicly available in the VectorBase expression browser (https://vectorbase.org, data sets from Strode et al. 2008; Marcombe et al. 2009; Poupardin et al. 2012; David et al. 2014). The majority of CNVs observed between resistant and susceptible populations were <2.5-fold, suggesting a predominance of duplications. However several genes displayed CNV >fivefold in particular resistant populations, suggesting that copy number may vary within continents. This was confirmed by qPCR on individual mosquitoes showing an important copy number polymorphism within and across resistant populations. Such polymorphism suggests that most amplifications are not fixed in resistant populations. The strong relationship between gene amplification and overtranscription was confirmed by RT-qPCR. Although, overexpression can also be triggered by *cis*/*trans* regulatory elements or post-translational events, the present study suggests that gene amplification is a common adaptive mechanism allowing mosquitoes to overexpress detoxification genes conferring resistance to insecticides. The importance of such mechanism may even be greater in species highly infected by transposable elements like *Ae. aegypti* because their presence is known to favor duplication events (Nene et al. 2007).

The present study revealed distinct gene amplification patterns among resistant populations according to their geographical origin. This supports that the selection of gene amplifications conferring resistance depends on the genetic background of populations, their population dynamics, and on the selection pressures they undergo. Most amplified genes encoded detoxification enzymes, suggesting that gene amplification is rather linked to metabolic resistance than resistance conferred by an altered transport/penetration of the insecticide.

The majority of detoxification enzymes affected by gene amplifications encoded P450s belonging to *CYP9J* and *CYP6* families that were frequently associated with insecticide resistance in mosquitoes (Vontas et al. 2012; David et al. 2013). The overrepresentation of P450s was expected because their overexpression is known to play a key role in pyrethroid resistance, and they are frequently affected by copy number variants (Feyereisen 2006, 2011). Several amplified *CYP9Js* were previously found overtranscribed in resistant populations and some of them (*CYP9J24*, *CYP9J28*, *CYP9J32*) have been validated as able to metabolize pyrethroids (Stevenson et al. 2012), supporting the importance of their amplification in resistance. Among the *CYP6s* amplified, most were previously found overtranscribed in resistant populations, and *CYP6BB2* was recently involved in pyrethroid metabolism (Kasai et al. 2014). In addition, some of their close orthologs in *Anopheles* (*CYP6P3*, *CYP6P7*, *CYP6P9*, *CYP6M2*, and *CYP6AA5*) are also capable of degrading pyrethroids (Boonsuepsakul et al. 2008; Müller et al. 2008; Duangkaew et al. 2011; Stevenson et al. 2011; Riveron et al. 2013). Altogether, these results confirm the key role of P450 in the resistance of mosquitoes to pyrethroids and their propensity to increase their expression level through gene amplification when undergoing a strong selection pressure from insecticides.

Three CCEs (*CCEAE3A*, *CCEAE4A*, and *CCEAE6A*) belonging to a single genomic cluster were highly amplified in Thai resistant populations leading to their strong overtranscription. Highest amplification levels were found in the Nakh R population which is also resistant to organophosphates (Poupardin et al. 2014). In this previous study, the overtranscription of *CCEAE3A* and *CCEAE6A* through gene amplification was associated with temephos resistance at the larval stage, although resistance to pyrethroids was also observed. Other studies performed on South American and Caribbean populations confirmed the association of some of these CCEs with temephos resistance (Marcombe et al. 2009, 2012; Saavedra-Rodriguez et al. 2014). Our data revealed higher CCE copy number in mosquitoes surviving deltamethrin exposure (R+ phenotype), supporting their role in pyrethroid resistance. Although the association between pyrethroid resistance and these CCE genes in Thailand might be due to multiresistant individuals carrying both pyrethroid- and organophosphate-resistance genes, the role of CCEs in pyrethroid metabolism has been demonstrated in mammals (Hodgson 2003; Nakamura et al. 2007) and mosquitoes (Somwang et al. 2011; Chandor-Proust et al. 2013). However, no individual mosquito CCE has yet been validated as a pyrethroid metabolizer, and insecticide sequestration may also confer resistance. Further functional studies are required for validating the precise role of these amplified CCEs in pyrethroid resistance.

Two sulfotransferases were amplified in Thai resistant populations. The overtranscription of these enzymes has been frequently described in insecticide resistant mosquitoes and deserve further attention (Poupardin et al. 2012; Marcombe et al. 2013; Nkya et al. 2013). Indeed, their involvement in insecticide degradation pathways has been validated in mammals (Lee et al. 2007).

Among GSTs, only *GSTE2* was found amplified in Thai resistant populations. This GST is known for its ability to metabolize DDT in various mosquito species (Ortelli et al. 2003; Lumjuan et al. 2005), and recent studies confirmed its role in pyrethroid resistance although pyrethroid metabolism has not been observed (Lumjuan et al. 2011; Riveron et al. 2014b).

A recent study suggested that the duplication of the gene encoding the *Ae. aegypti* voltage-gated sodium channel (VGSC) targeted by pyrethroids contributed to resistance by maintaining together wild-type allele and *kdr* mutations and reducing mutant allele deleterious effects (Martins et al. 2013). A similar role was suggested for the duplication of the acetylcholinesterase gene (carrying the *Ace1* mutation) in the resistance of *An. gambiae* to carbamates (Edi et al. 2014). Although such a mechanism is likely contributing to resistance in mosquitoes, no amplification of genes encoding proteins targeted by insecticides were detected in our study. In addition, a poor association was observed between the presence of *kdr* mutations and gene amplifications affecting detoxification enzymes in individual mosquitoes. Indeed, recombination events are probably countering such association, especially as the *VGSC* gene and amplified detoxification genes are located on distant genomic regions. In addition, fitness costs associated with both *kdr* mutations and gene amplifications have been identified (Kondrashov 2012; Brito et al. 2013; Katju and Bergthorsson 2013; Rinkevich et al. 2013).Therefore, although *kdr* mutations and gene amplifications of detoxification enzymes

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co-occur in resistant populations, additive fitness costs may contribute to segregate them at the individual level.

Polymorphisms associated with pyrethroid resistance

Apart from mutations affecting the proteins targeted by insecticides, very few nonsynonymous variants have been associated with insecticide resistance. In this context, the present study represents the first DNA-seq screening of variants associated with pyrethroid resistance in mosquitoes. Searching for selection footprints identified multiple genomic regions under selection, suggesting that pyrethroid resistance is polygenic. Several nonsynonymous variants were associated with deltamethrin resistance, but few were found in multiple geographic regions, confirming the strong influence of genetic background and population history on the selection of resistant alleles.

Although several nonsynonymous variants affecting P450s were identified from each region, those mostly associated with resistance were mainly detected in South American and laboratory populations. Some were previously identified in a permethrin-resistant strain by RNA-seq (David et al. 2014). In *Drosophila melanogaster*, functional studies demonstrated that nonsynonymous mutations in the P450 *CYP6A2* identified in the RDDT^R resistant strain have a prominent role in resistance by enhancing DDT metabolism (Amichot et al. 2004). In the mosquito, *Anopheles funestus*, directional selection footprints were found in the coding sequence of two *CYP6Ps* capable of metabolizing pyrethroids (Riveron et al. 2014a). Most nonsynonymous variants impacting CCEs were identified in Thailand with *CCEAE4A* and *CCEAE3A* impacted by multiple variants strongly associated with deltamethrin resistance. Elevated copy numbers of these genes were also identified in Thailand, suggesting that their amplification may have been followed by their neofunctionalization under insecticide selection pressure (Katju and Bergthorsson 2013). Interestingly, the best orthologous gene of *CCEAE4A* in the sheep blowfly, *Lucilia cuprina* (LcαE7), encodes an esterase associated with organophosphate resistance through a single point mutation enhancing its activity toward these chemicals (Newcomb et al. 1997). Later, it was shown that another mutation of this gene allowed this enzyme to metabolize synthetic pyrethroids (Heidari et al. 2005; Devonshire et al. 2007). More recently, multiple randomly generated nonsynonymous mutations of this gene were shown to enhance its activity toward various pyrethroids including deltamethrin (Coppin et al. 2012). Although additional work is necessary for validating the role of these nonsynonymous variants, our data support the selection of detoxification enzyme variants as being a key process in the adaptation of mosquitoes to chemical insecticides.

Finally, multiple nonsynonymous variants were detected in the gene encoding the voltage-gated sodium channel targeted by pyrethroids (*kdr* mutations). Their frequencies were consistent with those previously reported in Thailand and South America (Stenhouse et al. 2013; Linss et al. 2014). However, neither of the two mutations extensively used as resistance markers (V1016I/G) were retained as best candidates. Although the V1016I mutation showed high frequencies in South American resistant populations, it showed a poor association with deltamethrin survival phenotype. The V1016G mutation was associated with resistance in one Thai population but was not retained among the best candidates according to our criteria. Instead, two other less-known mutations (S989P and F1534C) showed a stronger association with deltamethrin resistance. Such a result supports the substantial contribution of other mechanisms in the resistance phenotype.

New tools for monitoring resistance genes in mosquito populations

Overall, the present study confirms that gene amplifications affecting detoxification enzymes are good markers of metabolic resistance in mosquitoes. Novel nonsynonymous variants potentially enhancing their ability to detoxify insecticides were also identified. Despite significant research efforts, developing molecular tests to track the overexpression of these enzymes in field populations has proved challenging in terms of affordability, throughput, and reliability; and current vector population monitoring tools do not include metabolic resistance markers (Bass et al. 2010). Detecting their increased copy number or the presence of specific variants conferring resistance through simple PCR-based DNA assays opens up new perspectives for managing insecticide resistance. Indeed, such novel resistance markers will be accessible to developing countries in terms of technology and affordability and will allow tracking down both target-site and metabolic-resistance alleles in individual mosquitoes and monitoring their frequencies through time and space among and within natural populations.

Methods

Mosquitoes

Nine *Ae. aegypti* populations of distinct genetic backgrounds were used. These included four populations susceptible to insecticides and five populations showing elevated resistance to deltamethrin. Two distant geographic areas where pyrethroid resistance is threatening mosquito control were studied: South East Asia (Thailand) and South America (French Guiana). Two resistant and one susceptible population having a close genetic background were studied in each area (see Table 2 for populations' information). In addition, one laboratory population selected with deltamethrin for five generations was used together in comparison to its susceptible "parental" population. The laboratory Liverpool population used for public genome sequencing (Liverpool population) (Nene et al. 2007) was used as an additional susceptible population. Resistant populations from Thailand and French Guiana were colonized for two generations in the laboratory without insecticide selection pressure before assessing resistance levels and sample collection.

Deltamethrin resistance levels

For each population, the exposure time necessary to knock down 50% of individuals (KDT_{50}) was determined on 2- to 4-d-old females using test tubes equipped with Whatman filter paper impregnated with 0.05% deltamethrin in silicone oil according to standard WHO procedure (WHO 2006). Then, a collection of Whatman filter papers impregnated with different concentrations of deltamethrin (from 0.005% to 0.75%) was used for evaluating the lethal dose for 50% of individuals (LD_{50}) of each population. Bioassays were conducted in duplicates of 40 2- to 4-d-old females, and mortality was recorded after 1 h exposure to deltamethrin and 24 h recovery without insecticide. For each resistant population, the impregnated paper killing 40%–60% mosquitoes after 1 h exposure was then used to segregate the most susceptible individuals (dead at LT_{25}: R– phenotype) from the most resistant ones

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Table 2. Sample descriptions

Population	Group	Full name	Origin	History (year colonized)	Deltamethrin resistance
Livp S	Genome	Liverpool (genome)	West Africa	Laboratory colony (1930s)	Susceptible
Bora S	Laboratory	Bora-Bora	French Polynesia	Laboratory colony (1990s)	Susceptible
Delta R	Laboratory	Deltamethrin R	French Polynesia	Bora S selected	Slightly resistant
Patg S	Thailand	Patthalung	South Thailand	Laboratory colony (2013)	Susceptible
Phet R	Thailand	Phetchaburi	SW of Bangkok	Field collected	Resistant
Nakh R	Thailand	Nakhon Sawan	Center Thailand	Field collected	Resistant
NwOr S	S. America	New Orleans	Louisiana	Laboratory colony (1980s)	Susceptible
Cayn R	S. America	Cayenne	French Guiana	Field collected	Highly resistant
StGe R	S. America	St-Georges de l'Oyapock	French Guiana	Field collected	Highly resistant

(survivors at LT_{75}: R+ phenotype) by adjusting the exposure time from 10 min to 3 h.

Sample preparation

All sampled mosquitoes consisted of 2- to 4-d-old non-blood-fed *Ae. aegypti* females grown in standardized laboratory conditions. For each population/phenotype, 200 individuals were collected and stored individually in silica gel. For each population, three pools of 30 females not exposed to insecticide were also collected and stored in RNAlater (Life Technologies) for gene expression analyses. Gene amplification screening by exon capture and mass sequencing was performed on gDNA extracted from two pools of 65 individuals per population/phenotype. Genomic DNA was extracted using the PureGene Core Kit A (Qiagen) following the manufacturer's instructions. For each population/phenotype, gDNA from each pool were then combined in equal quantity in order to be representative of a total of 130 individuals. Quantitative PCR validation of gene amplifications and genotyping of *kdr* mutations was performed on gDNA extracted from 12 single adult females per population/phenotype using the method described in Collins et al. (1987) and resuspended in 20 µL nuclease-free water. Gene expression analyses were performed on RNA extracted from three pools of 30 adult females per population (R and S populations) using the RNAqueous4PCR kit (Life Technologies) according to the manufacturer's instructions and resuspended in 50 µL nuclease-free water.

Capture of target regions and sequencing

Gene capture was performed by Hybrigenics-Helixio (Clermont-Ferrand, France) using the SureSelect target enrichment system (Agilent). Capture library consisted in 51,073 overlapping RNA probes of 120 bp (baits) targeting 3458 exons belonging to 789 genes. The mean coverage of target regions was 4×. These genes were chosen according to their putative role in insecticide resistance. These genes include all detoxification enzymes sensu lato, all cuticle proteins, all ABC transporters together with several ion channels, redox enzymes, and synaptic proteins (list of captured genes in Supplemental Table 5). Capture of target genes was performed according to "SureSelect Target Enrichment System for Illumina Paired-end Sequencing Library version 1.5" protocol. Briefly, 3 µg gDNA were fragmented using a Bioruptor (Diagenode), ligated to adaptors, and amplified by PCR using Herculase II DNA polymerase (Agilent). After QC analysis, libraries were hybridized to biotinylated baits and purified using Dynabeads MyOne Streptavidin T1 beads (Life Technologies). Captured DNA fragments were amplified, purified, and multiplexed before sequencing. Paired-end sequencing was performed on a Genome Analyzer IIx (Illumina) producing 76 bp reads. An average sequencing coverage of greater than 60× was expected for each sample.

Reads filtering and mapping

Sequenced reads were assigned to each sample (unplexing), and adaptors were removed. Overall read quality was checked for each sample using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were then filtered based on their length, pairing, and quality using Trimmomatic (Bolger et al. 2014). Parameters were set as follows: read length ≥76 bp; mean Phred quality score greater than 30; and Phred quality score greater than 30 across all reads (10-bp sliding window). Only paired reads were kept for mapping. Reads were mapped to the *Ae. Aegypti* genome (AaegL2 assembly) using BWA (Li and Durbin 2009) with default parameters implemented into a Galaxy pipeline (http://galaxyproject.org). Mapped reads were loaded into Genespring NGS version 12.5 (Agilent) and further filtered according to their mapping quality (alignment score of 95 or above). In case of multiple mapped reads, only primary hits were conserved.

Copy number variation analysis

Coverage of target genes was quantified for each sample and normalized according to the total number of filtered reads per library. Only genes showing more than 90 reads per kilobase exon model per million sequenced reads (RPKM) in all samples were considered for further analysis. Detection of copy number variation (CNV) was based on differential coverage between samples obtained from resistant and susceptible populations. Fold changes (FC) between resistant samples (R+ and R− phenotypes) and all susceptible populations (meanS) were computed for each gene. Genes satisfying the following criteria in any resistant population were considered affected by gene amplifications in association with deltamethrin resistance: [(R+)/(meanS) FC ≥ 1.5] and [(R+)/(R−) FC] > 1 (i.e., 1.5-fold more copies in resistant samples and more copies in LD_{75} survivors compared to LD_{25} dead).

Validation of gene amplifications on individual mosquitoes

Gene amplifications detected by previous CNV analysis were further studied by qPCR on 156 individual mosquitoes (12 from each population/phenotype). Nine candidate genes located in three different genomic clusters affected by gene amplifications were selected: Supercontig 1.1188: AAEL014614 (*CYP9J?*), AAEL014615 (*CYP9J23*), and AAEL014617 (*CYP9J28*); Supercontig 1.1327: AAEL014890 (*CYP6CC1*), AAEL014891 (*CYP6P?*), and AAEL014893 (*CYP6BB2*); Supercontig 1.142: AAEL05112 (*CCEAE3A*), AAEL005101(*CCEAE4A*), and AAEL005122

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(*CCEAE6A*). PCR primers targeting exonic regions were designed using NCBI Primer BLAST and checked for specificity against the whole *Ae. aegypti* genome (Supplemental Table 6). Two target genes showing a constant copy number across all samples from sequencing data (see above) were used for normalization (AAEL005950 and AAEL007808). Real-time quantitative PCR was performed on an iQ5 cycler (Bio-Rad). PCR reactions consisted of 3 µL gDNA template (see above), 3.6 µL nuclease free water, 0.45 µL of each primer (10 mM), and 7.5 µL of SYBR Green Supermix 2× (Bio-Rad). A dilution scale made from a pool of all gDNA samples was used for assessing PCR efficiency and quantification. All samples were amplified in triplicates (14 populations × 12 individuals × 3 replicates). After normalization, CNVs were expressed as mean relative gDNA quantity compared to one individual of the Livp S population.

Impact of gene amplifications on transcription levels

The link between gene amplifications and increased transcription levels was investigated on nine genes (see above) by RT-qPCR. Total RNA samples from each population were treated with DNase I (Invitrogen) to remove genomic DNA following the manufacturer's instructions. Reverse transcription and qPCR reactions were performed as described in Nkya et al. (2014). Data analysis was performed according to the $\Delta\Delta_{Ct}$ method taking into account PCR efficiency (Pfaffl 2001) and using the housekeeping genes encoding the ribosomal proteins L8 (AGAP005802) and S7 (AGAP010592) for normalization. Three technical replicates were performed per population, and results were expressed as mean transcription ratio in each resistant population ± SD relative to the mean transcription ratio of all susceptible populations.

Polymorphism calling and population structure

Polymorphisms (SNPs, multiple nucleotide polymorphisms [MNPs], and indels) were called against the reference genome using filtered reads by using Genespring NGS version 12.5 (Agilent). Calling parameters were as follows for each sample: calling score of 50 or above (P-value < 10^{-5}); ignore homopolymer 10 or higher and surrounding positions; locus coverage of 30 or more. Variants identified from each sample were further filtered based on their coverage and strand bias across all samples (coverage 30 or more; strand bias 140 or less). For each variant, allele frequencies were estimated for each population based on the number of reads supporting each allele, and genic effects were computed against the reference genome. A principal component analysis (PCA) based on allele frequencies of all polymorphic variants across all samples was used for inferring the genetic structure of the studied populations and confirming the relevance of the geographical groups.

Searching for selection footprint using a hierarchical Bayesian approach

BayeScan3 was used for identifying loci under natural selection through a hierarchical Bayesian model, taking into account population structure and usable on pools of individuals (Foll et al. 2014). This approach estimates the probability that each locus is subject to selection by discriminating population-specific and locus-specific components of the fixation index using a logistic regression. The posterior probability of a given locus being under selection is assessed by defining two alternative models, one including the locus-specific effect and the other excluding it. Departure from neutrality is supposed when the locus-specific component is necessary to explain the observed pattern of diversity using a reversible jump Markov chain Monte Carlo (MCMC). Because the high genetic proximity between R+ and R− phenotypes can bias the estimation of the neutral model, only S populations and R+ phenotypes from resistant populations were used. For the same reasons, loci showing allele frequency <5% or >95% in all samples were not considered. BayeScan3 was run with default parameters and the following hierarchical structure: Thailand (Patg S, Nakh R+, and Phet R+), South America (NwOr S, Cayn R+, StGe R+), and Laboratory (Bora S, Delta R+). Loci showing a F_{sc} Q-value ≤ 0.05 in any geographical group were considered under selection (i.e., outlier locus). These loci were further filtered based on their association with the resistance phenotype by retaining only those showing both [f(R+)-f(S)] and [f(R+)-f(R−)] metrics being positive (enrichment of variant allele) or negative (enrichment of reference allele). Because the *Ae. aegypti* genome is not assembled, selection footprints were looked for at the supercontigs scale. For each supercontig, the density of outlier loci was computed by dividing the number of outlier loci by the total length of captured regions within the supercontig. In order to avoid inferring selection footprint from limited genomic coverage, only supercontigs represented by not more than four genes or from which the captured region represented >10% of supercontig length were considered.

Identifying polymorphisms mostly associated with deltamethrin resistance

Allele frequency-based filtering was combined with the Bayesian approach for identifying polymorphisms most strongly associated with deltamethrin resistance. Frequency-based filtering consisted in retaining alleles satisfying the following conditions in any resistant/susceptible population pair from each geographical group: [f(R+)-f(S) ≥ 40%] and [f(R+)-f(R−) ≥ 5%] and both metrics being positive or negative. Alleles passing both the frequency-based filtering and the Bayesian approach were retained. In addition, the best candidates obtained by each approach were also retained. For the Bayesian approach, this included loci showing F_{sc} Q-values within the best 10th percentile from each region. For frequency-based filtering, this includes alleles passing frequency-based filtering and showing the five highest Abs{[f(R+)-f(S)]+[f(R+)-f(R−)]} metric from each region. Variants most associated with deltamethrin resistance were then filtered according to their genic effects and those being nonsynonymous (i.e., affecting protein sequence) were retained.

Validation of polymorphism data and association between *kdr* mutations and gene amplifications

Allele frequencies obtained by high-throughput sequencing were validated on *kdr* mutations (V1016I, V1016G, and F1534C) on 156 individuals (12 individual mosquitoes from each population/phenotype). Genomic DNA was extracted as described above, and *kdr* mutations were detected by qPCR following the method described in Saavedra-Rodriguez et al. (2007). Associations between the presence of *kdr* mutations and gene amplifications were tested across the five resistance populations for the three *kdr* mutations and the nine genotyped gene amplifications by testing linear correlations between the *kdr* gentoype (0: homozygote wildtype, 0.5: heterozygote, 1: homozygote resistant) and gene amplification levels.

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Data access

The sequence data from this study have been submitted to the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena) under accession number PRJEB7976.

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Author contributions: F.F. was involved in all aspects of the study and helped draft the manuscript. I.D. contributed to field sampling and molecular analyses. T.G. provided technical support for mosquito rearing and contributed to sample preparation. V.N. and F.B. contributed to data analyses and helped draft the manuscript. F.C., W.J., T.C., R.G., I.D., and V.C. contributed to study design and helped draft the manuscript. P.S., K.T., and R.P. contributed to field sampling. S.R. contributed to sample preparation and helped analyze data and draft the manuscript. J.P.D. conceived the study, contributed to field sampling and sample preparation, analyzed data, and wrote the manuscript.

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Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing

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Review

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Anopheles species diversity and distribution of the malaria vectors of Thailand

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In Thailand, seven *Anopheles* species within three species assemblages have been incriminated as important malaria vectors. Comprehensive maps showing the countrywide geographical distribution of primary and secondary malaria vector species have not yet been developed; the maps that do exist are typically restricted to specific areas or are out of date. In addition, with the advent of molecular-based species-identification tools, the geographical locations of various sibling species have been more clearly defined in the country. This review has compiled published data on the known distribution of important malaria vectors in Thailand based on genetic and molecular identification assays from 1994 to 2014, with maps of relevant species distribution provided herein.

Introduction

Malaria remains one of the most important parasitic diseases in Thailand, and has long been one of the major causes of morbidity/mortality, especially along the international borders with Cambodia, Myanmar, and Malaysia [1]. Many previous studies on malaria vector species distributions are based on morphological identification alone [2,3], including techniques on the study of morphological variations, use of cross-mating experiments, examining mitotic and meiotic karyotypes, heterochromatin variations of polytene chromosomes, electrophoretic protein variations, and the use of cuticular hydrocarbons. Unfortunately, all earlier ecological and behavioral studies based exclusively on morphologically identified specimens are questionable with regard to the precise species observed. In the past decade, more genetics and molecular-based species identification techniques have been developed and applied for the detailed study of anophelines, leading to the recognition of additional species and species complexes [4–6]. These advanced laboratory and molecular techniques have used cytogenetic/polytene chromosome compatibility and cross-mating experiments, isoenzyme protein markers, species-specific DNA probes, PCR technologies, DNA sequencing of the ribosomal ITS2 (internal transcribed spacer) and D3 explansion segments, mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) sequences, and allele-specific PCR (AS-PCR) and random amplified polymorphic DNA (RAPD) markers to discern and accurately differentiate individual species from one another [7].

The Ministry of Public Health in Thailand has classified malaria control areas based on the risk of acquiring infection (A1, A2, B1, and B2 in decreasing order) and the presence or absence of malaria vector species. The accurate identification of malaria vector species is essential to gain a better understanding of the role of each species in malaria transmission and to improve the effectiveness and proper targeting of disease control program activities. This review presents the current known distribution for all implicated (confirmed) malaria vector species in Thailand as identified by molecular methods.

Anopheles vectors of human malaria parasites

At least 74 species of *Anopheles* mosquitoes have been documented in Thailand, comprising 73 formally named species and a closely related species of *Anopheles gigas* Giles [8,9]. Moreover, three additional species in the *An. barbirostris* complex are being formally named (R.E. Harbach, personal communication). Several of the most important malaria vectors in Thailand are members of closely related sibling species that may differ considerably in their biology, behavior, and epidemiological characteristics related to disease transmission, including susceptibility to malaria parasites [10] and to insecticides used to control the vector. The various sibling species show distinct distribution patterns and population densities that can often vary according to seasonal climatic changes and other factors (e.g., land use).

The most important malaria vectors are in the subgenus *Cellia*, and include members in the Leucosphyrus Group

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(Neomyzomyia Series), Maculatus Group (Neocellia Series), and the Minimus Subgroup (Myzomyia Series) 11–15]. Collectively, these include *Anopheles baimaii* Sallum & Peyton [4,12,16], *Anopheles dirus* Peyton & Harrison [12,15], *Anopheles minimus* Theobald [4,17], *Anopheles maculatus* Theobald [12,17], *Anopheles pseudowillmori* (Theobald) [12], *Anopheles sawadwongporni* Rattanarithikul & Green [14,17,18], and *Anopheles aconitus* Doenitz [11–13]. However, some species far exceed others in their overall capacity to transmit malaria, and their relative importance and role may vary depending on locality, season, and epidemiological circumstances. In addition, *Anopheles epiroticus* Linton & Harbach and *Anopheles campestris* Reid have been incriminated as secondary/incidental vectors in the country [4,19]. This review focuses on the distribution of these species and the methods used in their identification.

The Leucosphyrus Group is represented by 10 species in Thailand, including *An. baimaii, An. dirus, Anopheles cracens* Sallum & Peyton, *Anopheles hackeri* Edwards, *Anopheles introlatus* Colless, *Anopheles latens* Sallum & Peyton, *Anopheles macarthuri* Colless, *Anopheles nemophilous* Peyton & Ramalingam, *Anopheles pujutensis* Colless, and *Anopheles scanloni* Sallum & Peyton. *Anopheles baimaii* and *An. dirus* are considered to be primary malaria vectors in Thailand [4,20]. *Anopheles dirus* is the only species in the group that is found throughout Thailand and occurs in sympatry with *An. baimaii* in Kanchanaburi Province (western Thailand) and along the Thai–Myanmar border [21,22]. The remaining eight species in this group are regarded as non-vectors and occur primarily in southern (peninsular) Thailand [16,23].

For the Funestus Group, which includes the Minimus Subgroup, only *An. minimus* has been implicated as an important vector in Thailand [4]. The subgroup contains the *Anopheles* (*Cellia*) *minimus* complex, Theobald 1901, initially recognized as a complex of five sibling species, provisionally designated A, B, C, D, and E. The Minimus Complex was first described by Green *et al.* [24]. The two species, subsequently formally named *An. minimus* (species A) and *Anopheles harrisoni* Harbach & Manguin (species C), were also initially studied by Green *et al.* [24]. Currently, very limited information is available on species B [25] and species D [23]. Species B is considered to be the morphological variant of *An. minimus* [26]. *Anopheles minimus* [27,28] is the most common and widespread species of the complex throughout Thailand. *Anopheles harrisoni* appears to be restricted to two districts of Kanchanaburi Province in western Thailand and Chiang Mai Province in northern Thailand, and also occurs in sympatry with *An. minimus* in both areas [4,29]. This species has also been found in Tak and Chiang Mai Provinces [4]. The fifth species, *An. minimus* species E, appears to be restricted to small island groups in southern Japan [30] and was subsequently named *Anopheles yaeyamaensis* Somboon & Harbach [31].

Four members of the Maculatus Group are important malaria vectors in the greater Oriental Region that includes Thailand, Nepal, Indonesia, Malaysia, and the Philippines [32]. This group contains at least nine closely related species that are differentiated based on variability in morphological, behavioral, and genetic characters [33–40]. In Thailand, seven species have been identified, including *An. maculatus*, *An. pseudowillmori*, *An. sawadwongporni, Anopheles dravidicus* Christophers, *Anopheles notanandai* Rattanarithikul & Green, *Anopheles willmori* [37,41], and *Anopheles rampae* Harbach & Somboon [9].

Recent observations on the biting cycles and host preferences have been made on molecularly identified vector species within the Dirus and Minimus Complexes and the Maculatus and Leucosphyrus Groups in Thailand [22,29,42,43]. The two species of Minimus Complex, *An. minimus* and *An. harrisoni*, have been described in detail from two malaria endemic areas in Tak [42] and Kanchanaburi [29] Provinces, Thailand. Sungvornyothin *et al.* [29] used a one-shot PCR method [44] for *An. minimus* and *An. harrisoni* species identification, and studied the trophic (host and blood-feeding) behavior of these two species. Kongmee *et al.* [45] described the larval habitats of sympatric *An. minimus* and *An. harrisoni* in Kanchanaburi Province. Vector species diversity, biting activity patterns, and trophic behavior of the Minimus Complex were also observed in the Mae Sot District, Tak Province [42] that involved two closely related malaria vectors, *An. minimus* and *An. aconitus*, in the same locality. With the availability of molecular-based identification tools, more investigations on population biology and behavior of sympatric malaria vector sibling species should be carried out to determine the precise epidemiological role of each in malaria transmission.

Geographical distribution of malaria vectors

Currently, 481 formally named *Anopheles* species have so far been recognized throughout the world (Mosquito Taxonomic Inventory: http://www.mosquito-taxonomic-inventory.info), and approximately 80 species are regarded as important vectors of malaria parasites, filarial nematodes, and a few arthropod-borne viruses [46]. Of 25 described *Anopheles* complexes worldwide, 11 occur in Asia, 10 of which are recognized in Thailand [47,48]. Because individual species within a complex may differ significantly in biological, ecological, and behavioral characteristics, which can greatly influence their potential as disease-transmitting agents, incorrect species identification of individual complex members may result in failure to distinguish between a vector and non-vector species, and thus lead to misdirected and ineffective vector control programs [49,50].

Illustrated morphological keys exist to identify both female adult and larval stages of *Anopheles* mosquitoes in Thailand [4]. In addition, numerous studies have been published describing molecular techniques that separate sibling species and that have also generated distribution maps [51]. We have taken all available published information, together with new location data generated in this study, to produce a series of tables and distribution maps illustrating the currently known distribution of important malaria vectors in Thailand. Our study samples were collected from 10 provinces and are presented in Tables 1 and 2. All-night human-landing collections were used, including indoor and outdoor captures, together with cattle-baited outdoor trap collections. For details on specific handling and molecular mosquito identification methods used please refer to Tainchum *et al.* [43].

Table 1. *Anopheles* mosquito collection sites and dates in present study

Province	District	Observation period	Geo-coordinates
Chiang Mai	Mae Wang	11–12 March 2011	18°36′ N 98°46′ E
	Mae Teang	20–21 May 2011	19°7′ N 98°56′ E
Mae Hong Son	Sop Moei	22–23 February 2011	17°52′ N 97°57′ E
	Mae Sariang	19 July 2011	18°9′ N 97°56′ E
Tak	Mae Sot	28–29 October 2010	16°40′ N 98°40′ E
Kanchanaburi	Sai Yok	20–21 November 2010	14°17′ N 99°11′ E
Prachuap Khiri Khan	Hua Hin	11–12 September 2010	12°30′ N 99°31′ E
Chumphon	Tha Sae	25–27 January 2012 23–25 April 2012 28–30 April 2012	10°39′ N 99°10′ E
	Phato	20–22 February 2012 18–20 July 2012	10° 42′ N 99°19′ E
	Lamae	15–17 March 2012	9°46′ N 99°5′ E
Ranong	La-un	10–11 February 2012	10° 6′ N 98°45′ E
Surat Thani	Khiri Rat Nikhom	13–16 February 2012	9°1′ N 98° 57′ E
Phangnga	Thap Put	12–14 January 2012 7–8 March 2012 2–3 April 2012	8°30′ N 98°38′ E
	Mueang	7 March 2011 25–27 June 2011	8°26′ N 98°31′ E
Phuket	Thalang	17 May 2012	8°1′ N 98°20′ E

Dirus Complex

The Dirus Complex belongs to the Leucosphyrus Group [16] that contains at least four important malaria vectors present in South and South-East Asia, including *An. dirus, An. baimaii, Anopheles balabacensis* Baisas, and *Anopheles leucosphyrus* Doenitz. However, the taxonomy of this diverse and widely distributed group remains difficult to discern. In 1989, 20 known species and two forms had been morphologically identified in the Leucosphyrus Group [52]. Peyton and Ramalingam [53] provided the morphological and geographical descriptions of the Dirus Complex for the first time (1988). Evidence of sibling species within the Dirus Complex began with cytological observations [16,23,52,54,55] and the use of the mitotic karyotypes to demonstrate variations in the structure of sibling species within the Dirus Complex [16,20,23,52,54,55]. Using DNA probes, Panyim *et al.* [56] reported four species-specific DNA sequences, and Audtho *et al.* [57] developed DNA probes and successfully identified *An. dirus*, *An. cracens*, *An. scanloni*, and *An. baimaii* from field collections.

ITS2 sequence analysis revealed two different sequences for *An. scanloni* from northern and southern Thailand [58]. The population genetic analysis using 11 microsatellite markers of northern and southern populations showed significant differences in the genetic structure between the two populations [58,59]. From the sequence analysis of the ITS2 region, Walton *et al.* [58] found *An. baimaii* different from species D from China [60], suggesting that the latter may represent an additional species in the complex.

Several techniques and diagnostic features are available for the identification of sibling species in the Dirus Complex. Twenty different restriction enzymes were used to study restriction fragment length polymorphisms (RFLP) [61] in *An. dirus*, *An. cracens*, *An. scanloni*, and *An. baimaii*. Seven enzymes (*Ava*II, *Alu*I, *Bgl*II, *Hae*III, *Hin*fI, *Mbo*I, and *Sau* 3AI) produced unique patterns for each species while other enzymes produced specific patterns for one or two species only. An AS-PCR assay [60] using three primers, one derived from highly-conservative 5.8S coding sequences and two from the ITS2 region of ribosomal DNA, could clearly distinguish *An. dirus* from *An. baimaii*, with 374 bp and 663 bp amplicons, respectively. Walton *et al.* [58] used a multiplex PCR assay with primers designed from rDNA ITS2 sequences of the different sibling species that could clearly identify *An. dirus*, *An. cracens*, *An. scanloni, An. baimaii*, and *An. nemophilous* from Thailand using a single reaction.

Manguin *et al.* [62] and Huong *et al.* [63] also developed PCR assays which distinguished *An. dirus*, *An. cracens*, *An. scanloni*, and *An. baimaii*. Microsatellite markers have been used for population analysis [64] of *An. dirus* and to assess polymorphisms in *An. scanloni* and *An. baimaii*. Walton *et al.* [65] used these same markers to study gene flow in populations of *An. dirus*, *An. scanloni*, and *An. baimaii*, wherein all three species were found to be well differentiated using specific microsatellite loci. Based on these findings using described techniques, the distributions of *An. dirus* and *An. baimaii* are presented in Tables S1 and S2 in the supplementary material online, and are illustrated in Figure 1A,B, respectively.

Anopheles dirus is found throughout the country (Figure 1A), whereas *An. baimaii* appears to have a more restricted distribution, being found only in more focal, central, southern and western parts of the country, particularly along the Thai–Myanmar border (Figure 1B). The other members of this complex, *An. cracens*, *An. scanloni*, and *An. nemophilous*, have been reported primarily in western and southern Thailand (Figure 1C) (Tables S3 to S7 in the supplementary material online). From our collections along the Thai–Myanmar border, with identification based on AS-PCR assays, higher numbers of *An. baimaii* were found compared to *An. dirus*, with only two specimens of *An. nemophilous* being detected from Ranong Province.

Table 2. Molecular identification of mosquito populations within the Minimus and Dirus Complexes and the Maculatus Group from difference study sites in present study

Location	Sample size by species										
	An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*	*An. maculatus*	*An. sawadwongporni*	*An. dravidicus*	*An. pseudowillmori*	*An. dirus*	*An. baimaii*	*An. nemophilous*
Mae Hong Son	265	–	8	11	67	203	15	6	2	4	–
Tak	1164	–	7	51	236	59	30	4	15	3	–
Kanchanaburi	97	271	–	–	92	48	–	–	2	1	–
Prachuap Khiri Khan	86	–	15	–	2	4	–	–	–		–
Chumphon	211	–	–	–	16	–	–	–	4	5	–
Ranong	1	–	4	–	3	–	–	–	–	3	2
Phangnga	2	–	–	–	–	–	–	–	–	5	–
Phuket	11	–	–	–	–	–	–	1	–	–	–
Surat Thani	89	–	–	–	–	–	–	–	–	–	–
Chiang Mai	2	–	–	–	–	–	–	–	–	–	–
Total	1928	271	34	62	416	314	45	11	23	21	2

Minimus Complex and closely related species

Initially, the use of allozymes served as the gold standard to separate the two species, *An. minimus* and *An. harrisoni* (species A and C, respectively) within the Minimus Complex [24]. In 1999, two PCR methods, AS-PCR of the D3 variable region of the 28S rDNA gene and SSCP-PCR of the D3 amplified region, were developed to identify *An. minimus* and *An. harrisoni*, and two other closely related species, *An. aconitus* and *Anopheles varuna* Iyengar [66]. AS-PCR was able to distinguish *An. minimus* from *An. harrisoni*, whereas the SSCP-PCR was used to separate *An. varuna* from *An. aconitus* [66]. In 2000, a PCR-amplified ITS2 technique was developed by Van Bortel *et al.* [67]. In 2004, RFLP-PCR and AS-PCR methods were developed, the latter being a one-shot PCR method differentiating members of the Minimus Complex from other related species [44,68]. Based on the use of these assays, the distributions of *An. minimus*, *An. harrisoni*, and the three closely related species, *An. aconitus*, *Anopheles pampanai* Büttiker & Beales, and *An. varuna*, are listed in Tables S6 to S10 in the supplementary material online and illustrated in Figure 2.

The most common and widely distributed species of the complex in Thailand is *An. minimus* (Figure 2A, Table S6 in the supplementary material online). *Anopheles harrisoni* has been reported once in Chiang Mai but otherwise appears to be restricted within Kanchanaburi Province, western Thailand (Figure 2B, Table S7 in the supplementary material online). The related species, *An. aconitus*, *An. varuna*, and *An. pampanai*, have been found mainly in northern, western, and southern regions of Thailand (Figure 2C, Tables S8 to S10 in the supplementary material online). Of note, *An. pampanai* has been found to be infected with *Plasmodium vivax* in Vietnam by Durnez *et al.* [69] and Ngo *et al.* [70], but has yet to be implicated as a vector in Thailand.

Maculatus Group

Anopheles maculatus was deemed to be a member of larger species assemblage, creating the Maculatus Group, after studying the polytene and mitotic chromosomes. The members of the Maculatus Group can be easily separated from each other by examining polytene chromosomes for paracentric inversions on the autosomes [71]. Glucose 6-phosphate dehydrogenase (6-*Pgd*) isoenzyme allelic variations can also be used to distinguish *An. sawadwongporni* (species A), *An. maculatus* (species B), and *An. pseudowillmori* (species I) [71]. Species B, E, and F can be distinguished by gas–liquid chromatographic analysis of cuticular lipids using a multivariate principal component analysis [72]. Based on interspecific variation in the ITS2 region, a diagnostic PCR assay was developed that can distinguish five members of the complex found in China (*An. sawadwongporni*, *An. maculatus*, *An. willmori*, *An. dravidicus*, and *An. pseudowillmori*) [73]. Another PCR-based diagnostic assay was developed to facilitate field research in northern Thailand, which can distinguish *An. maculatus*, *An. dravidicus*, *An. pseudowillmori*, *An. sawadwongporni*, and *An. rampae* (species K) [6]. Approximately 23 microsatellite markers were identified from *An. maculatus* [74], seven of which were used to study the genetic variations in

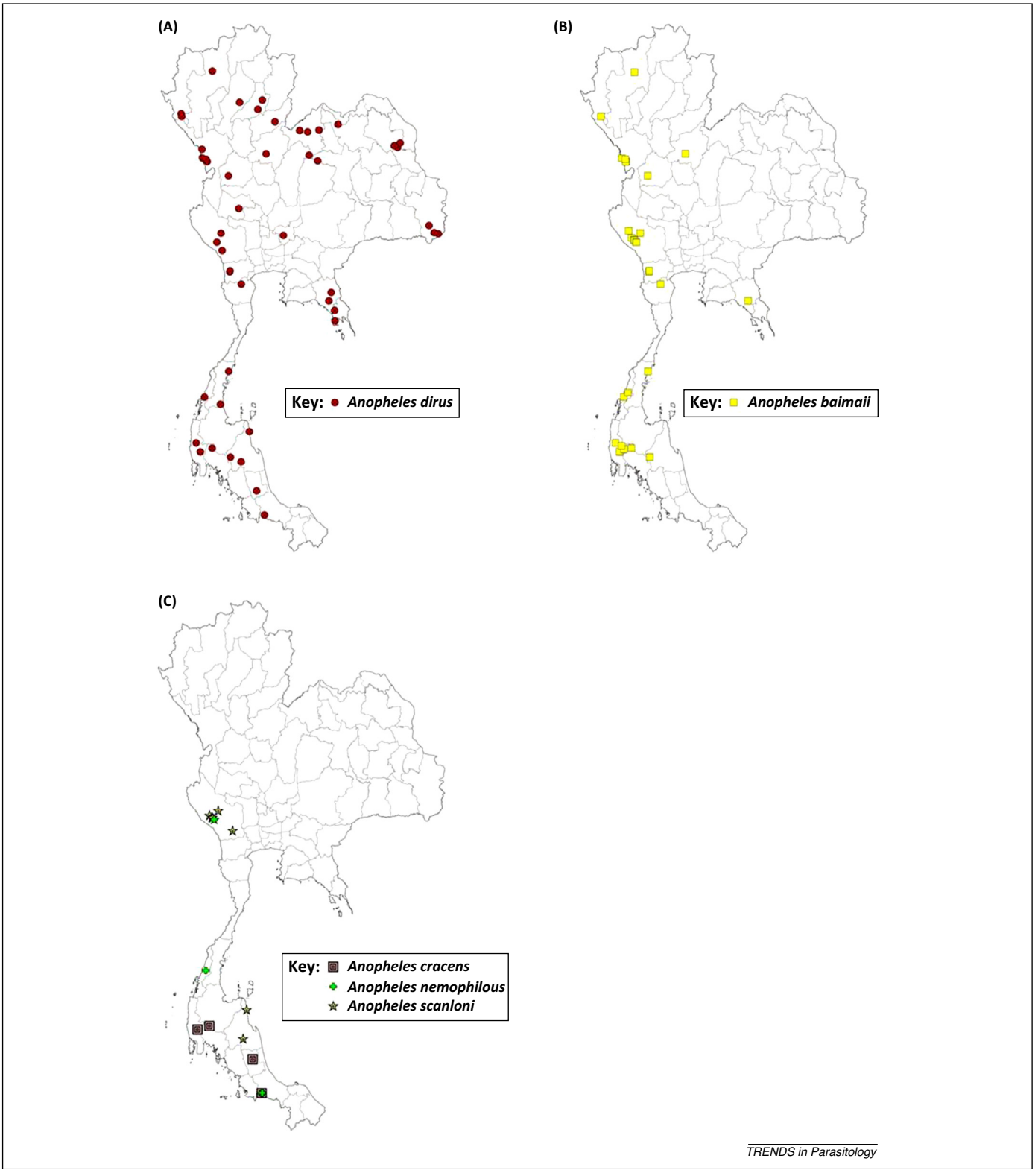


Figure 1. Known geographical distributions of **(A)** *Anopheles dirus*, **(B)** *Anopheles baimaii*, and **(C)** three related species in the Dirus Complex in Thailand based on molecular species identification.

eight widely dispersed localities in western and peninsular Thailand [75]. Species E and F are genetic forms or cryptic forms, and not all members have been identified by molecular methods. The molecular profile of *An. notanandai* is not known. However, based on molecular identification assays, the distributions of the seven species of the Maculatus Group present in Thailand are provided in Tables S11 to S17 in the supplementary material online, and are illustrated in Figure 3.

In Thailand, the most commonly encountered species in the group is *An. maculatus* (Figure 3A, Table S11 in the supplementary material online), followed by *An. sawadwongporni* (Figure 3B, Table S12 in the supplementary material online). *Anopheles pseudowillmori* is also

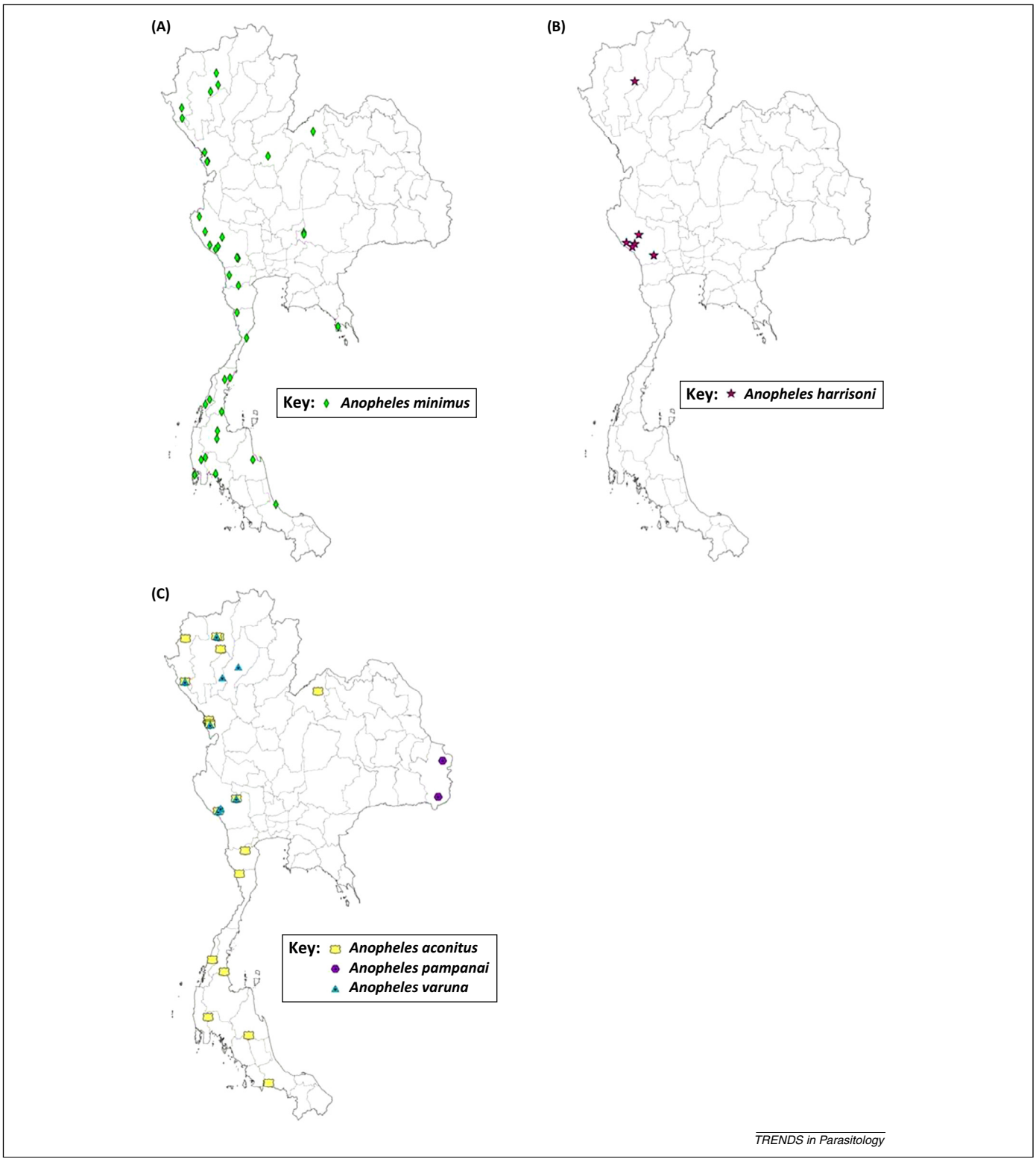


Figure 2. Known geographical distributions of **(A)** *Anopheles minimus*, **(B)** *Anopheles harrisoni*, and **(C)** three related species in Thailand based on molecular species identification.

regarded an important malaria vector in parts of northern Thailand [12] (Figure 3B, Table S13 in the supplementary material online). The other member species (*An. willmori*, *An. dravidicus*, *An. rampae*) have been studied far less frequently and relatively little is known about their bionomics and transmission potential (Figure 3B,C, Tables S14 to S16 in the supplementary material online).

Sundaicus Complex (Anopheles epiroticus)

The Sundaicus Complex is commonly found near coastal zones, and is typically associated with brackish water habitats. The valid species or genetic forms include species A, B, C, D, and E (Dusfour *et al.* [76] and http://mosquito-taxonomic-inventory.info/sites/mosquito-taxonomic-inventory.info/files/Subgenus%20Cellia%

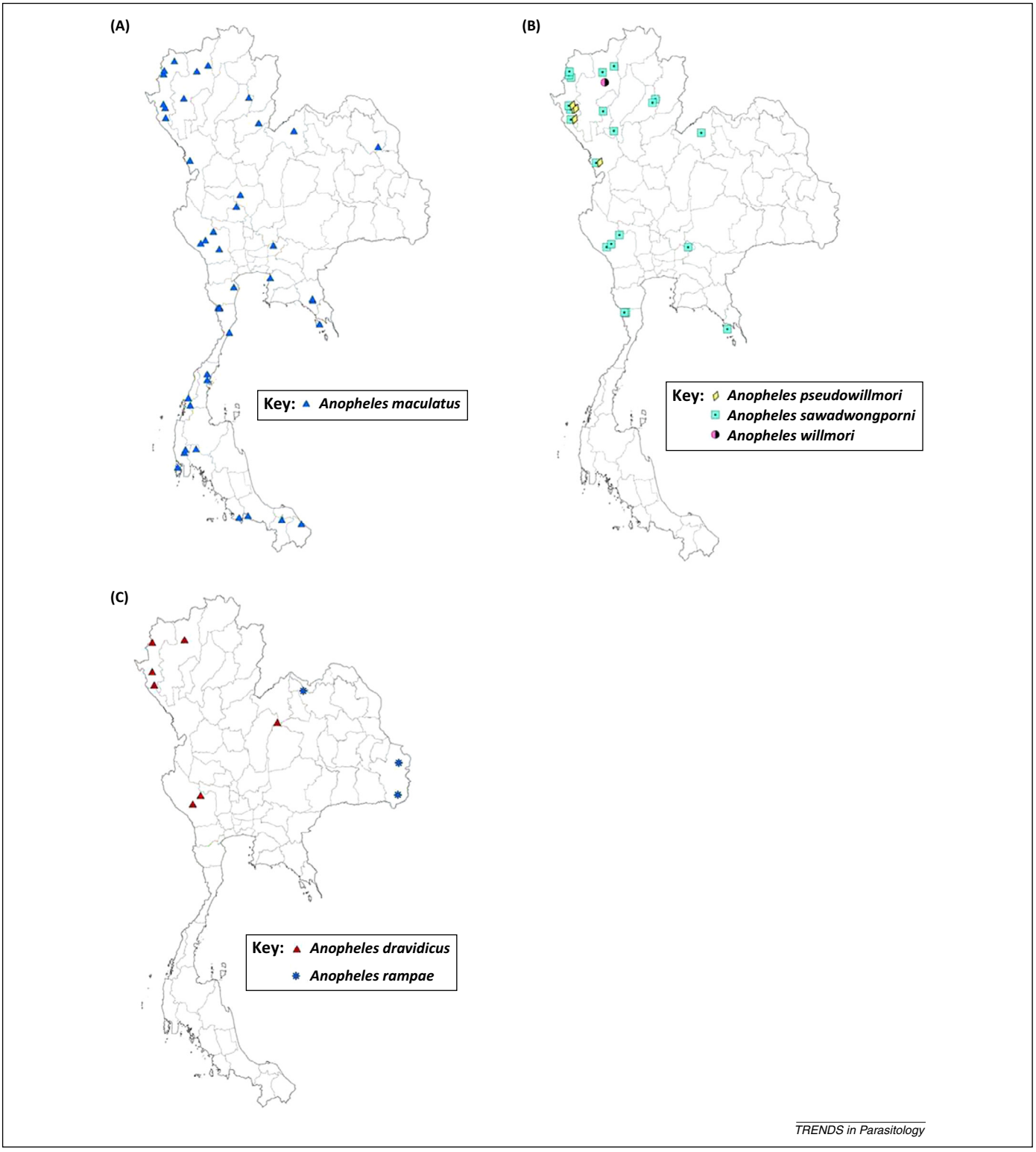


Figure 3. Known geographical distributions of **(A)** *Anopheles maculatus* and **(B,C)** five other members of the Maculatus Group in Thailand based on molecular species identification.

20classification_14.pdf). The possible existence of a sibling species complex was first reported by Sukowati and Baimai [77] based on chromosomal variations among three identified cytological forms (A, B, and C) distributed between Thailand and Indonesia. In Thailand (Trat and Phangnga Provinces), only the A form was initially detected. In 1999, mannose phosphate isomerase (*Mpi*) locus isoenzyme variations were used to differentiate the three putative species [78] in the complex. Dusfour *et al.* [79] later analyzed *Anopheles sundaicus* complex, comparing the mitochondrial DNA sequences of partial regions of cytochrome *b* (CYTB) and cytochrome oxidase I (COI) that resulted in identifying two sibling species, *An. sundaicus* (Rodenwaldt) from eastern Malaysia (Borneo), and *An. sundaicus*

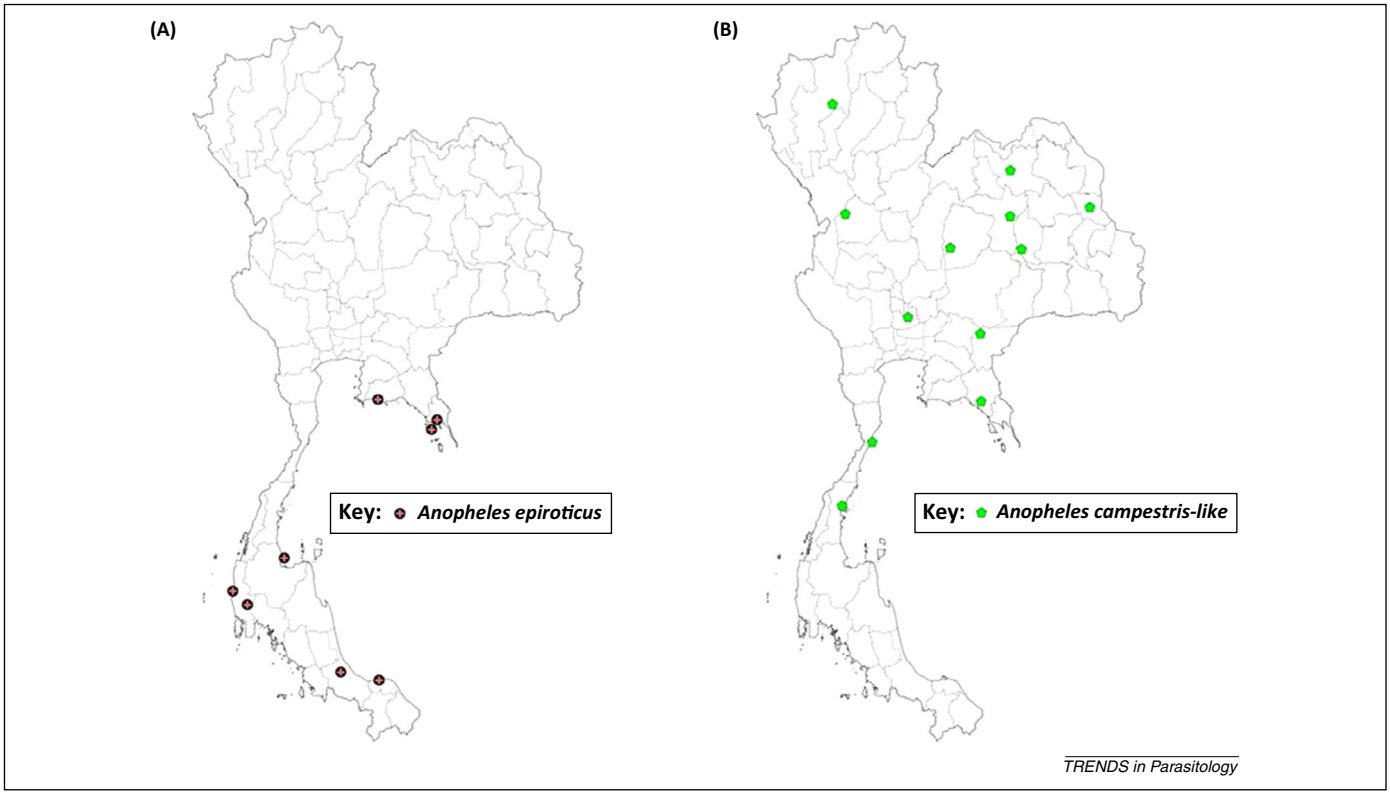


Figure 4. Known geographical distributions of **(A)** *Anopheles epiroticus* and **(B)** *Anopheles campestris*-like species in Thailand based on molecular species identification.

species A from Vietnam and Thailand. *Anopheles sundaicus* species A was subsequently named *An. epiroticus* [80] based on ITS2, CYTB, and COI gene sequences. Dusfour *et al.* [81] developed two multiplex PCR assays, one consisting of four species-specific SCAR (sequence characterized amplified region) primers derived from random amplified polymorphic regions (CYTB and COII), and the other based on sequences of CYTB and COI that allowed unambiguous identification of *An. sundaicus*, *An. epiroticus*, and *An. sundaicus* species E present in Indonesia [76]. Recently, Ritthison *et al.* [82,83] reported that *An. epiroticus* was a common species in coastal areas of eastern and southern Thailand (Figure 4A, Table S17 in the supplementary material online). Recent work on the Sundaicus Complex showed eight genetic and geographical groupings in the Indo-Oriental region, with evidence for recent gene flow among them [84]. It is apparent that more work is needed on this important species complex involved in malaria transmission along coastal zones.

Barbirostris Subgroup

The Barbirostris Subgroup (Subgenus *Anopheles*, Myzorhynchus Series) includes *An. campestris*, *An. campestris*-like, and *An. barbirostris* complex. At least five sibling species have been identified within the Barbirostris Group, in other words *An. campestris*-like and *An. barbirostris* species A1, A2, A3, and A4. This grouping of isomorphic species was identified based on cytogenetic and molecular evidence, and on crossing experiments [85–88]. In addition, the systematics of *Anopheles barbirostris* van der Wulp and a sibling species of the Barbirostris Complex has been reported from Java, Indonesia by Townson *et al.* [89]. The molecular phylogeny of the *Anopheles barbirostris* subgroup was studied by Paredes-Esquivel *et al.* [90,91]. Previously, anthropophilic species within the *An. barbirostris*/*campestris* assemblage have been incriminated as potential natural vectors of *Plasmodium vivax* in Aranyaprathet District, Sa Kaeo Province [92,93], Sangkhlaburi District, Kanchanaburi Province [12], and Chantaburi Province [94], Thailand. The distribution map of *Anopheles campestris*-like species (Figure 4B, Table S18 in the supplementary material online) indicates it is scattered across much of the country.

Concluding remarks

This review has compiled data regarding the known distribution of important malaria vectors in Thailand based on molecular identification assays from 1994 to 2014. Using the various non-morphological tools developed over the course of the past three decades, geo-referenced maps have been generated from both published data, combined with more recent data collected in this study, to generate an updated, countrywide distribution of primary and secondary/incidental malaria vector species in Thailand. With the advent and increasing use of molecular-based species identification methods, the distributions of the various sibling species have been more clearly defined in Thailand. More precise geographical distributions can now be coupled with more accurate descriptions of the behavior and ecology of specific species to provide a far greater understanding of the epidemiological importance of each member. This effort has also helped to illustrate the current limitations of sampling efforts and how little we understand the distribution and diversity of anopheline species in large areas of

Thailand that have either not been sampled or have had material identified using less-accurate laboratory methodologies. Other noted limitations in many published studies include use of relatively low sample sizes, low numbers of collections attempted, temporal (seasonal) and spatial (geographical) boundaries of sampled locations, and the collection techniques used that can bias observations on the true diversity of mosquito populations in an area. Nevertheless, the tables and maps presented in this review provide the first update on the distribution maps of selected species published by Rattanarithikul *et al.* in 2006 [4], the principal difference being that all locations in the maps contained herein are based on methods other than morphological identification alone, including cytogenetic and molecular-based techniques. Recently, Saeung [95] also provided a distribution map of species complexes in Thailand. We provide 11 maps showing the distribution of 18 species and 20 tables with GPS coordinates on *Anopheles* species distribution of public health importance in Thailand. We anticipate as more research and mosquito faunistic surveys are performed using non-morphological methods for definitive (and confirmatory) species identification, the anopheline map of Thailand will become more complete and useful to the operational and academic workers in this field.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pt.2015.01.004.

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RESEARCH Open Access

Targeting educational campaigns for prevention of malaria and dengue fever: an assessment in Thailand

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Abstract

Background: The current study assessed the knowledge, attitudes, and practices (KAP) of at-risk populations for malaria and/or dengue fever in relation to mosquito exposure and household mosquito control practices. Specific objectives included comparison of individual and household level health practices between a rural and urban setting in Thailand. Findings are intended to guide Thailand Ministry of Health educational campaigns targeting arthropod-borne disease.

Methods: A mixed method design was employed using a forced choice and open-ended questionnaire to assess KAP of participants seeking point-of-care treatment for malaria and/or dengue fever at government health-care facilities. Following informed consent, household construction characterization (percent eave gap, floor, wall, and roof material) and mosquito collections both indoors (using aspiration) and outside (using traps) were conducted at a subsample of participant homes. All mosquitoes were identified to genus and anopheline and aedine samples processed for potential pathogen infection.

Results: A total of 64 participants were recruited from both study sites; 62 categorized as malaria symptomology and 2 categorized as dengue across all study healthcare facilities. Significant associations between study site and household construction were indicated. Trends also identified household level practices and both occupation and household construction regarding type of mosquito control products purchased and the abundance of mosquitoes in sampled homes.

Conclusion: Overall, Ministry of Health information from education campaigns regarding malaria and dengue fever strategies is reaching the intended target populations at the study sites. Participants are aware of the presence of mosquitoes and that they serve as the potential vector for transmitting malaria and dengue fever diseases. However, specific knowledge gaps were also identified in each study site that may influence exposure to infected mosquitoes. Findings from this study are intended to guide future health education campaigns in these study settings to address specific community needs.

Keywords: Malaria, Dengue fever, Education campaigns, Vector-control, Knowledge, Attitudes, and Practices (KAP), Thailand

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Background

Vector-borne diseases, such as malaria and dengue fever, account for an estimated 17% of the global burden of infectious diseases [1]. Dengue fever, caused by a flavivirus of four dengue serotypes: DEN-1, DEN-2, DEN-3, and DEN-4, is transmitted by the *Aedes aegypti* mosquito and represents one of the world's fastest growing vector-borne diseases [2]. Acquiring one serotype will provide lifelong immunity to that particular serotype; however infections by subsequent serotypes can increase the risk of developing severe dengue symptoms [2]. Symptoms of dengue fever range from asymptomatic to mild fever, severe headache, muscle and join pain, rash to more severe hemorrhagic manifestations [2]. Malaria, another vector-borne disease of global importance, was responsible for an estimated 627,000 deaths in 2013 alone [3]. Transmitted by the bite of specific anopheline mosquitoes, which feed predominately in the evening or nighttime, infection with any of the four *Plasmodium spp.* human parasites cause symptoms of fever, headache, and vomiting which typically appear between 10–15 days after an infective mosquito bite [3]. In the absence of a marketed vaccine for either malaria or dengue fever, vector control continues to be the primary strategy to mitigate pathogen transmission.

Thailand, an endemic setting for both malaria and dengue fever disease, has been undergoing a transition from a rural and agriculture-based economy to a more urban and industrial society over the last decade [4]. This rapid urbanization has introduced new susceptible human populations into disease settings, which in turn require vector-control programs to adjust to changes in social behavior and migration patterns from rural to urban areas which may contribute to potential dengue outbreaks [5]. Dengue fever, which has had more than a 30-fold increase in incidence across the globe, caused 78,000 cases and 80 deaths in 2012 in Thailand [6]. Although a substantial decrease over recent years due to enforced vector control efforts like indoor residual spraying (IRS) and increased availability of personal protection tools like insecticide treated bed nets (ITNs) [7], malaria caused a reported 24,897 cases and 43 deaths in Thailand in 2012; of which *Plasmodium falciparum* represents 40% of cases and *Plasmodium vivax* 60% of reported cases across the country [8]. Interventions targeting the mosquito vector, such as those incorporating mosquito species-specific behaviors, have also shown to reduce the number of malaria cases [9]. One example is introducing small larvivorous fish into intra-domestic water containers for malaria vectors like *Anopheles stephensi,* which are a known to inhabit geographical regions in Thailand [9].

Commonly found in sub-tropical and tropical regions, transmission of malaria parasites and dengue virus, and subsequently health practices to reduce risk of disease, is dependent on mosquito species abundance, underlying vector ecology, as well as socioeconomic status and environmental factors [10]. In Thailand, peak transmission seasons of both malaria and dengue fever occur from June to August and from October to November which coincides with the rainy season when elevated precipitation leads to an increase in habitable breeding sites for mosquitoes [10]. This ignites many public health facilities to begin campaigning for disease prevention by performing active case detection and/or vector control measures. Insecticide treated bed nets (ITNs) are a very common form of vector-control used in Thailand and represented approximately 12.5% of the government expenditure in 2012 for malaria control [11]; however, dengue vectors are daytime-biters which make bed nets inadequate for full protection against infective mosquitoes as generally bed nets are used within the home during the nighttime hours.

Anthropogenic degradation of the environment can also contribute to changes in mosquito biodiversity (i.e., species types present) and therefore shifts in exposure of communities to varied arthropod-borne pathogens [12]. For instance, as land areas become more urban, mosquito diversity within the environment has been found to decrease [12]. Similarly, an increase/decrease in distance from mosquito breeding habitats to human blood-sources (i.e., clearing of forests), specifically in the case of malaria vectors, may alter pathogen transmission by shifting overall vector feeding patterns, vector density, and/or longevity of the mosquito all which can lead to an increased risk of arthropod-borne disease and influence health practices at the individual and/or household level [12].

Socioeconomic variables are also known to affect vector-borne disease transmission intensity. Common migration patterns within Thailand include that from rural to urban areas, and have led to urban population projections to increase from 39% of the total population in 2007 to 47% by 2027 [13]. Urbanization provides many opportunities for suitable breeding habitat of *Aedes aegypti* due to the presence of available containers for which this species is adapted (waste, tires, water storage bins) [14]. Migration also impacts housing density within communities, which has been found to increase mosquito density and play a significant role in the risk of exposure to infected vectors in crowded urban areas. In Thailand, villages with twice as many houses per unit area have been found to have significantly more *Aedes aegypti* adults and pupae than houses in villages with fewer houses per unit area [15]. Additionally, household construction characteristics are often linked to socioeconomic status and can reflect influential determinants of risk of exposure to mosquito bites [16]. For instance, the presence of window screens are typically associated with

discretionary spending of the homeowner and can serve to reduce entry of mosquitoes [16]. In Thailand, tin-roofed houses have been found to have an increased risk for *Aedes aegypti* larval infestation among rural locations, whereas thatched-roofed houses have shown negative risk factors [17].

The use of qualitative information, like that of an open-ended questionnaire, can serve as a tool to gauge health practices, knowledge, and perceptions within a community to better understand gaps in use, uptake, and acceptance of vector-control programs. This information can then be shared with public health officers to guide 'best practices' for modifying current interventions or creating more effective strategies to include education campaigns. A study looking at dengue fever reported an increase in knowledge which led to better preventative behavior and thus a reduced burden of dengue fever disease [18]. Even more, the source of knowledge an at-risk person acquires health information from can be influential in the results observed in disease control practices. Commonly, poor and low-income families receive their information from teachers, health care workers, television, or parents [18]. Penetrating the information system to provide better knowledge will prevent wrong beliefs and common misconceptions that traditionally pass from parent to child [18].

In addition to active case detection within communities, various qualitative measures have been incorporated into the current Thai intervention and campaign schemes for vector-borne diseases. These include the use of school-based programs which target education at children, verbal education aimed at improving knowledge, radio announcements, or pamphlets, to increase campaign awareness [18-21]. Rural and urban areas have been shown to have separate campaign strategies with varied success in each [19]. For example, community-based interventions in Thailand have in past years been targeted for rural areas, and up until 2012 had not been successfully implemented in an urban setting [19]. By gaining a better understanding of individual health behaviors and/or household protection practices, these strategies can be enhanced to address specific barriers that influence the desired behavior seen at the individual or community level and thereby enhance acceptability and/or increase usage of effective protective measures. Indeed, interventions that utilize a community participatory approach to carry out mosquito control interventions have shown greater sustainability as compared to using 'outsiders' non-familiar to the community in a governmental top-down approach [20]. For this reason it is vital to characterize the relationship between human and coexisting vector populations in urban versus rural settings in order to better target intervention and educational campaigns in at-risk locations.

The goal of the current study was to assess the knowledge, attitudes, and practices (KAP) of at-risk populations for malaria and/or dengue fever in relation to mosquito exposure and household mosquito control practices. Specific objectives included comparison of individual and household level health practices between a rural and urban setting in Thailand. Findings are intended to guide Thailand Ministry of Health educational campaigns targeting arthropod-borne disease.

Methods

Ethical approval was granted by The Institutional Review Boards at The University of Notre Dame, USA and Kasetsart University with informed consent of study participants conducted accordingly (Review Number:14-03-1630). A total of 18 days were spent in each study site rotated in two 9-day blocks. This schema allowed five days for conducting hospital surveys and approximately five days to perform both household mosquito collections and household construction surveys during a single site visit.

Study sites and catchment

Two political districts, Pong Nam Ron and Phanom, were selected as study sites based on endemicity for malaria and/or dengue fever and working relationships between the Ministry of Health and study personnel. Sites were characterized as either rural (Pong Nam Ron District) or urban (Phanom District) according to land use, population density, and average monthly income based on 2011 Thai government data and criteria outlined by The Royal Institute, Thailand [21-23]. Two healthcare facilities within each of the study districts were selected to serve as base field stations and location for participant recruitment. To capture both target populations; one district level hospital and one district level malaria clinic were selected. Hospital facilities were aimed at recruiting both malaria and dengue fever participants while malaria participants were captured at malaria clinic facilities. The catchment area of facilities reflected incoming populations at the sub-district and village level (Figure 1).

Pong Nam Ron District, characterized as the rural study site, is located 191 miles east of Bangkok in northwest Chanthaburi Province. Chanthaburi Province is mostly comprised of mountains, high plains, and includes several large rivers, waterfalls and natural wildlife preserves. Pong Nam Ron has a population of 42,587 people, a density of 67.17 people per mi^2 and covers over 350 mi^2, lying along the Cambodian border, it is divided into 5 sub-districts and 47 villages [23]. The main occupation is agricultural farming including durian, longan, potato, and corn. In 2011, the average monthly income per household was 24,278 baht (USD 755.15) [23]. Currently, there is 1 public hospital in Pong Nam Ron District and one health promoting hospital located in each of the 5 subdistricts within Pong Nam Ron

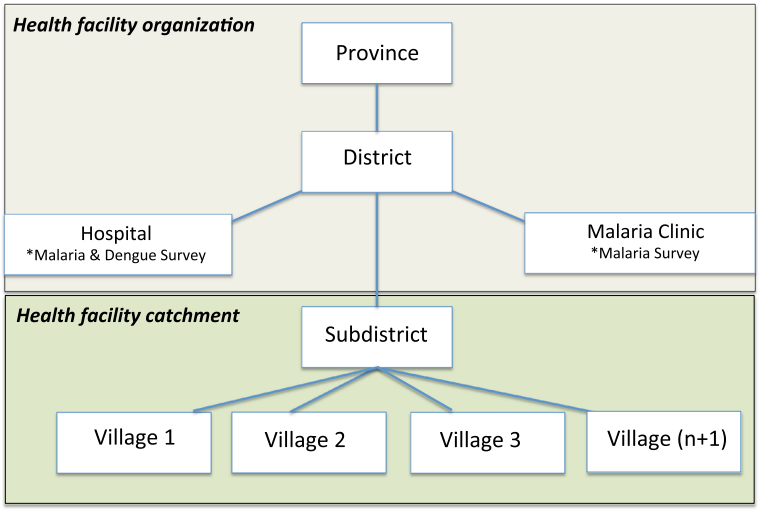


Figure 1 Overview of study healthcare facility organization within provincial divisions of Thailand. *Type of survey researchers performed at the healthcare facility.

District. In 2013, Pong Nam Ron had 61 cases of malaria and 5 cases of dengue hemorrhagic fever [24].

Phanom District, characterized as the urban study site, is located 441.5 miles south of Bangkok in Southern Thailand within Surat Thani Province. Phanom has a total population size of 37,950 people, a density of 85.09 people per mi^2 and over 4977 mi^2 of land [25]. Largely made up of dense forests and high mountain ranges; major industries within this district include rubber plantation, palm oil, coffee and fruit farms. Phanom is further divided into 6 sub-districts comprised of 56 villages [25]. In 2011 the average monthly income per household in Surat Thani was 34,417 baht (USD 1070.51) [25]. Currently, Phanom Province has 11 public hospitals in total. There is 1 hospital located in Phanom District and 9 health promoting hospitals located within 9 sub districts of Phanom District. In 2013, Phanom had 418 cases of malaria, 62 cases during the months of May and June, and 38 cases of dengue fever, 10 cases in May and 6 in June-making Phanom one of the top endemic districts within Thailand [24].

Recruitment

Participants were recruited from study healthcare facilities using passive case detection of persons seeking point-of-care treatment for symptoms related to malaria or dengue fever. Inclusion criteria consisted of male and female adults aged 18 years and older who presented with non-life-threatening symptoms consistent with malaria and/or dengue fever, and did not require emergency medical attention. A positive diagnosis was irrelevant to participant eligibility, allowing all persons who consented to participate. Local Thai technical staff participated in recruiting eligible persons for study enrollment. A verbal explanation of the study protocol was provided in local Thai dialect, translated from English, which explained the study goals and experimental procedures. A request to survey participants' homes for the purpose of house characterization and to collect mosquitoes was explained at the time of recruitment. One consent form was used for the KAP survey, household construction characterization survey, and mosquito collections.

Hospital questionnaire

Convenience sampling was used to recruit eligible participants at designated study healthcare facilities. One healthcare facility was visited each day for approximately 5–7 hours over a 5 day period starting at varying times (i.e., morning, midday, and afternoon) which controlled for gender and/or age-specific biases that might occur as the result of when health care is sought (i.e. outside school, when market is closed, and/or field-work). Consenting subjects participated in a questionnaire pertaining to their KAP on malaria or dengue fever, depending on reasoning for clinic visit. Questions focused on participant's demographic information, history of clinic attendance, individual knowledge regarding prevention of malaria and/or dengue fever disease, and individual household mosquito protection behaviors. Questions were designed based on previous KAP surveys in Thailand regarding malaria and/or dengue fever disease and were reviewed for completeness [26,27]. Each survey consisted of approximately 35 questions.

Mosquito collections

Permission to access homes to conduct a household construction survey and to perform mosquito collections was

obtained from those participants consenting to enroll in the KAP questionnaire. Household mosquito collections were performed both inside and within the immediate peri-domestic area of consenting households. Participants were provided advance notice before the house visit was made. Homes were not sampled if the head of household was absent, unless permission from a primary adult resident was granted as per informed consent during recruitment. Healthcare clinic personnel accompanied Thai technical staff and the primary research investigator to all sampled homes to facilitate collections.

Indoor mosquito collections were performed by Prokopack aspiration tool which has been proven to collect different mosquito species under different entomological conditions [28]. Sampling was performed both in the morning and evening hours to maximize probability to capture vectors based on expected resting behaviors exhibited from *Aedes* spp. and *Anopheles* spp. Prokopack collections were conducted for up to a total of 30 minutes or approximately 3 minutes per 9 m^2. Collections were made from all rooms, if permitted, and occurred between 6:00–9:30 AM and 4:30–8:30 PM. One of two outdoor mosquito trapping methods was used depending on symptomology of the study participant. An unbaited® BG-Sentinel (Biogents AG, Regensburg, Germany) mosquito trap (BGS) was used to maximize outdoor collection of dengue vectors and an un-baited Black-hole UV light trap was used to maximize capture of outdoor malaria vectors. A single BGS trap was placed outdoors in close proximity to the entrance of the home where individuals were commonly aggregating and was operated continuously between 6:00 AM –6:00 PM. UV light traps were hung from roofs generally within 5 meters from the entrance of the home and operated continuously between 7:00 PM – 7:00 AM. Captured mosquitoes from all collection methods were held in individually labeled cartons by house code then killed on-site for identification to *Aedes* spp., *Culex* spp., *Anopheles* spp., *Armigeres* spp., or *Mansonia* spp. using morphological keys [29]. Numbers were recorded on entomologic survey forms by household, collection method, indoor or outdoor location, genus, and gender.

Household construction characterization

Household construction characteristics were surveyed at all homes where mosquito collections were performed. Targeted variables of interest included materials used for roof, wall, and floor construction. The percentage of eave gaps, defined as the opening between the wall and roof which would allow movement of insects from outside to inside the home (i.e. exposure) was also evaluated. Additionally, the presence and condition of window screens and total amount of windows available was also recorded.

Mosquito processing

Mosquito processing for malaria parasite or dengue virus infection status occurred at Kasetsart University in Bangkok, Thailand. A nested PCR amplification process was conducted for the detection of each *Plasmodium falciparum* and *Plasmodium vivax* within all captured female *Anopheles* spp. mosquitoes using previously established protocols [30]. The DEN-K050VectorTest® Dengue Antigen Assay (Thousand Oaks, CA) was used for assessing dengue virus infection in all female *Aedes spp.* samples following manufacturer's specifications [31].

Data analysis

Data was digitally entered into a master key using Microsoft Excel software and transferred to SPSS software package (SPSS version 21, Chicago, IL). Trends and patterns associated with KAP survey responses, mosquito abundance, genus type, and household construction variables were evaluated in each Phanom and Pong Nam Ron study locations. Sample data was explored for normality using box plot and histogram outputs. Statistical associations including geometric mean and median reporting were based on the deviation of sample data from a normal distribution and were performed with analyses not sensitive to normality assumptions and small sample size. Categorical data was analyzed using a Chi-Square Test of Independence or Fisher's Exact Test. A Mann–Whitney Test was used to compare continuous variables verses categorical variables. Statistical outcomes with a p-value of 0.05 were considered significant.

Results

Demographics

In total, 64 persons who presented at the healthcare facilities with either malaria or dengue fever symptoms participated in the study between May-June 2014 (Table 1). Eleven participants were from Pong Nam Ron, all of whom displayed symptomology relating to malaria and 53 were from Phanom, 51 of whom displayed symptomology relating to malaria and two related to dengue fever. Seventy-three (8/11) percent of the participants in Pong Nam Ron were male, had a median age of 32 (IQR = 21–50), and identified as having a primary school education. Fifty-eight percent (31/53) of the participants in Phanom were male, had a median age of 39 (IQR = 32–47) and half identified as having up to a secondary education. One participant was unable to select his education. Almost all of the participants (73%; 7/11) within Pong Nam Ron had a farming occupation. Similarly in Phanom participants' primary occupation was farming (62%; 33/53), followed by government officer (13%; 7/53) and other (11%; 6/53). The two participants seeking dengue fever diagnoses were aged 18 and 53, both having a secondary education and an occupation as either a

Table 1 Demographic information of study participants from Phanom (urban) and Pong Nam Ron (rural) study Districts in Thailand

Participant characteristic	Phanom (urban)	Pong Nam Ron (rural)	Total	P-value
	n (%)	n (%)	n (%)	
Total	53 (83)	11 (17)	64 (100)	
Disease				
Malaria	51 (96)	11 (100)	62 (97)	
Dengue	2 (4)	0 (0)	2 (3)	
Gender				0.505
Male	31 (58)	8 (73)	39 (60)	
Female	22 (41)	3 (27)	25 (39)	
Age				0.627
18-29	11 (21)	5 (45)	16 (25)	
30-45	26 (49)	3 (26)	29 (45)	
46-60	13 (25)	3 (27)	16 (25)	
>61	3 (5)	0	3 (5)	
Education				0.168
None	1 (2)	1 (9)	2 (3)	
Primary	21 (40)	8 (73)	29 (46)	
Secondary	26 (50)	2 (18)	28 (44)	
Bachelor	3 (6)	0 (0)	3 (5)	
Missing	1 (2)	0 (0)	1 (2)	
Occupation				0.044*
Farmer	33 (62)	7 (64)	40 (62)	
Business	1 (2)	0 (0)	1 (2)	
Employer	3 (6)	4 (36)	7 (11)	
Government officer	7 (13)	0 (0)	7 (11)	
Unemployed	3 (6)	0 (0)	3 (5)	
Other	6 (11)	0 (0)	6 (9)	

*Significant p-value.

government officer or other, respectively. Both study sites had a median of 4 persons living within their household (IQR = 3–5) and over 50% (Pong Nam Ron: 6/11 and Phanom: 33/53) of the respondents had no history of an infected case within their home. Occupation was the only variable significantly different between the study sites, however interpretation should be cautioned as the small sample size led to a sensitive test and significance may not be due to actual association (χ^2 = 11.38, df = 5, N = 64, $p < 0.05$).

Over half the participants (64%; 7/11) in Pong Nam Rom had never been to the selected healthcare facility before the study visit; however more than half (66%; 34/53) of the participants in Phanom had been to the selected healthcare facility for related testing, most often between 1–2 times prior. When asked why the participant visited the clinic, most from Pong Nam Ron responded that a friend, family member, or employer had recommended it. Participants from Phanom traveled to their designated facility based on close proximity to the house or for "other" reasoning. One participant seeking malaria diagnoses at the Phanom facility reported having received a negative result at the nearby malaria clinic and came to the hospital for diagnostic verification. Additional statements included that the Phanom hospital was able to test for more diseases as compared to the malaria clinic. In both locations, over 70% (49/64) of participants traveled to the clinic by driving.

Knowledge, attitudes, and practices relating to malaria and dengue fever

Results indicated a significant difference between study sites and the frequency of participants having heard of malaria and/or dengue fever prior to their visit to the facility ($p = 0.002$). Sixty three percent (7/11) of participants within Pong Nam Ron confirmed to having heard of malaria or dengue fever before their visit in comparison to 98% (52/53) in Phanom. Over half (58%; 31/53) of participants from Phanom had suffered from malaria at a prior time and one out of the two dengue fever participants had suffered from dengue fever previously. In contrast, about a quarter (27%; 3/11) of the respondents in Pong Nam Ron had suffered from malaria at least once prior to their visit (Table 2).

When participants in Pong Nam Ron were asked to identify how they acquired malaria or dengue fever, 82% (9/11) of the responders correctly identified 'mosquito' as the vector of transmission. Responses of 'dirty water' and 'another person' were also selected as causes of malaria and dengue at this study site. Asked the same question, 96% (51/53) of the participants in Phanom correctly identified the 'mosquito' as the vector. Responses of 'contaminated food', 'dirty water', and 'another person' were also selected as causes either separately or in conjunction with the belief of the mosquito vector (Table 2); of which the belief in 'dirty water' was the only variable of significant difference between study sites ($p = 0.023$). Further exploration revealed that some participants from both study sites who identified mosquitoes as the vector for malaria or dengue fever, could also state the genus. Twelve participants reported aedine mosquitoes as the vector responsible for causing malaria disease, seven participants correctly named anopheline mosquitoes as the vector for malaria, and five could match both aedine and anopheline to the appropriate disease. The KAP survey also revealed a significant difference in the source of information from whom participants' received their knowledge specific to malaria and/or dengue fever ($\chi^2 = 24.3$, df = 6, N = 64, $p < 0.001$). However, interpretation should be cautioned as the small sample size led to a sensitive test and significance may not be due to actual association.

Table 2 Knowledge of vector-borne diseases from participants in Phanom (urban) and Pong Nam Ron (rural) study Districts in Thailand

Survey question	Phanom (urban) n (%)	Pong Nam Ron (rural) n (%)	P-value
"Have you heard of malaria or dengue fever?"			0.002*
Yes	52 (98)	7 (64)	
No	1 (2)	4 (36)	
"Have you suffered from M/D before?"			0.096
Yes	31 (58)	3 (27)	
No	22 (42)	8 (73)	
"Do you know how you can get malaria and/or dengue fever?"**			
Contaminated food	2	0	1.0
Bite from a mosquito	51	9	0.410
Another person	3	1	0.508
Dirty water	1	2	0.023*

*Significant p-value.
**Forced choice question; multiple responses recorded for each respondent.

Table 3 Knowledge, attitudes, and practices from participants in Phanom (urban) and Pong Nam Ron (rural) study Districts in Thailand

Survey question	Phanom (urban) n (%)	Pong Nam Ron (rural) n (%)	P-value
Who do you get your information about malaria and dengue from?			<0.001*
None	1 (2)	4 (36)	
Television	4 (8)	0 (0)	
School teacher	1 (2)	0 (0)	
Government official	31 (58)	2 (18)	
Family member	5 (9)	4 (37)	
Other/More than one	11 (21)	1 (9)	
"I am protected from malaria if I sleep under an insecticide treated bed net"			0.933
True	46 (87)	10 (91)	
False	5 (9)	1 (9)	
Don't Know	1 (2)	0 (0)	
Missing	1 (2)	0 (0)	
"I am only at risk of getting bit by a mosquito at night"			0.505
True	30 (56)	8 (73)	
False	21 (40)	3 (27)	
Missing	2 (4)	0 (0)	
"How often do you sleep under a bed net?"			0.220
Every night	32 (60)	8 (73)	
A few times a week	4 (8)	2 (18)	
Rarely	17 (32)	1 (9)	

*Significant p-value.

In Pong Nam Ron, family members were the main source of malaria and dengue fever information whereas over half of the participants in Phanom reported a government official or village health volunteer as the main source of information (Table 3).

When participants were assessed on their attitude and knowledge of protection from malaria by sleeping under an insecticide treated bed net, all but one participant in Pong Nam Ron answered 'true'. Similarly, 87% (46/53) of participants in Phanom also believed the statement was true. Two participants were unable to definitively answer this question. A response from one participant in Phanom stated that while the statement is true "*it only protects you from malaria 90% of the time*". About three-quarters of participants (73%; 8/11) in Pong Nam Ron perceived their only risk to getting a mosquito bite was during the night. In Phanom, 57% (30/53) believed this to be true and one participant even stated "*there are no infective mosquitoes during the day time*". Some participants within Phanom perceived most of their risk to occur at night, but only sometimes during the day. These participants ultimately answered 'false' to 'sleeping under an insecticide treated bed net will protect me from malaria' (Table 3). Overall, there were no significant differences indicated in both knowledge of bed net use ($p = 0.933$) and attitudes towards risk from mosquito bites ($p = 0.505$) between study sites. Additionally, no significant difference existed with regard to the frequency participants reported sleeping under a bed net ($p = 0.220$). When participants in Pong Nam Ron were asked how often they sleep under a bed net, 73% (8/11) responded with 'every night', followed by a 'few times a week' (18%; 2/11) and 'rarely' (9%; 1/11). When asked the same question in Phanom, 60% (32/53) responded with 'every night' and 32% (17/53) responded 'rarely' (Table 3). At both study sites, 10% of participants reported owning 0 bed nets, with a median of 2 bed nets owned by each participant. As one participant stated, "*I do not believe in sleeping under an impregnated bed net, if your hand touches the bed net you can still get bit*".

When participants were asked to list prevention methods they knew would protect them against diseases caused by mosquitoes, bed nets and repellents were the most common responses from participants in Pong Nam Ron, followed by wearing long sleeves, pants and burning coils. Other prevention methods mentioned included using fire or smoke, a fan, treating containers with temephos (a larvacide), and fish. Participants in Phanom also commonly responded with bed nets and repellents as methods to prevent diseases caused by mosquitoes as

well as destroying containers, temephos, and coils followed by fire or smoke, long pants and sleeves, IRS or fogging, a fan, bug zapper, fish, eating healthy, and exercise. One urban participant reported that placing salt water in cups underneath table legs was an appropriate control method (data not shown).

In Pong Nam Ron, when asked how the participant could tell if another person was sick with malaria, 73% (8/11) of patients stated they didn't know and only 27% (3/11) were able to correctly mention one or more correct symptoms, with headache and fever being most frequently mentioned. When participants in Phanom were asked the same question, 82% (42/51) were able to correctly name one or more corresponding symptoms with headache, fever, and chills most commonly mentioned. Additionally, both dengue fever participants were able to correctly identify at least one symptom related to dengue, mentioning a high fever, headache and rash; with one of the dengue fever participants stating that malaria and dengue fever had the same symptoms, mentioning headache, cyclic fever, and chills.

Household mosquito control practices

When participants were asked about protective measures they use in their home to control mosquitoes, 54% (6/11) of participants within Pong Nam Ron and 90% (45/50) in Phanom responded that they paid for a personal protective tool outside of what might have been given to them by the Thai MOH and/or other organizations. Of the products purchased, repellent creams and coils were the most frequently named products in both Pong Nam Ron and Phanom, although in Phanom, bed nets were also included in the most frequently named products (Additional file 1). However, one participant in Phanom stated, "*The officer gave me a bed net, but I do not use because it is too small so I bought a new one*". Another participant declared, "*I don't like the bed net with chemical, I bought one without chemicals*". The majority (81%; 52/64) of participants within both study sites considered mosquitoes to be a problem at their house and 90% (58/64) reported seeing mosquitoes inside their home every day. When asked whether the participant would buy additional products to protect themselves out of fear of disease or to protect against annoying mosquito bites, the overwhelming response in both study sites was to prevent mosquito bites (Pong Nam Ron: 8/9 and Phanom: 38/45).

Overall, participants from both study sites reported having personnel come out to their household to discuss ways to prevent malaria and/or dengue fever. Participants from Pong Nam Ron reported government officers as the most common personnel to visit and in Phanom both government officers and voluntary health workers were regularly mentioned. Reported activities performed by these personnel included: IRS spraying, verbal education, providing bed nets, temephos treatment of containers, and performing blood testing.

Household mosquito collections

In total, 53 participants gave consent to study personnel for household construction characterization and mosquito collection. Of those, a total of 34 households were selected; 8 in Pong Nam Ron (rural) and 26 in Phanom (urban). Final sample size was a result of both logistical feasibility and accessibility to enter homes at time of survey. All 8 homes characterized in Pong Nam Ron were associated with participants seeking malaria treatment. In Phanom, 25 homes were from participants seeking malaria diagnoses and 1 participant for dengue fever. A total of 149 *Anopheles* spp., 209 *Aedes* spp., 438 *Culex* spp., 38 *Armigeres* spp., and 7 *Mansonia* spp. were captured from all trapping methods throughout the study period. The total number of mosquitoes captured indoors by prokopack across both study sites was 501 (Pong Nam Ron: 162 and Phanom: 339) from a total of 6 and 24 homes, respectively. The total number of mosquitoes collected by outdoor UV light trap was 414 across both sites (Pong Nam Ron: 44 and Phanom: 370) from a total of 6 and 24 homes, respectively. Seven mosquitoes were captured by outdoor BG trap from the 1 home sampled in Phanom.

Overall, the total number of mosquitoes collected inside the homes at Pong Nam Ron was higher than outside while collections made in Phanom was highest outside surveyed homes (Additional file 2). The geometric mean number of mosquitoes captured indoors in homes within Pong Nam Ron was 6.8 and outside the home was 3.7. In Phanom, the geometric mean number of mosquitoes collected indoors was 5.43 and outside the home was 10.75. At both study sites, *Culex* spp. was the dominant mosquito genus collected from outdoor traps as well as within homes at Pong Nam Ron. Over 50% of the collections performed within homes at Phanom were *Aedes* spp. The majority (14%) of anophelines captured outside in Phanom were from the UV light trap whereas in Pong Nam Ron (rural) equal proportions of anophelines (27%) were collected using Prokopack aspiration (inside) and the UV light trap (outdoors). A significant difference was indicated in the total number of mosquitoes found outside the home between study sites ($U = 134$, $n_1 = 7$, $n_2 = 25$, $p < 0.05$). However, the total number of mosquitoes found within the home was not ($p = 0.560$).

Household characterization

Of the 8 homes surveyed in the rural site of Pong Nam Ron, the majority were constructed with a tin roof, a wood floor, wood or mixed walls, and 50% had an eave

gap of >75%. On the contrary, the majority of surveyed homes in Phanom had a concrete roof, tile floor, cement walls, and an eave gap < 25% (Table 4). Results indicated a significant difference in roof ($\chi^2 = 25.8$df = 3, N = 34, $p < 0.001$), wall ($\chi^2 = 21.2$, df = 3, N = 34, $p < 0.001$), floor construction ($\chi^2 = 23.6$, df = 3, N = 34, $p < 0.001$), and eave gap ($\chi^2 = 15.23$, df = 3, N = 34, $p = 0.002$) between the two study sites; however interpretation should be cautioned as the small sample size led to a sensitive test and significance may not be due to actual association.

Mosquito collections, household eave gap and relation to mosquito control practices

In Pong Nam Ron mosquitoes were collected from participant homes that represented eave gap classifications of <25%, 25-50%, and >75%. Homes with < 25% eave gap had an overall higher abundance of mosquitoes inside homes from Pong Nam Ron in comparison to Phanom (Figure 2). Homes in Pong Nam Ron that had an eave gap > 75% had a geometric mean of 5.8 mosquitoes collected from indoors in comparison to 8.4 mosquitoes which was the geometric mean for homes <25%. Alternatively, households within Phanom represented eave gap classifications of <25%, 25-50%, and 50-75%. Homes with an eave gap of 50-75% had a geoemetric mean of 26.5 mosquitoes collected indoors in comparison to 4.6 mosquitoes collected from homes <25% eave gap (Additional file 3).

Table 4 Frequency of household construction characteristics of study participant homes in Phanom (urban) and Pong Nam Ron (rural) study Districts within Thailand

	Phanom	Pong Nam Ron	Total	P-value
	n (%)	n (%)	n (%)	
Total	26 (76)	8 (23)	34 (100)	
Roof construction				<0.001*
Concrete/Masonry	24 (92)	1 (13)	25 (74)	
Tin	0 (0)	6 (75)	6 (17)	
Mixed	1 (4)	1 (12)	2 (6)	
Other	1 (4)	0 (0)	1 (3)	
Floor construction				<0.001*
Concrete	5 (20)	3 (38)	8 (23)	
Tile	21 (80)	0 (0)	21 (62)	
Dirt	0 (0)	1 (12)	1 (3)	
Other	0 (0)	4 (50)	4 (12)	
Wall construction				<0.001*
Cement	24 (92)	1 (12.5)	25 (74)	
Bamboo	0 (0)	1 (12.5)	1 (3)	
Wood	0 (0)	2 (25)	2 (6)	
Mixed	1 (4)	2 (25)	3 (8)	
Other	1 (4)	2 (25)	3 (9)	
% of Eaves open				0.002*
Less than 25%	14 (54)	3 (38)	17 (50)	
25-50%	10 (38)	1 (12)	11 (32)	
50-75%	2 (8)	0 (0)	2 (6)	
More than 75%	0 (0)	4 (50)	4 (12)	

*Significant p-value.

Specifically, *Culex* spp. was collected in greatest abundance inside homes with < 25% eave gap in Pong Nam Ron (rural) whereas *Aedes* spp. was found in greater abundance within homes with similar eave gap classifications in Phanom (urban). The abundance of *Anopheles* spp. was greatest in Pong Nam Ron as compared to collections in Phanom with similar eave gap openings (Figure 2). The greatest abundance of outdoor mosquitoes represented *Culex* spp. in both study sites (Figure 3). In Phanom, homes with < 25% eave gap had higher outdoor trap collections as compared to homes with a greater eave gap. *Aedes* spp. were found in greater abundance outside of homes within Phanom as compared to Pong Nam Ron. *Anopheles* spp. were trapped in higher abundance from homes in Phanom verses Pong Nam Ron; although not influenced by eave gap characterization (Figure 3).

Among participants from Pong Nam Ron, bed nets, temephos, repellents, and other household products were reportedly used for household protection from malaria and/or dengue fever. Participants in Phanom mentioned bed nets, coils, repellents, and eliminating mosquito breeding sites. Participants from homes within Pong Nam Ron that had >75% eave gap reported having used more products in general than homes with a 0-50% eave gap (Figure 4). In Phanom, participants reported purchasing more products overall as compared to the rural study site. Repellent and coil usage were methods reported more often from participants whose homes had a 0-50% eave gap verses homes with an eave gap > 50%. In both study sites, participants that reported having no household control methods were from houses with <25% eave gap (Figure 4). In addition, as homes were characterized with less of an eave gap (0-50%), there was a matched participant survey response of lack of bed net use; 100% of participants residing within homes that had an eave gap of >75% responded with the use of a bed net (Additional file 4).

Mosquito infection processing

A total of 84 female anophelines out of the149 collected by both Prokopack and UV light trap methods were processed for the detection of *P. falciparum* and *P. vivax* infection status. Of those tested, 29 were confirmed negative: 3 from collections in Pong Nam Ron and 26 from collections in Phanom. The remaining 55 *Anopheles* spp. samples were collected from Phanom and indicated a false positive infection by malaria nested PCR

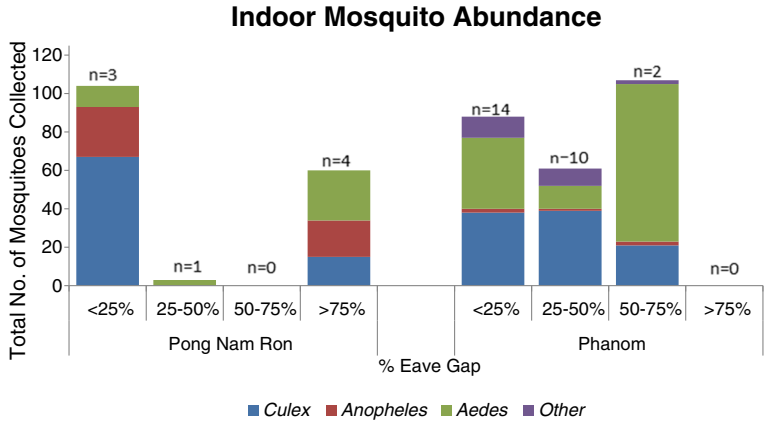


Figure 2 Indoor mosquito abundance at participant households by eave gap classification (n = homes sampled).

protocol. These samples will be retested under a separate nested PCR protocol using alternative primers.

The sampled household from the dengue fever participant resulted in zero female *Aedes spp*. mosquitoes and thus RDT dengue detection was not applicable.

Discussion

Key determinants of successful vector control programs include implementation of site-specific strategies that strengthen community buy-in required for long-term sustainability [10]. The current study employed a mixed method approach to assess relationships among household vector control activities, housing construction characteristics, and individual KAP in relation to malaria and/or dengue fever. Overall, while statistical evidence should be interpreted with caution, results revealed important trends between the two study sites, one urban and one rural, in Thailand that may directly impact household disease prevention practices.

Participants' perceptions of risk of acquiring malaria and dengue fever were similar within both sites. Over 75% of the participants in Pong Nam Ron and 90% of those in Phanom were correctly able to identify the mosquito as the primary vector responsible for transmitting malaria and dengue fever. However, participants' were unable to name the genus of the mosquito vector (*Anopheles or Aedes)*, or incorrectly identified the mosquito genus associated with a particular disease etiology despite government officers and healthcare personnel from study healthcare facilities confirming that educational campaigns provide the proper genus name of the mosquito responsible for disease transmission. This confusion with regard to mosquito identification highlights the potential gap in absorbing information presented during educational campaigns and how this may directly translate to inappropriate actions used to protect themselves from malaria and dengue fever in their home. The inability to recall that malaria and dengue diseases are transmitted by two

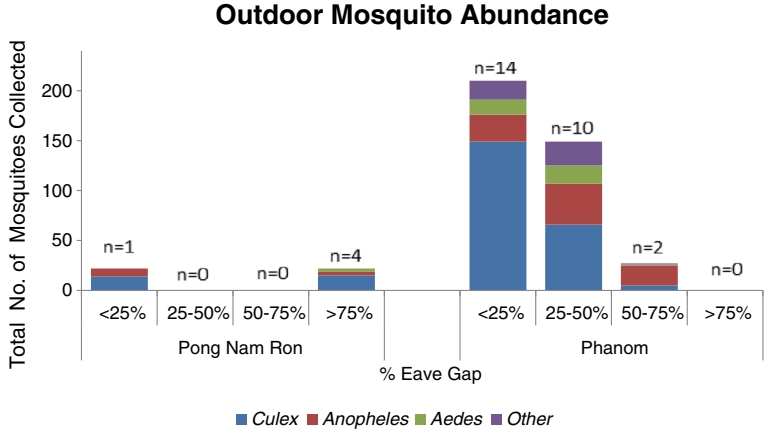


Figure 3 Outdoor mosquito abundance at participant households by eave gap classification (n = homes sampled).

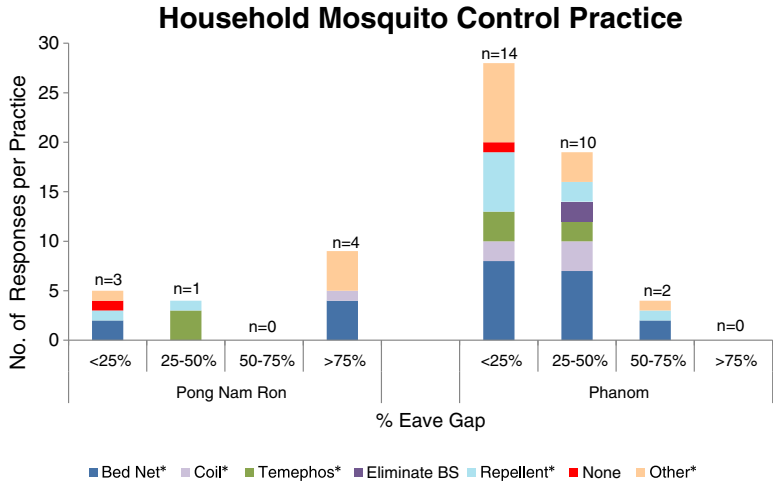


Figure 4 Mosquito control products utilized at participant households by eave gap classification (n = homes surveyed). *Product mentioned was purchased; "other" refers to bug zappers and fans.

different mosquitoes which exhibit different behaviors and biology, most importantly that *Aedes aegypti* dengue vectors bite during the day and *Anopheles spp.* malaria vectors bite predominately at night, plays a crucial role in the personal protective behaviors practiced by the participant. Without this basic understanding, a participant's perception of risk and how best to prevent exposure to these pathogens may be compromised. This may be what underlies the shared belief from more than half of participants that mosquitoes are only capable of biting in the evening, with one participant even stating, "*there are no infective mosquitoes during the day time*".

Trends in participants' knowledge related to prevention methods for mitigating malaria and dengue fever similar among the two study locations. This was surprising as increasing socioeconomic status often leads to more access to education and potential access to knowledge surrounding these diseases. In most cases, more than one prevention method was mentioned by each participant, suggesting education campaigns are adequately addressing this issue. However, there were some shared knowledge gaps in understanding why these products are used as one participant explained, "*I use a coil in my home, but I do not know why*". These knowledge gaps may be due, in part, to current practices of community education. Specifically, malaria clinics are primarily responsible for implementing intervention campaigns for malaria and dengue fever campaigns and household intervention methods, such as distribution of temephos and elimination of breeding sites, are the responsibility of separate healthcare facilities. Both approaches incorporate home visits, resulting in residents being exposed to intervention and campaign work for both diseases which may create an overabundance of information thereby making it difficult for residents to distinguish appropriate household preventive measures for each malaria and dengue fever. The result is a potential false sense of practiced protection. Motivation for practicing mosquito control methods in both study sites was predominately due to annoyance from mosquito bites versus a concern for disease transmission. This suggests that no relationship yet exists between the understanding that even one mosquito is enough to cause a health concern and transmit disease. Even after performing mosquito collections at participants' homes, such expressions like "*that is not too many*" highlights the continued need for health education.

Nearly every participant considered mosquitoes to be a problem at their home and purchased products for household mosquito control outside out of what was given to them for free. The fact that over half of the participants from Phanom reported having suffered from malaria at a prior time may have contributed to the numbers and types of products purchased. The choice of products purchased between study sites showed trends related to occupation. Participants from Phanom (urban) were most often associated with farming occupation, particularly rubber plantation farming, which predisposes participants being exposed to anopheline vectors during the nighttime hours because this is when latex is harvested. Results revealed not only a higher abundance of mosquitoes found outside the home in comparison to indoors, but specifically a high abundance of *Anopheles* spp. Related occupations from this area may have led to participants' to have perceived mosquito annoyance outside the home and therefore influenced products and behaviors practiced such as using topical repellents and temephos.

In comparison, participants from Pong Nam Ron (rural), where agriculture farming particularly fruit orchard farming,

identified themselves as an employee or farmer. Such occupations predispose participants to daytime biting mosquitoes when fruit it typically harvested. Indeed, overall mosquito abundance and anophelines in particular, was greater indoors in comparison to outside the home. Participants from Pong Nam Ron not only reported a more frequent practice of sleeping under a bed net every night, but also mentioned the purchase of coils and fans that are used inside the home. This difference in occupation, and/or associated discretionary spending, may have influenced motivation for products purchased and household practices performed. Lastly, while the majority of participants stated they practiced personal protection by sleeping under a bed net, a number of participants revealed that the bed net used was not the recommended ones provided by healthcare officials. Sometimes participants would purchase their own untreated nets citing negative attitudes towards chemicals or the small bed net size. Further investigation into the beliefs and perceptions of treated versus untreated bed nets is warranted and would be beneficial to better inform on safety of insecticides to humans, patterns of net distribution, re-treatment instruction as well as proper usage of nets in these target populations. Of particular interest, participants who reported 'rarely' sleeping under a bed net came from both study sites in housing construction with a 0-50% eave gap (Additional file 4) suggesting that as housing structure improves, participant's individual health practices are influenced.

Household construction characteristics were also shown to be associated with mosquito density trends that may have influenced survey responses between sites. For instance, in general there was improved housing construction observed in Phanom, the urban setting, whereby concrete was used for roof and wall construction, with flooring being tiled. The homes also exhibited eaves gaps representing <25%, which may have mitigated exposure to mosquitoes inside the home. However, when comparing entomological data from homes with similar eave gap percentage between study sites, we did not see strong trends suggesting greater mosquito density within homes from Pong Nam Ron whose housing construction materials were overall poorer. What the data did reveal was that nearly twice as many mosquitoes were collected on average within homes in Pong Nam Ron as compared to outside the home. This was a stark difference as compared to Phanom where nearly twice as many mosquitoes were collected on average outside the home as compared to within. A larger sample size (i.e., community level) is necessary to further explore relationships among housing, vector exposure, and KAP.

There were several limitations of this study that should be noted. First, the study period occurred during the rainy season, which resulted in heavy and prolonged rain lasting several days. Small participant sample sizes might have resulted from difficulty traveling to healthcare facilities and/or fewer persons being able to seek medical attention and not necessarily representative of actual disease presence within the study site. Additionally, as the study design employed passive compared to active surveillance, this most likely further reduced the number of potential participants. However, based on MOH data the year prior to the study, it was anticipated that cases of both malaria and dengue fever would be presenting at study facilities during the months of May and June [24].

Potential biases in mosquito collections may have also occurred as a result of providing advance notice of when household visits would occur and/or the accompaniment of government officers to the participant homes. Both scenarios may have altered homeowner mosquito control practices prior to study personnel arrival.

Lastly, limitations occurred in the total number of mosquito collections and household characterization surveys that were able to be performed which increased the chance for bias due to low replication. Larger study teams that can be used to cover more area and greater effort to sensitize the participant on link of mosquito collections to overall program goal are recommended for future studies to increase willingness to participate and household sample size. The socioeconomic status of participants influenced the placement of UV light traps used for mosquito collections (i.e., access to electricity) that resulted in mosquito trapping being performed up to 500 meters from the home. In those instances, captured mosquitoes may not represent densities and/or species immediately outside the participant's home and potentially encountered by the participant. Finally, there were also two households that had a fire with smoke during mosquito collections that may have decreased mosquitoes inside the homes thereby misrepresenting actual densities.

Findings from this pilot study have identified several areas where government campaigns and interventions could be targeted to enhance individual health behavior and household mosquito control practices. This includes initiating an open platform collaborating educational campaign for both malaria and dengue fever among healthcare facilities. Combining efforts simultaneously may allow for a more cohesive intervention program. A mobile device platform could be used to track activities of village health volunteers and Ministry of Health personnel from their respective campaigns. Data tracking the home visited, intervention actions performed, education discussed, and/or any positive cases reported by active surveillance could be collected. This mobile platform could be monitored in real-time and be accessed by both parties to provide a more comprehensive approach of education, outbreak, or clusters of positive cases in addition to filling any gaps in intervention strategies or education needed to tackle both

vector-borne diseases. One possible intervention action to be performed during household visits by healthcare workers may be to incorporate occupation linked vector control strategies. Occupation-based interventions, such as DEET impregnated soap, insecticide treated hammocks and personal clothing, have been shown to reduce the number of malaria cases in countries such as Pakistan, Afghanistan, and Vietnam [32]. Similar approaches may prove useful within the current study communities based on trends associating occupation to the type of vector control products purchased by homeowners identified in our surveys.

Another collaborative approach could reflect a "Village Health Day" where available medical services are held within the community. Opportunities to increase access to mosquito control tools such as repellent or coils could be distributed through such organized events. Sample data suggests that participants from urban areas purchased more mosquito control vector tools in comparison to participants from the rural setting. Subsidizing these products during select campaigns would benefit communities with less access to these products, and provide an opportunity for direct outreach and information exchange. Either of these community-level approaches could greatly benefit resource-limited facilities as well as streamline vector-borne disease approaches for better up-take. Finally, increasing capacity to detect multiple vector-borne diseases at a single healthcare facility, or increasing the number of point of care treatment access sites would greatly benefit community members. Convenience to clinics factored into participants' treatment seeking behavior. Increasing the number of clinics available for diagnostic testing may encourage more frequent facility visits and therefore reduce probability of disease outbreaks due to human reservoirs. A specific example would be the introduction of dengue RDT kits to malaria clinics.

Conclusion

Overall, education from malaria and dengue fever intervention campaigns is reaching the intended target populations. Target populations are aware of the presence of mosquitoes and that they serve as the potential vector for transmitting malaria and dengue fever diseases. However, data from this pilot study suggests there are gaps in knowledge and perception of risk across participants in both study sites. The current pilot study serves as a platform for future longitudinal studies to assess individual perceptions and household practices related to the prevention of malaria and dengue fever. Continued monitoring of the variables measured here would be expected to facilitate an understanding of disease transmission trends as the study locations and serve as a tool to guide vector control strategies, including educational campaigns, within communities at-risk for these diseases. Combined, this would inform on best approaches to household mosquito control practices for protection against arthropod-borne disease. Further goals include improving the KAP of endemic populations, reducing misconceptions of risk, increasing overall individual health practices and understanding of preventative tools.

Additional files

Additional file 1: KAP questionnaire responses from participants whose households were sampled for mosquito collections.

Additional file 2: Total abundance of mosquitoes collected both inside and outdoors of participant households (n= homes sampled).

Additional file 3: Geometric mean number of mosquitoes collected inside participant households by eave gap classification (n = homes sampled).

Additional file 4: Reported household bed net use at participant households by eave gap classification (n= homes sampled).

Abbreviations

KAP: Knowledge, attitudes, and practices; MOH: Ministry of Health; IRS: Indoor residual spraying; ITN: Insecticide treated bed nets; BGS: An unbaited ®BG-Sentinel (Biogents AG, Regensburg, Germany) mosquito trap; PCR: Polymerase chain reaction; IQR: Interquartile range; RDT: Rapid detection test.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB participated in the study design, participated in data collection, analysis, interpretation, and write-up, drafted the manuscript and revised the manuscript. JG participated in study design and critically revised the manuscript. NP participated in study design and critically revised the manuscript. TC designed the study, study implementation, and critically revised the manuscript. WT participated in the implementation of the study design. TG participated in implementation of the study design and participated in the field collection. NA designed the study, participated in the study implementation, and critically assessed the manuscript. All authors read and approved the final manuscript.

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The effects of plant essential oils on escape response and mortality rate of *Aedes aegypti* and *Anopheles minimus*

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ABSTRACT: The High Throughput Screening System (HITSS) has been applied in insecticide behavioral response studies with various mosquito species. In general, chemical or natural compounds can produce a range of insect responses: contact irritancy, spatial repellency, knock-down, and toxicity. This study characterized these actions in essential oils derived from citronella, hairy basil, catnip, and vetiver in comparison to DEET and picaridin against *Aedes aegypti* and *Anopheles minimus* mosquito populations. Results indicated the two mosquito species exhibited significantly different ($P<0.05$) contact irritant escape responses between treatment and control for all tested compound concentrations, except with the minimum dose of picaridin ($P>0.05$) against *Ae. aegypti.* Spatial repellency responses were elicited in both mosquito species when exposed to all compounds, but the strength of the repellent response was dependent on compound and concentration. Data show that higher test concentrations had greatest toxic effects on both mosquito populations, but vetiver had no toxic effect on *Ae. aegypti* and picaridin did not elicit toxicity in either *Ae. aegypti* or *An. minimus* at any test concentration. Ultimately, this study demonstrates the ability of the HITSS assay to guide selection of effective plant essential oils for repelling, irritating, and killing mosquitoes.
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Keyword Index: Aedes aegypti, *Anopheles minimus*, contact irritant, spatial repellent, toxic.

INTRODUCTION

Repellents have been used as personal protection against mosquitoes for decades. DEET (*N,N*-diethyl-3-methylbenzamide) was developed by the U.S. Army in 1946 and marketed for public use in 1957. DEET is still considered the most effective and common ingredient in insect topical repellent products but reported side effects including rash, skin irritation, blisters, and eye irritation have occurred (Miller 1982, Amichai et al. 1994, Patel et al. 2012). Picaridin, 2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropylester, also known as KBR3023, was developed by Bayer® AG in 1980 as a DEET alternative. Picaridin was found to be as effective as DEET at repelling mosquitoes (Yap et al. 1998). This compound is considered less irritating to skin and does not damage plastics or fabrics like DEET. In 2000, the World Health Organization (WHO) recommended picaridin as a topical repellent for malaria prevention and the Environmental Protection Agency found no toxic effects using animal testing (Katz et al. 2008). In previous studies, Barnard et al. (2002) reported the repellent effect of four synthetic repellents, DEET, picaridin, p-menthane 3,8-diol (PMD), and IR3535 and determined picaridin displayed the highest repellency effect against the black salt marsh mosquito, *Aedes taeniorhynchus*. Furthermore, Frances et al. (2004) revealed that picaridin had similar repellent properties as DEET against *Anopheles* spp.

Plant-derived essential oils are considered non-toxic, alternative insect repellents for humans (Barnard 1999, Trongtokit et al. 2005, Misni et al. 2009). Efficacious, naturally derived mosquito repellents offer the potential to elicit less offensive side effects to consumers when used properly. Citronella is the most common ingredient used in plant-based mosquito repellents. Phasomkusolsil and Soonwera (2010) reported that citronella had repellent activity against *An. minimus*, *Culex quinquefasciatus,* and *Ae. aegypti* when applied on human skin. Moreover, Bernier et al. (2005) revealed catnip was a good spatial repellent against *Ae. aegypti*, *An. albimanus,* and *An. quadrimaculatus*. Various plant essential oils have been proven to be effective against many mosquito species (Tawatsin et al. 2001, Zhu et al. 2006, Phasomkusolsil and Soonwera 2010). Essential oils obtained from plants can potentially play an important role in repelling vector mosquitoes, therefore potentially reducing pathogen transmission. However, a full characterization of the chemical actions of either synthetic or natural products being considered for use as mosquito repellents must be conducted to select an optimal candidate for product development.

This study used the HITSS assay to investigate contact irritancy, spatial repellency, knock-down, and toxicity responses of *Ae. aegypti* and *An. minimus*, the main dengue fever and malaria vectors in Thailand, respectively, against citronella, hairy basil, catnip, and vetiver oils in comparison with the standard topical repellent DEET.

MATERIALS AND METHODS

Mosquitoes

Susceptible laboratory colonies of *Ae. aegypti* (USDA strain) and *An. minimus* were used for this study. *Aedes*

aegypti eggs were obtained from the U.S. Department of Agriculture, Gainesville, FL. This species has been maintained in the USDA laboratory for over 40 years (Juntarajumnong et al. 2012). The *An. minimus* colony originated from the Bureau of Vector-Borne Diseases, Department of Disease Control, MOPH, Thailand. Both colonies have been continuously maintained in the entomological laboratory insectary at Kasetsart University, Bangkok at 25 ± 5˚ *C* and 80 ± 10% RH with a 12:12 *L:D* photoperiod. Larvae were fed with fish food. Adults were kept in screened cages and provided a 10% sugar solution. Three to five-day-old unfed female mosquitoes were used in this study.

Essential oils

Four plant essential oils of 100% purity were used in behavioral response studies: citronella (*Cymbopogon nardus*), hairy basil (*Ocimum americanum*), catnip (*Nepeta cataria*), and vetiver (*Vetiveria zizanoides*). Three essential oils were provided by Thai-China Flavours and Fragrances Industry Company Limited and catnip oil (*Nepeta cataria*) was received from the USDA Chemicals Affecting Insect Behavior Lab, Beltsville, MD.

Repellents

DEET (*N, N-diethyl-3-methylbenzamide*) and picaridin (2-(2-hydrozyethyl)-1-piperidinecarboxylic acid 1-methyl-propyl ester) were used as standard repellents for comparison. DEET (97% purity) was obtained from the USDA, Beltsville, MD. Picaridin (98.4% purity) was provided by Bayer® Thai Company Limited.

Net impregnation

Essential oils were tested against *Ae. aegypti* and *An. minimus* using concentration ranges between 0.5-3.0% of active ingredient in absolute ethanol. Final test concentrations used were based on the knock-down and mortality response from preliminary studies. Nylon netting was cut into 11 × 25 cm strips. A volume of 1.5 ml test solution was applied evenly to the net strip using a 1000 µl micropipette with a tip. Treated nets were dried for 15 min before being attached to the metal test cylinder. Control nets were treated with only ethanol.

Knock-down and mortality responses assay

Knock-down and mortality were evaluated using both the WHO susceptibility test (WHO 1998) and the HITSS assay (Grieco et al. 2005). Test were conducted for all essential oils, using four concentrations (1, 3, 5, and 10%). For the WHO assay, 25 female mosquitoes, aged three to five days were introduced into holding tubes then transferred to the exposure tube with a treated filter paper. After 1 h in the exposure tube, mosquitoes were returned to the holding tube and provided a 10% sugar solution. Knock-down after the 1-h exposure and mortality at 24 h were recorded. Concentrations selected for HITSS tests were based on initial dose-response assays for both *Ae. aegypti* and *An. minimus* test populations. Based on these results, selected HITSS test concentrations included 0.5, 1, 2, and 3%.

High-Throughput Screening System

The HITSS assay was used to evaluate test compounds for contact irritancy, spatial repellency, and toxicity responses. Standard operating protocols followed that described previously (Grieco et al. 2005, 2007, Achee et al. 2009).

Contact irritancy assay (CIA)

The CIA configuration consisted of a single clear cylinder attached to a metal cylinder, with each end of the cylinders capped. The cylinders were connected with a butterfly valve to allow mosquitoes to escape. Ten female mosquitoes were released into the metal cylinder that contained either an oil treated net or carrier only (matched control). Afer introduction, mosquitoes were allowed to rest for 30 s, then the butterfly valve was opened for 10 min, after which the valve was closed. The number of mosquitoes that escaped into the clear cylinder, those remaining in the metal cylinder, and knocked-down mosquitoes within both chambers were recorded. Six replicates were conducted for each test oil and concentration.

Spatial repellency assay (SRA)

The SRA configuration consists of one clear cylinder attached to two metal cylinders containing either control or treatment netting (see Grieco et al. 2005, Thanispong et al. 2010). Twenty female mosquitoes were introduced into the clear cylinder and allowed to rest for 30 s after which the butterfly valves were opened for 10 min. Following the exposure period, the number of mosquitoes in each metal and clear cylinders was recorded, including knocked-down specimens. Nine replicates for each essential oil and concentration were conducted.

Toxicity assay (TOX)

The TOX assay consisted of a metal cylinder fitted with an end cap on one end and funnel section on the opposite end. The treated net was fixed inside the cylinder of each treatment and matched control assay. Twenty female mosquitoes were transferred to test cylinders and exposed for 1 h. After the 1-h exposure, knocked-down mosquitoes were recorded and all mosquitoes were transferred to clean plastic cups containing a 10% sugar solution. Mortality was monitored and recorded after a 24-h holding period. Six replicates were performed for each treatment and control test, essential oil, and concentration.

Statistical analysis

Contact irritancy data were analyzed using the two-sample Wilcoxon test to evaluate the number of escaped mosquitoes between the treated and control cylinders. A spatial activity index (SAI), based upon the oviposition activity index, as described by Kramer and Mulla (1979), was applied to evaluate repellency response in the SRA where the $SAI = (N_c - N_t) / (N_c + N_t)$; N_c is the number of mosquitoes in the control cylinder and N_t is the number of mosquitoes in the treatment cylinder. The SAI values range from -1 to 1, with zero representing no response. A SAI value close to -1 indicates a larger number of mosquitoes in the treatment

cylinder, suggesting an attractant response. A SAI value close to 1 indicates a larger number of mosquitoes entered the control cylinder, suggesting a repellent response. Spatial repellency data were also analyzed by the nonparametric Wilcoxon signed ranks test to compare the SAI for each test treatment. Toxicity data, to include percent knock-down and mortality at 24 h, was corrected using Abbott's formula and transformed to arcsine square root for analysis of variance (ANOVA). The knock-down and mortality for treatments at each concentration was compared and separated using Tukey's honestly significant difference test at P=0.05 (SAS 2002).

RESULTS

WHO Assay - Knock-down responses and mortality responses

Aedes aegypti showed high knock-down and mortality responses (80-100% to test concentrations of 3% to 10% DEET. Similar results were seen using hairy basil (100%) (Figure 1). Citronella and catnip both demonstrated high knock-down (100%) using the 3% test concentration with mortality ranging from 48.6% using citronella and 2.6% for catnip. Knock-down and mortality responses were not observed against picaridin, and few mosquitoes were knocked down and no mortality was observed when exposed to 3% vetiver oil. At 3-10% DEET and hairy basil both elicited 100% mortality in test populations of *An. minimus*. Citronella and catnip concentrations between 5%-10% resulted in 100% *An. minimus* knock-down and mortality. All concentrations of vetiver produced between 87-100% knock-down and 91-100% mortality. Picaridin tested on *An. minimus* and *Ae. aegypti* showed 0% efficacy in knock-down and mortality rates (Figure 2).

HITSS Assay - Contact irritancy responses

Escape response of *Ae. aegypti* and *An. minimus* varied dramatically depending on the essential oil tested, concentration (0.5-3%), and mosquito species (Figure 2) evaluated. Most *Ae. aegypti* tests indicated significant differences between treatment and control escape responses (P=0.0022), but no significant difference was observed using 0.5% picaridin (P>0.05). All *An. minimus* data showed statistically significant (P<0.05) differences between the treatment and control groups (Figure 3).

HITSS Assay - Spatial repellency responses

Significant differences (P< 0.05) in repellent responses were found between all treatment and control pairs for catnip, vetiver, and DEET against *Ae. aegypti* (Figure 3). Citronella and hairy basil showed significant differences at 2% and 3% test concentrations. No significant difference in spatial repellent response was observed at 0.5% and 1% citronella and hairy basil or with 0.5% picaridin. DEET exhibited SAI values between 0.97 to 1 for all concentrations whereas 0.5% picaridin had SAI negative value of -0.33.

Anopheles minimus results indicated no significant difference (P > 0.05) in 2 and 3% catnip, which also was found in picaridin 0.5%. All concentrations of DEET had SAI values = 1, while picaridin had values of SAI less than 1. Citronella at 1-3% indicated SAI = 1 and hairy basil had SAI values between 0.96 to 1. Catnip showed SAI close to 1 only at 0.5% while 3% catnip produced a negative SAI value (Figure 3).

HITSS Assay - Toxicity responses

In general, knock-down and mortality rates for both mosquito species increased with higher test concentrations of test oils. Citronella, hairy basil, and catnip produced high mortality rates (Figure 4). DEET exhibited the highest knock-down and mortality rates with all concentrations for both mosquito species. Vetiver showed toxicity in *An. minimus* with 59-94% mortality but was non-toxic to *Ae. aegypti* using the test concentrations.

In *Ae. aegypti* 2-3% citronella and catnip showed 100% knock-down and 76-100% mortality. Hairy basil at 2-3% gave high knock-down (98-100%) and mortality rate (99-100%) in *Ae. aegypti*. The results of *An. minimus* indicated that at 1-3% citronella, hairy basil, and catnip displayed between 99-100% knock-down and 82-100% mortality. No toxicity, knock-down, or mortality was detected with picaridin in either mosquito species.

DISCUSSION

Most studies evaluating repellent properties of plant oils against mosquitoes have used a cage or hand–in-cage method to monitor reduction in bite rates (Barnard 1999, Misni et al. 2009, Phasomkusolsil and Soonwera 2010). An excito-repellency test system developed by Chareonviriyaphap et al. (2002) investigate mosquito responses to chemicals, natural or synthetic, in more detail. This includes escape response in the form of non-contact repellency and contact irritation actions. With this assay, Polsomboon et al. (2008) discovered catnip had contact irritant and non-contact repellent actions against *Ae. aegypti* and *An. harrisoni* with different concentrations and Suwansirisilp et al. (2012) found *Cx. quinquefasciatus* displayed stronger escape responses to citronella and clove than *Ae. aegypti*. In addition, the HITSS assay has been developed to characterize the spatial repellency, contact irritancy, and toxicity chemical actions against mosquitoes as part of a chemical screening program (Grieco et al 2005). Achee et al. (2009) used the HITSS to evaluate synthetic insecticides for spatial repellency, contact irritancy, and toxicity responses. Similarly, the HITSS assay was used by Thanispong et al. (2010) to demonstrate contact irritant and spatial repellent responses to six *Ae. aegypti* field strains from Thailand.

The current study is one of few reports characterizing three major chemical actions from plant essential oils using the HITSS. Understanding the types of repelling action against mosquitoes is important for selecting appropriate essential oils for mosquito control. This study found that catnip was a strong contact irritant to *Ae. aegypti* and *An. minimus*. Hairy basil was a better contact irritant to *An. minimus* than *Ae. aegypti*. Citronella and DEET were moderate irritants to *Ae. aegypti,* while picaridin displayed the least irritancy to

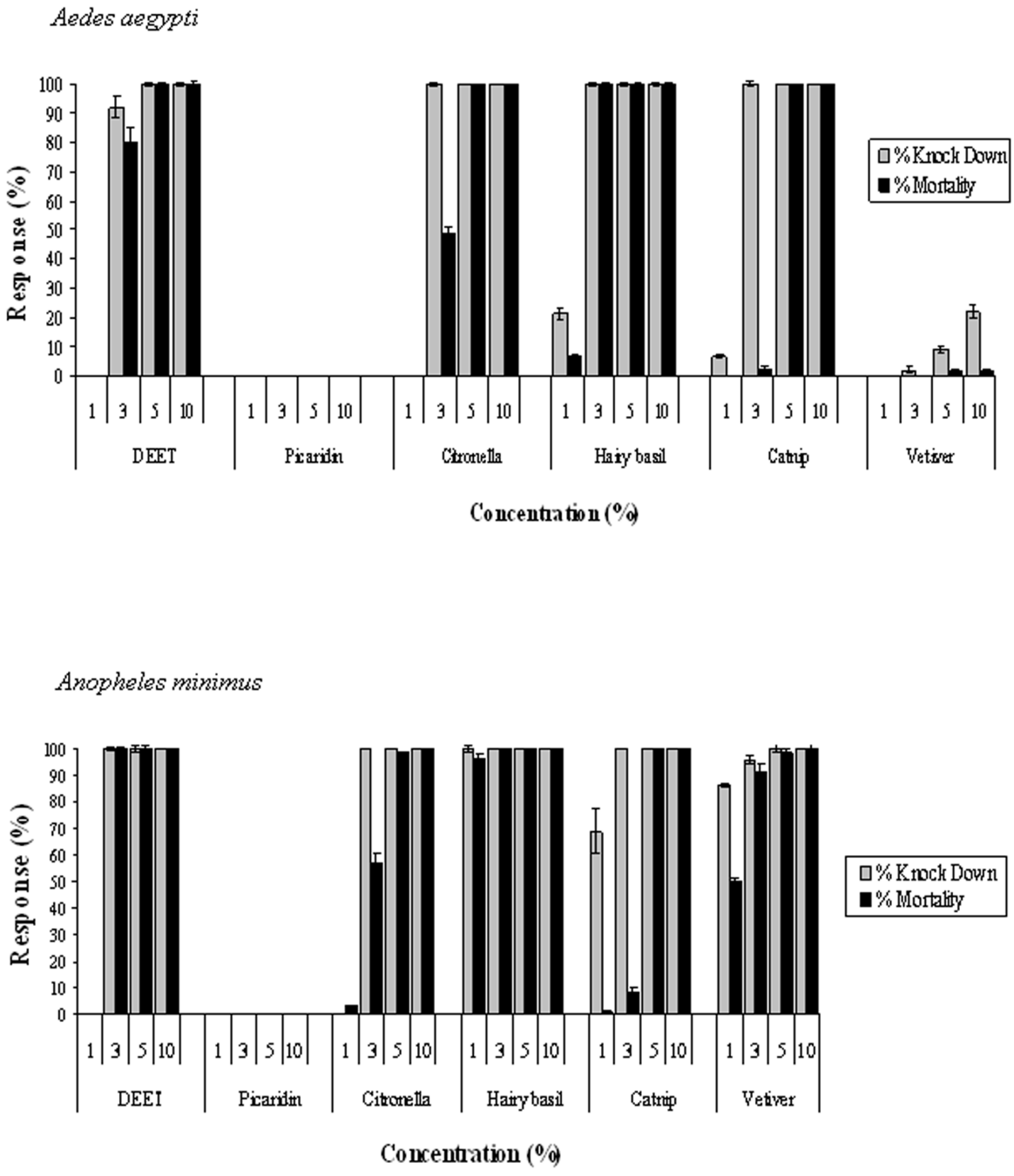


Figure 1. Percent knock-down (KD) and mortality of *Aedes aegypti* and *Anopheles minimus* laboratory strains exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET, and picaridin.

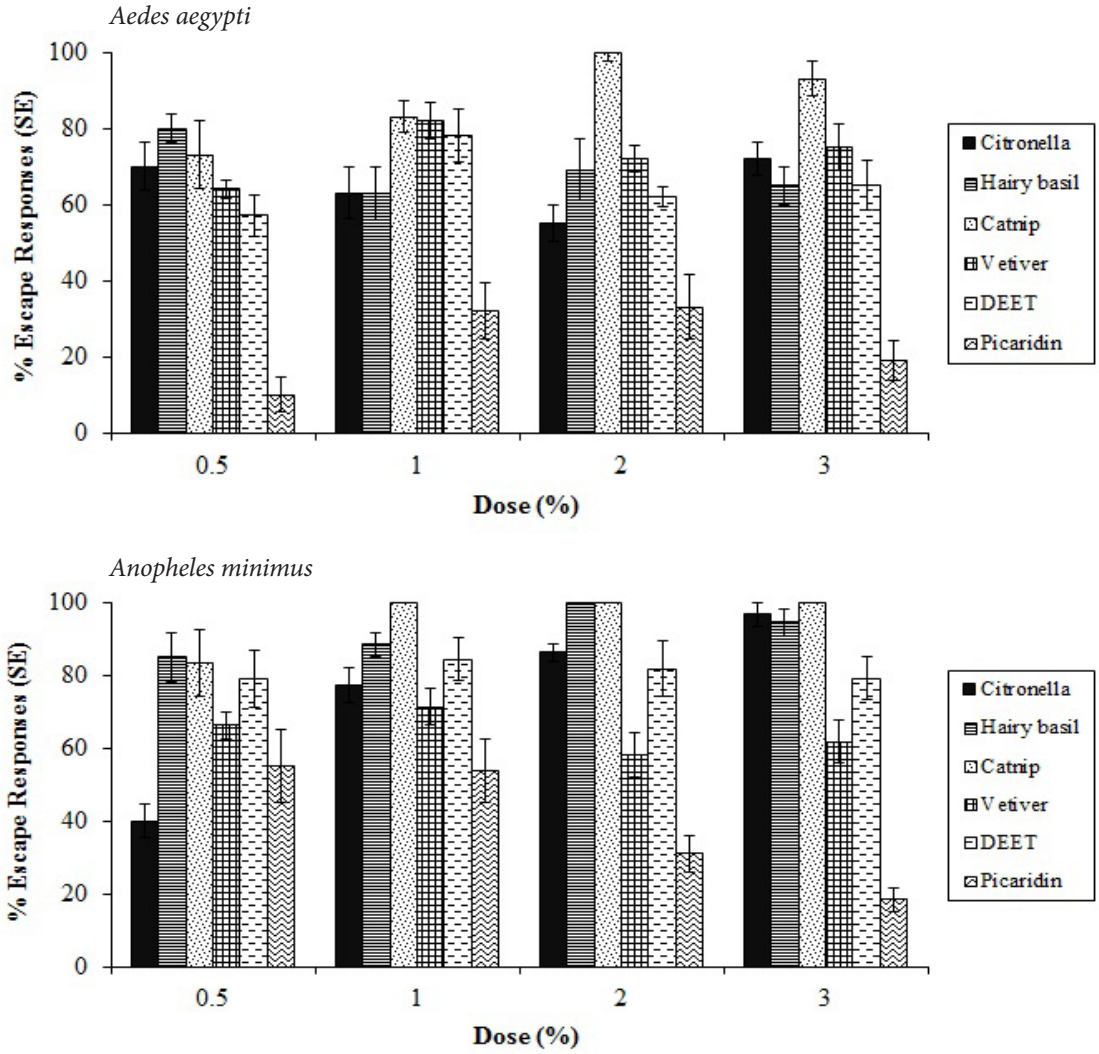


Figure 2. Contact irritancy response (CIA) with standard error (SE) of *Aedes aegypti* and *Anopheles minimus* laboratory strains, exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET, and picaridin.

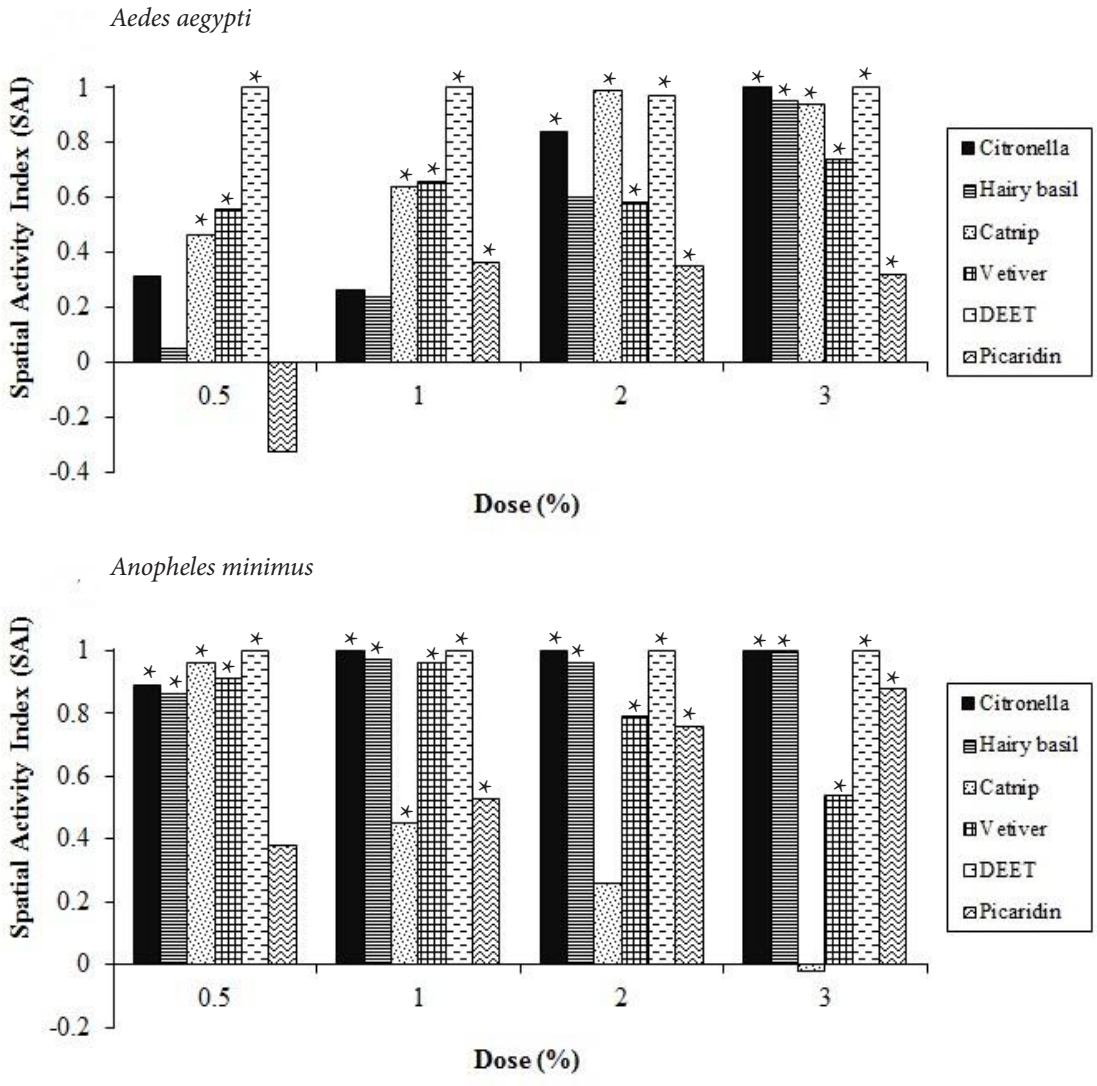


Figure 3. Spatial repellency response (SRA) of *Aedes aegypti* and *Anopheles minimus* laboratory strains exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET, and picaridin. *Denotes significant ($P < 0.05$) repellent response between treatment and matched control treatments.

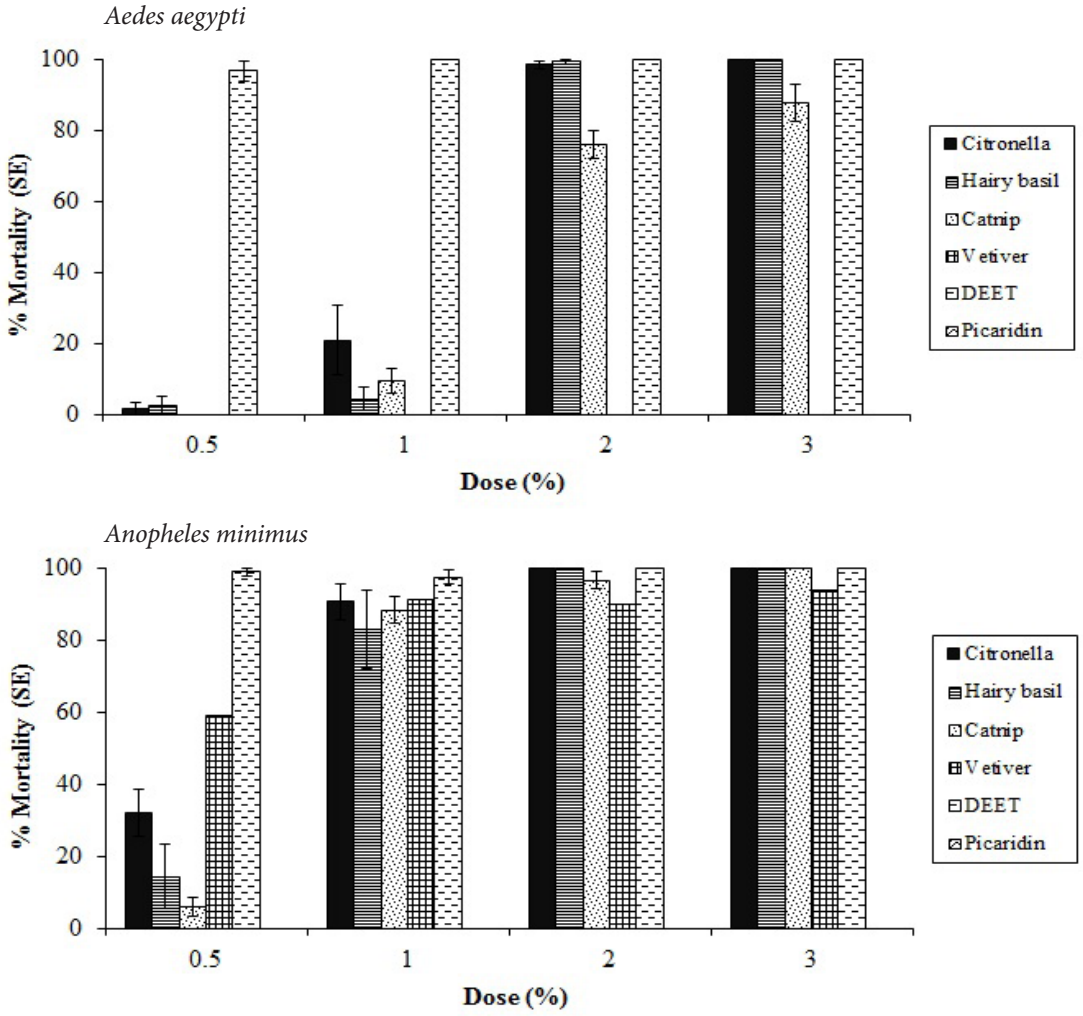


Figure 4. Percentage 24-hour mortality rates (TOX) with standard error (SE) in *Aedes aegypti* and *Anopheles minimus* laboratory strains exposed to four concentrations of citronella, hairy basil, catnip, vetiver, DEET, and picaridin.

Ae. aegypti and *An. minimus*. For spatial repellency, DEET exhibited the highest SAI in both mosquito species. Citronella and hairy basil at 1-3% concentrations gave a similar effect as DEET when tested against *An. minimus* with SAI values close to 1, while only the maximum concentration of hairy basil showed good repellency to *Ae. aegypti*. Deletre et al. (2013) tested the spatial repellency action of 20 plant extracts against *An. gambiae* using HITSS and found citronella was one of twelve plants that had a repellent effect. Our results showed that catnip had the highest repellency against *Ae. aegypti* at 2-3% concentrations but the greatest repellency to *An. minimus* was found at 0.5%. Bernier et al. (2005) used an attraction and attraction inhibitor bioassay and found catnip was an effective spatial repellent. Polsomboon et al. 2008 reported primary chemicals of catnip oil to be E, Z-nepetalactone, Z,E-nepetalactone, and β-caryophellene, all of which were effective against the *Aedes* spp. and *Anopheles* spp. mosquitoes used in evaluation. The dominate chemicals of citronella, hairy basil, and vetiver oils have also been identified (Nuchuchua et al. 2009). Those of citronella oil are limonene and citronellal, while those of hairy basil are 3-carene and caryophylleneare. An active constituent of vetiver oil is vetiveric acid. Picaridin showed the weakest repellent effect to both mosquitoes in our study using SAI values as an indicator and indicated attractancy using a 0.5% concentration with *Ae. aegypti*. Additional results for toxicity assays found DEET was the most toxic compound to *Ae. aegypti* and *An. minimus,* similar to other reports (Licciardi et al. 2006). At high concentrations (2-3%), citronella and hairy basil were also toxic to both test species. There were no toxic effects of picaridin against either species, or vetiver against *Ae. aegypti*. These observations reflect findings from previous studies using picaridin (Licciardi et al. 2006). In contrast, Phasomkusolsil and Soonwera (2011) demonstrated, using the WHO test kit (WHO 1998), that lemongrass and citronella had lethal effects against *Ae. aegypti*, *Cx. quinquefasciatus,* and *An. dirus* while Deletre et al. (2013) found cinnamon, citronella, savory, and thyme elicited toxic effects in *An. gambiae*. DEET was highly effective in eliciting spatial repellency, irritancy, and toxicity in our *Ae. aegypti* and *An. minimus* test populations while these same responses could only be elicited using the highest test concentration of citronella, hairy basil, and catnip *against* both *mosquito* species.

Overall, findings reported here indicate the natural products tested are warranted for development as potential mosquito repellents and insecticides as well as providing contributory data on the mosquito-repellent property of plants against *Ae. aegypti* and *An. minimus*. Other plant-based oils that are registered as mosquito repellents include lemon eucalyptus, geranium, neem, and clove (Barnard 1999, Novakand and Gerberg 2005, Paluch et al. 2010). However, plant essential oils are short-lived in their effectiveness because they can evaporate more quickly than synthetic active ingredients such as DEET. A previous study by Yang and Ma (2005) revealed that eucalyptus oil with 5% vanillin added gave longer protection time against *Ae. albopictus*. Likewise, Kongkaew et al. (2011) reported that citronella with 5% vanillin added was effective for up to six h. Moreover, nanotechnology applied to plant-based repellents has improved the mosquito repellent capacity and extended protection time (Nuchuchua et al. 2009). The data obtained from these laboratory tests should be confirmed in field studies to further support the effectiveness of the tested compounds as human and environmental factors could impact repellent efficacy.

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Comparison of Field and Laboratory-Based Tests for Behavioral Response of *Aedes aegypti* (Diptera: Culicidae) to Repellents

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ABSTRACT The repellent and irritant effects of three essential oils—clove, hairy basil, and sweet basil—were compared using an excito-repellency test system against an insecticide-resistant strain of *Aedes aegypti* (L.) females from Pu Teuy, Kanchanaburi Province. DEET was used as the comparison standard compound. Tests were conducted under field and controlled laboratory conditions. The most marked repellent effect (spatial noncontact assay) among the three test essential oils was exhibited by sweet basil, *Ocimum basilicum* L. (53.8% escaped mosquitoes in 30-min exposure period) under laboratory conditions while hairy basil, *Ocimum americanum* L. and clove, *Syzygium aromaticum* (L.) Merill et. L.M. Perry from laboratory tests and sweet basil from field tests were the least effective as repellents (0–14%). In contrast, the contact assays measuring combined irritancy (excitation) and repellency effects found the best contact irritant response to hairy basil and DEET in field tests, whereas all others in laboratory and field were relatively ineffective in stimulating mosquitoes to move out the test chambers (0–5.5%). All three essential oils demonstrated significant differences in behavioral responses between field and laboratory conditions, whereas there was no significant difference in contact and noncontact assays for DEET between the two test conditions ($P > 0.05$).

KEY WORDS *Aedes aegypti*, excito-repellency, response, essential oil, DEET

Aedes aegypti (L.) is a common and widespread mosquito in Thailand. This species has a high proclivity for biting humans and is the primary vector for dengue virus transmission in the country (Ponlawat et al. 2005). Each year, many thousands of people in Thailand are infected and develop acute dengue fever that can progress to a much more serious condition, dengue hemorrhagic fever and dengue shock syndrome. The use of personal protection methods to prevent bites from *Ae. aegypti* is an important measure for avoiding infection from dengue and other arboviruses (e.g., chikungunya and Zika) associated with this vector. One commonly available method is the application of topical synthetic repellent products containing DEET (diethyltoluamide) or other active ingredients (e.g., picaridin) that can be effective and relatively long-lasting depending on the concentration and formulation used (Frances et al. 1996, 2004, 2010). Additionally, some plant-based topical repellents can provide protection from mosquito bites but typically of shorter effective duration (Tawatsin et al. 2006, Zhu et al. 2006, Webb and Russell 2007, Moore et al. 2007). Both toxic and nontoxic substances in plants have been found useful in repelling mosquitoes (Das et al. 2003, Singh et al. 2012). The nontoxic or sublethal properties of plant-based chemicals can be divided into those that act as contact irritants and noncontact spatial repellents (Grieco et al. 2007). Examples of studies in Thailand that have examined the behavioral responses to various compounds, including synthetic repellents, insecticides, and natural plant compounds against different mosquito species include Chareonviriyaphap et al. (2001), Kongmee et al. (2004), Polsomboon et al. (2008), and Suwansirisilp et al. (2012).

This study was designed to assess the two principal behavioral responses (repellent and irritant actions) of *Ae. aegypti* to three plant-based essential oils, clove, hairy basil, and sweet basil, while being compared with DEET under two different test conditions (indoor or laboratory and outdoor or field setting) using an excito-repellency test chamber. The purpose of the experiments was to investigate the possible effects of natural environmental factors such as air movement, sunlight, ambient temperature, humidity that might significantly impact the behavioral response of *Ae. aegypti* compared to more controlled laboratory settings.

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Materials and Methods

Study Sites. The behavioral studies using two test conditions (laboratory and field locations) occurred during the same time period (December 2011 to January 2012). Laboratory tests were performed in a room with environmental controls for lightening, air temperature, and relative humidity (RH), at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The field site was in Pu Teuy Village, Sai Yok District, Kanchanaburi Province, a rural area located ~250 km west of Bangkok (14° 20' N, 98° 59' E, at 400 m above sea level). The test area was near a small, open grass field, surrounded by agricultural land, dense bamboo forests, and shrub-land, with numerous perennial small streams. Air temperature and RH readings were recorded during all test periods.

Mosquitoes. The F2–F3 generation of an insecticide-resistant strain of *Ae. aegypti* from Pu Teuy Village was used for tests. Larvae and pupae were collected from the field and were reared in insectary conditions at 25 ± 5°C and 80 ± 10% RH, with a photoperiod of 12:12 (L:D) h. Three- to five-day-old adult females (mated, nonblooded) were used for susceptibility and excito-repellency (ER) tests in both laboratory and field settings.

Test Compounds. The extracted essential oils (100% purity) of clove (*Syzygium aromaticum*), hairy basil (*Ocimum americanum*), and sweet basil (*Ocimum basilicum*) were supplied by the Thai-China Flavours and Fragrances Industry (TCFF) Co., Ltd., Thailand. The information on the principal chemical composition of each oil extract was also provided by TCFF Co. Ltd. The oils were obtained from select plant parts using a steam distillation method and their chemical composition was analyzed by gas chromatography and mass spectrometry. The main components of the clove bud and flower oil were 97.3% of eugenol, while hairy basil leaf oil was 33.76% of E-citral and 20.61% of Z-citral. The sweet basil oil extracted from leaf and flower top contained 86.89% of methyl chavicol (estragole) and 4.27% of 1,8-cineol. Each essential oil was compared with the standard synthetic mosquito repellent DEET (*N*, *N*-diethyl-meta-toluamide). The technical grade DEET (97% purity) was obtained from the United States Department of Agriculture, Beltsville, MD. Chemical-treated papers (Whatman No.1 filter paper) were cut into rectangles of size 14.7 by 17.5 cm^2. Each test compound was prepared as a 5% solution in absolute ethanol. A precise volume of 2.8 ml of test solution was spread evenly throughout the filter paper by hand using a pipette. Papers were treated in the morning (0800–0900 hours) and were allowed to air dry at least 1 h before use.

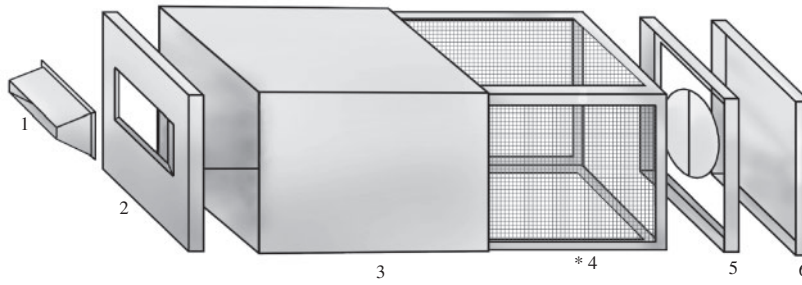
Susceptibility Tests. Insecticide-treated papers containing standard diagnostic doses of deltamethrin (0.05%), permethrin (0.75%), cypermethrin (0.05%), malathion (0.8%), and propoxur (0.1%) were prepared based on World Health Organization recommendations (WHO 1998). The WHO susceptibility test kits and insecticide-treated papers (and blank controls) were acquired from the Vector Control Research Unit, University Sains, Malaysia. In the laboratory, each active ingredient was tested in four replicates of 25, mated and nonblooded, sugar-fed female mosquitoes (derived from immature stages). The number of knockdown mosquitoes was recorded immediately after the 1-h exposure period and final mortality at 24 h (WHO 1998).

The susceptibility status of *Ae. aegypti* to each insecticide was evaluated on the following WHO revised criteria (WHO 2013). For final mortality between 98 and 100%, a test population is considered fully "susceptible"; mortality between 90 and 97% is treated as "suggestive of the existence of resistance" and requiring further investigation (i.e., additional bioassays or determination of mechanism(s)); and mortality below 90% is an indication of outright "resistance" in the population. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed. Lastly, if mortality is <90%, confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary provided a minimum of 100 mosquitoes of each species (population) is tested.

Behavioral Tests. The excito-repellency (ER) test system was divided into two designs: the "contact system," which is designed to evaluate an escape response ("irritancy" and repellency) by mosquitoes following direct contact with a chemical-treated surface, and the "noncontact system," to determine the behavioral response (spatial repellency only) of mosquitoes without physical contact with a chemical-treated surface (Fig. 1). The complete test system arrangement consists of four chambers; two containing target treatments of active ingredient (contact and noncontact chambers, respectively), paired with two control chambers containing papers without active ingredient. Each chamber was linked with an individual receiving box for collecting mosquitoes that escaped during the observation period. Fifteen, 3- to 5-d-old, nonblooded female mosquitoes (and sugar starved for ~24 h before testing) were released into each test chamber and allowed to adjust to the darken, internal environmental conditions for ~3 min before the exit (escape) door was open and observations begun. The number of escaped mosquitoes in each receiving box was recorded at 1-min intervals for 30 min. After the exposure period, both escape and nonescape (those that remain inside the test chamber) mosquitoes were kept separately in clean plastic holding cups and provided with 10% sugar solution soaked on a cotton ball that served as nutrition during the 24-h holding period. Knockdown (KD) at 30 min and mortality at 24 h postexposure were recorded. Each test compound was tested in four replicates. All tests were conducted during a 6-h daytime period (0900 and 1500 hours) in both laboratory and field settings.

Data Analysis. A Kaplan–Meier survival analysis was used to analyze the behavioral response data (Roberts et al. 1997). The log rank test was used for evaluating comparisons of laboratory and field condition test data (Mantel and Haenzel 1959). The time to

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ER test chamber is consisted of six parts: 1) Exit portal 2) Front panel 3) Outer chamber 4) Screened inner chamber 5) Plexiglas® panel with rubber latex door, and 6) Rear door cover. *The screen inner chamber or exposed wall surface area: contact assay, the treated filter papers were attached on the inward facing surface of each of 4 inner wire mesh screen walls, and for the noncontact assay, the treated papers were placed behind each the mesh screen thus preventing direct contact by landing mosquitoes.

Fig. 1. Blow-up diagram of excito-repellency (ER) test chamber.

escape was computed in 1-min intervals and analyzed for determination of the time required for 25, 50, and 75% of the test mosquitoes to depart the chamber measured as mean "escape time" (ET_{25}, ET_{50}, and ET_{75}). In susceptibility tests and excito-repellency trials, 24-h mortality and escape at 30 min were adjusted based on responses of mosquitoes in matched untreated chambers when control mortality or escape percent was between 5 and 20% (Abbott 1925). The crude contact escape percent (a measure of combined irritancy and repellency) was further adjusted to better reflect escape more likely due to contact irritancy alone by using the following equation to account for any unequal sample sizes between pairings: (1 – # contact in test × # noncontact escape/ # noncontact in test × # contact escape) × 100. This equation is the reciprocal of the standard Henderson–Tilton formula designed to measure the effects of toxic chemicals on arthropod populations (Henderson and Tilton 1955), wherein we compare those mosquitoes that successfully escaped the chambers in paired contact and noncontact designs in order to exclude the repellency effect and provide a better estimate of true contact irritancy.

Results

In susceptibility tests, *Ae. aegypti* from Pu Teuy Village demonstrated very high resistance to the organophosphate (malathion), with mortality of 21%, and a carbamate (propoxur), with mortality of only 4%. This population also showed clear resistance to three pyrethroid insecticides—deltamethrin (86% mortality), permethrin (82.5%), and cypermethrin (64.4%; Table 1). At 24 h, control mortality in all tests was below 5% (2.02% mortality); therefore, no correction factor was needed to derive the final percent mortality.

In the behavioral response trials, contact tests in the field found clove (40.7% escape) and hairy basil (65.4% escape) with significantly greater escape activity compared to 11.0 and 3.4% escape, respectively, in laboratory tests (Table 2). In noncontact repellency trials, clove and hairy basil showed similar and significant differences in escape between field and laboratory observations (48.3 vs. 14% and 45.5 vs. 1.7%, respectively). Comparisons between laboratory and field conditions for percent escape response, and knock down at 30 min and percent mortality at 24 h for nonescaping *Ae. aegypti* exposed to one of three essential oils or a synthetic repellent are illustrated for nonadjusted contact tests (Fig. 2) and noncontact tests (Fig. 3). For example, sweet basil displayed better repellent and irritant effects under laboratory conditions compared to field tests, yet showed little difference in mortality between the two settings. On the other hand, hairy basil showed very strong and significant escape responses in both contact and noncontact tests in the field compared to very low escape in the laboratory. Conversely, both KD and mortality were significantly greater in the poorly responsive laboratory trials compared to the field.

Table 1. Mortality of Kanchanaburi (Pu Teuy) population of *Ae. aegypti* to diagnostic concentrations of deltamethrin, permethrin, cypermethrin, malathion, and propoxur

Insecticide	Concentration (%)	No. of mosquitoes	% Knockdown after 1 h	% Mortality at 24 h
Deltamethrin	0.05	93	93.5	86.0
Permethrin	0.75	103	85.4	82.5
Cypermethrin	0.05	101	81.2	64.4
Malathion	0.8	100	1.0	21.0
Propoxur	0.1	100	1.0	4.0

However, when contact (irritancy + repellency) escape responses were adjusted with matched noncontact (true repellency) responses in laboratory and field trials, respectively, escape due to contact excitation was effectively excluded as a causal stimulus in four of the eight side-by-side test comparisons. Only lab- and field-based hairy basil and field-based sweet basil and DEET trials retain evidence of a small to modest

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Table 2. Escape response and mortality rates of *Ae. aegypti* in the contact and noncontact assays exposed to 5% of Clove (CL), Hairy basil (HB), Sweet basil (SB), and DEET (DT)

Assay	Test location	A.I.	% Escaped[a,b]				% Mortality[a]			
			Treated		Control		Treated		Control	
			N	% Esc[a] ([b])	*N*	% Esc	Esc	Not	Esc	Not
Contact	Laboratory	CL	57	11.0 (0)	60	13.3	1.8	43.4	0	10
		HB	58	3.4 (1.7)	60	20	1.7	94.5	5	38.3
		SB	60	39.2 (0)	60	15	10	6.7	3.3	16.7
		DT	58	9.8 (0)	61	19.7	1.7	3.4	1.6	1.6
	Field	CL	59	40.7 (0)	60	3.3	0	0	0	1.7
		HB	59	65.4 (19.9)	58	6.9	0	3.4	0	0
		SB	58	5.5 (5.5)	59	5.1	0	8.6	0	0
		DT	60	36.4 (5.5)	60	8.3	0	0	0	3.3
Noncontact	Laboratory	CL	58	14.0	60	11.7	0	34.5	0	6.7
		HB	58	1.7	60	18.3	1.7	98.3	6.7	30
		SB	58	53.8	59	6.8	13.8	6.9	1.7	16.9
		DT	58	32.3	60	8.3	0	0	0	3.3
	Field	CL	58	48.3	57	1.8	3.4	0	0	0
		HB	58	45.5	59	5.1	0	15.5	0	0
		SB	57	0	56	5.4	0	3.5	0	0
		DT	59	30.9	59	6.8	0	0	3.4	1.7

A.I., active ingredient.
[a] Treatment escape (30 min) and mortality data at 24 h corrected for paired control responses using Abbott's formula where appropriate.
[b] Contact escape percent adjusted using paired noncontact escape response (see Materials and Methods).

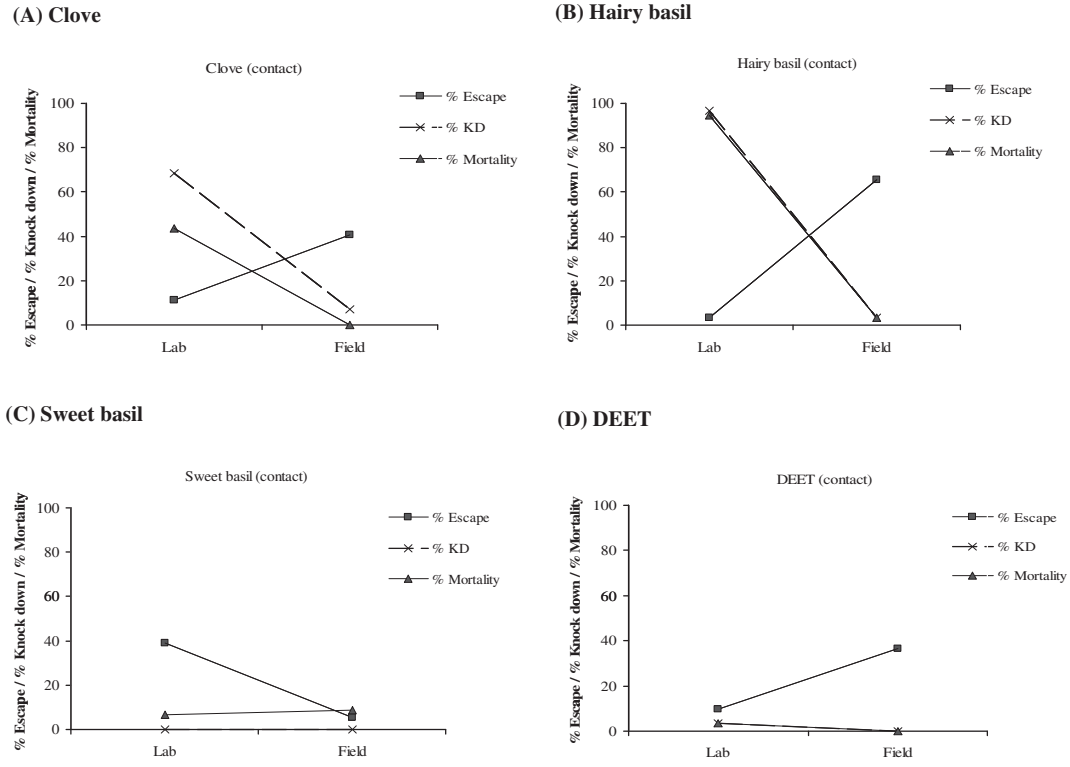


Fig. 2. Comparisons between laboratory and field test conditions for percent escape response (% Escape), percent knock down (% KD) at 30 min, and percent mortality at 24 h for nonescaping *Ae. aegypti* exposed to one of three essential oils or a synthetic repellent—(A) Clove, (B) Hairy basil, (C) Sweet basil, and (D) DEET in contact trials. Data were not adjusted to remove spatial repellent effects based on matched noncontact tests.

contact response. By excluding repellency as a response confounder, the crude contact escape due to irritancy decreased by 50% for laboratory-based hairy basil, and 69.6 and 84.7% for hairy basil and DEET, respectively, in field tests. The 24-h mortality rates for escape and nonescape mosquitoes are presented in Table 2. Higher mortality was seen in laboratory contact and noncontact trials compared to field tests for all test compounds.

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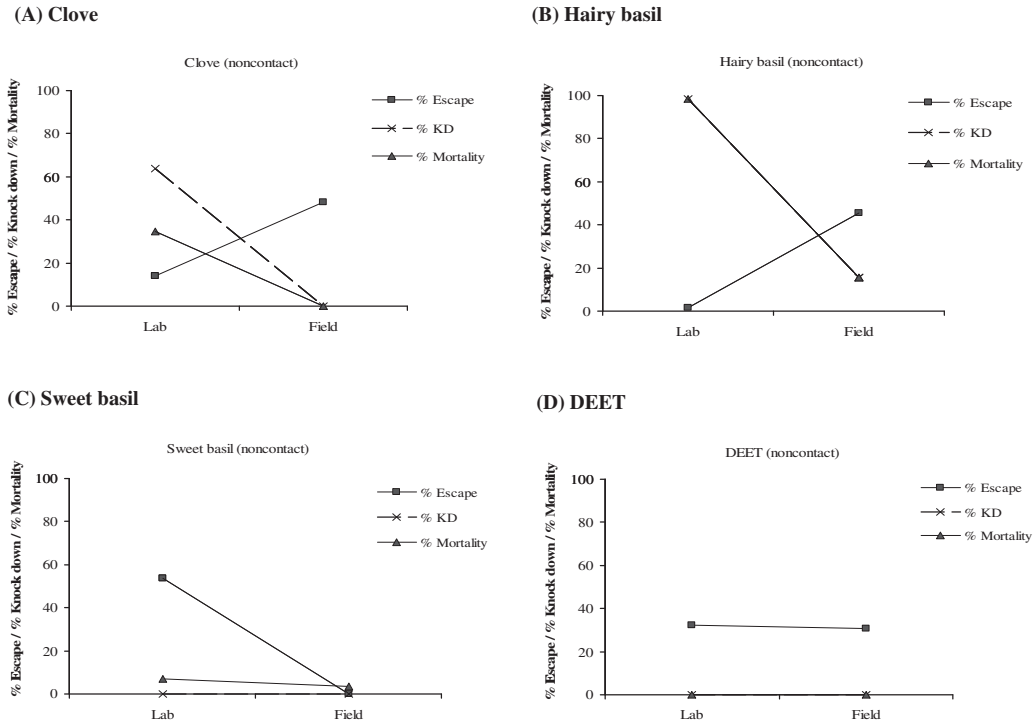


Fig. 3. Comparisons between laboratory and field test conditions for percent escape response (% Escape), percent knock down (% KD) at 30 min exposure, and percent mortality at 24 h for nonescaping *Ae. aegypti* exposed to one of three essential oils or a synthetic repellent—(A) Clove, (B) Hairy basil, (C) Sweet basil, and (D) DEET in noncontact (spatial repellency) trials.

There were no significant differences in unadjusted escape responses between laboratory and field-based contact and noncontact tests for DEET.

The mean escape time in minutes (ET) for *Ae. aegypti* in contact field tests for clove, hairy basil, and DEET showed ET_{25} at 1–2 min, with sweet basil having an ET_{25} of 2 min in the laboratory. Similar ET_{25} results were obtained in noncontact field trials with clove and hairy basil (1–2 min), and sweet basil in the laboratory (2 min). The ET_{50} was possible only for hairy basil (field contact trials at 10 min) and sweet basil (laboratory noncontact at 3 min). None of the active ingredients, including DEET, produced sufficient irritant or repellency escape responses within the 30-min exposure time to allow a determination of the ET_{75}.

Statistical comparisons of *Ae. aegypti* escape patterns between laboratory and field tests for each compound in contact and noncontact trials is shown in Table 3. The Kaplan–Meier method with log-rank comparisons of escape data showed similar results for the three essential oils showing significant differences in activity between laboratory and field tests ($P < 0.05$) while there were no significant differences between the two test conditions and DEET. Survival curves of those mosquitoes remaining inside the test chambers in contact and noncontact trials are shown in Figures 4 and 5. Hairy basil was shown the greatest irritant in field tests, whereas sweet basil displayed the most marked repellency in laboratory trials.

Table 3. Log-rank tests comparing escape activity between laboratory and field conditions in contact and noncontact assays for each compound

Compound	Laboratory and field site comparisons (*P*)	
	Contact	Noncontact
Clove	0.0262*	0.0031*
Hairy basil	0.0004*	0.0272*
Sweet basil	<0.0001*	<0.0001*
DEET	0.1032	0.7470

*$P < 0.05$ shows significant differences in escape behavior patterns.

Discussion

This study demonstrated marked differences in the behavioral escape responses of *Ae. aegypti* exposed to essential oils (clove, hairy basil, and sweet basil) and DEET, depending upon the conditions (laboratory or field) in which tests were conducted. Clove and hairy basil appear to illicit significant activity in both irritant and repellent effects in field tests as compared to laboratory tests where both essential oils produced moderate to high mortalities from mosquitoes failing to escape the exposure chamber. This was especially true for hairy basil which produced mortality exceeding 90% in both contact and noncontact designs. The reasons behind the relatively high mortality seen for clove and hairy basil in the laboratory but not field tests is unclear.

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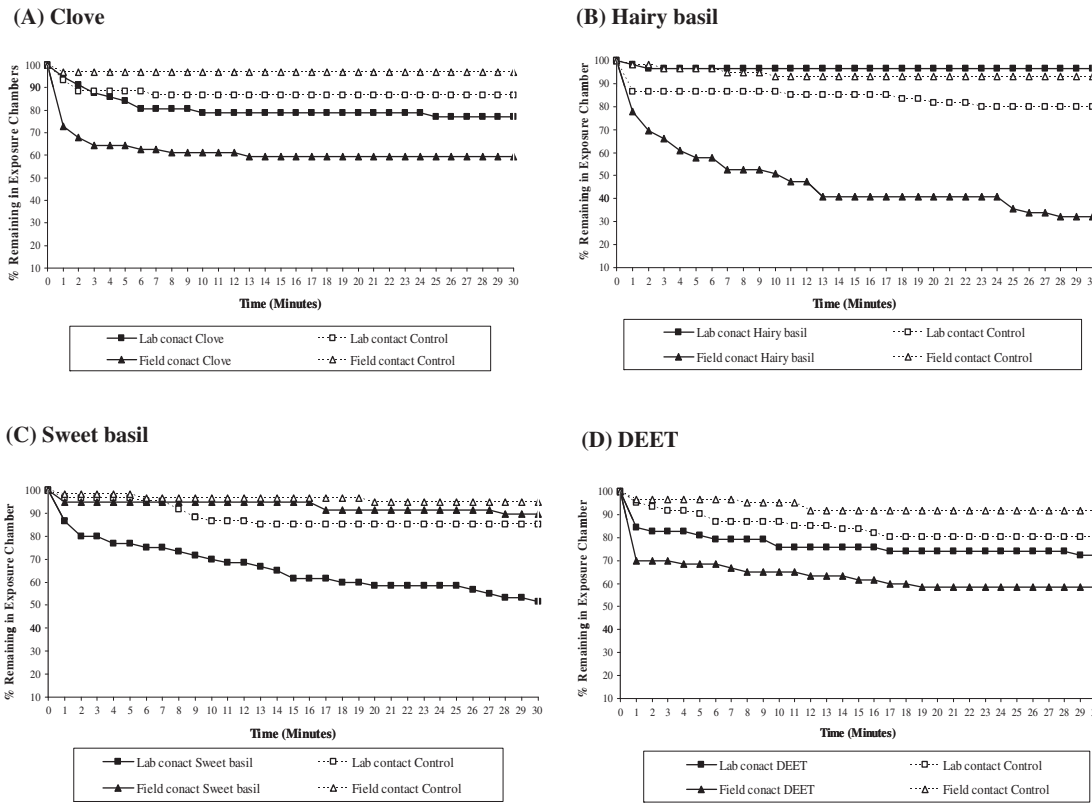


Fig. 4. Kaplan–Meier survival curves for *Ae. aegypti* in laboratory and field tests in contact trials exposed to (A) Clove, (B) Hairy basil, (C) Sweet basil, and (D) DEET.

In contrast, sweet basil was shown to have greater repellent and irritant effects on the mosquito in the laboratory tests compared to the field. The reasons for this are unclear; possibly sweet basil has a higher vapor pressure than clove and hairy basil so that it is more readily available in the laboratory setting at room temperatures, whereas it may volatize slower in warmer, more humid field condition than other oils. An opposite phenomena may have occurred with the clove and hairy basil oil, therefore it would be of interest to examine more closely the physical and chemical properties of each oil and determine the influence of ambient temperature and humidity on each. Another factor that may have influenced the performance of the active ingredients was the paper impregnation process wherein treated papers were air dried indoors for the laboratory tests and outdoors (exposed to wind but not direct sunlight) in the field possibly increasing evaporation and influencing the final amount of active ingredient on the paper at the time of testing. The potential loss of essential oils in the field may have been affected by natural environmental factors such as the light, wind, and indirect sunlight during the drying time and testing periods.

There were some limitations to the study design that may have influenced the behavioral and mortality responses. All tests took place in the daytime (during daylight hours) and variably over a 6-h period from 0900 to 1500 hours. Wild populations of *Ae. aegypti* typically show marked periodicity patterns (circadian rhythms) of resting and host foraging activity based on time of day and photoperiod (Ponlawat et al. 2005). Therefore, for better comparison, a narrower window of test times might provide advantages for more accurate or consistent responses. It is unclear what impact resistance to insecticides (organophosphates, carbamates, and pyrethroids) in *Ae. aegypti* had on behavioral responses, as we did not compare with an insecticide-susceptible strain. In another study, resistance was associated with excito-repellency responses (Sathantriphop et al. 2014). Likewise, it would have been beneficial to have tested the essential oils and DEET with the standard WHO susceptibility test kits to measure direct contact toxic responses to each.

Because the ER design in the contact test is as likely to measure irritancy excitation following contact with a treated surface as with noncontact, spatial repellency (i.e., combined effects), another way of calculating and interpreting the results is to adjust the contact escape response using the paired noncontact response to better ascertain the "true" irritancy effect (Table 2). In this study, we have introduced this adjustment (a reciprocal of the original Henderson–Tilton equation) for the first

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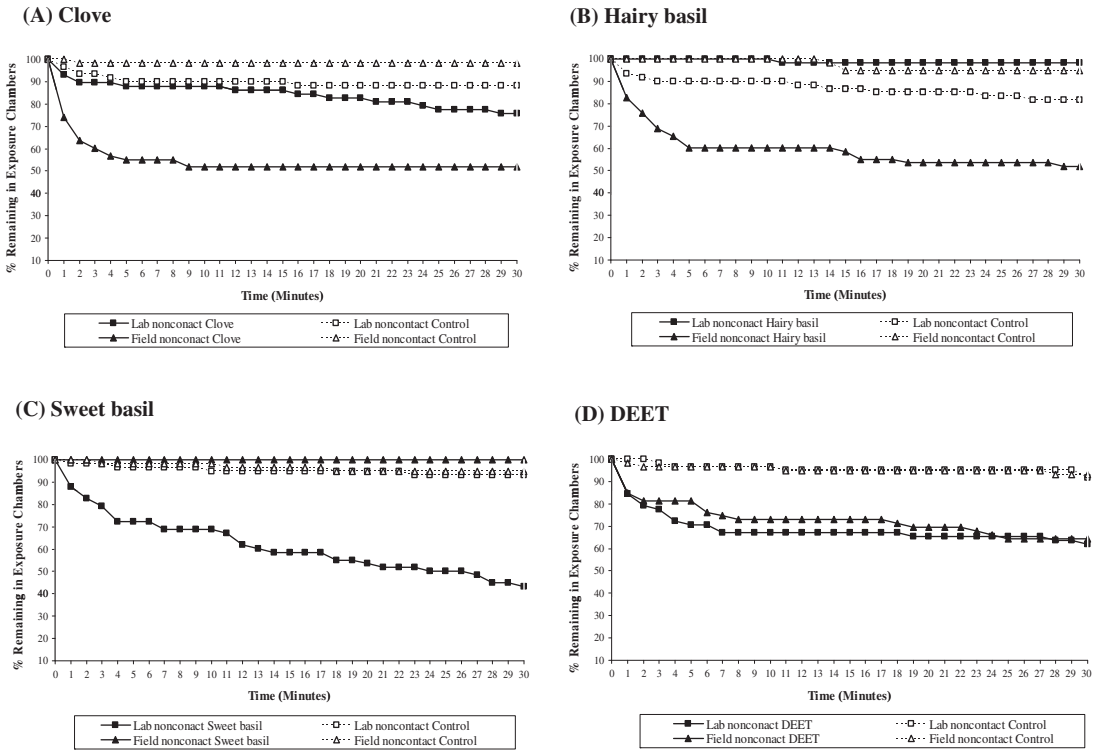


Fig. 5. Kaplan–Meier survival curves for *Ae. aegypti* in laboratory and field tests in noncontact trials exposed to (A) Clove, (B) Hairy basil, (C) Sweet basil, and (D) DEET.

time in the analysis of data collected in the ER system. In cases where greater repellency was observed compared to contact escape, the irritancy effect is nullified entirely. This is likely an oversimplification of what is actually happening inside the contact chamber, as it was apparent that toxicity also played a role, especially in nonescape responses and knockdown in both contact and noncontact laboratory tests using clove and hairy basil oil. Based on these adjusted data, it would appear none of the essential oils or DEET were performing as strong contact irritants, but rather as much stronger repellents.

DEET showed no statistically significant difference in mosquito response between laboratory and field test conditions (Table 3). DEET was a relatively low-level irritant in the laboratory compared to the field but had comparable repellency in laboratory and field tests against the Pu Teuy population. For repellent tests on human skin, Schreck et al. (1985) reported that 10% DEET was effective for up to 2 h when exposed to *Anopheles albimanus* and 20% DEET would prevent biting for 4–4.5 h. Frances et al. (2010) showed 35% DEET formulated in a gel offered 2 h protection, whereas 40% DEET in ethanol provided only 1 h of protection (>95%) against *Anopheles farauti* in the field. Thus, the effectiveness of DEET to repel mosquitoes depends on the target species, the concentration of active ingredient, product formulation and likely other factors. Most commercial products containing DEET that is directly applied on human skin vary in concentration from 5–30% (Koren et al. 2003).

This study indicates that hairy basil had good irritant and repellent properties in the field and also proved quite toxic to mosquitoes in the laboratory. Boonyuan et al. (2014) also reported that hairy basil performance under laboratory conditions using the ER test system showed high knockdown and mortality of *Ae. aegypti* in both contact and noncontact trials. The hairy basil oil used in this study was rich in methyl cinnamate, d-camphor, and citral (E & Z forms) and combined appear as the active components affecting *Ae. aegypti*. Dekker et al. (2011) reported that methyl cinnamate from *Ocimum forskolei* was very effective in repelling *Ae.* aegypti and *Anopheles arabiensis* mosquitoes. Tropical origin cultivars of basil oil were found to have methyl cinnamate as a major constituent and this component was considered a good mosquito repellent (Marotti et al. 1996). Makhaik et al. (2005) reported citral in lemongrass oil (*Cymbopogon flexuous*) was also an effective repellent against *Ae. aegypti* and *Culex quinquefasciatus*. In Kenya, live hairy basil plants have been recognized as having repellent properties and commonly used against biting insects, including mosquitoes (Seyoum et al. 2002). In the current study, this promising essential oil appears suitable for further development into a usable formulation and possibly an alternative to DEET as a topical repellent. Alternatively, various forms of hairy basil might have

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commercial potential as a natural bio-pesticide to kill larval and adult mosquitoes (Cavalcanti et al. 2004, Kamsuk et al. 2007). Previous work with hairy basil combined with 5% vanillin was shown to significantly extend the effective protection time against *Ae. aegypti*, *Anopheles dirus*, and *Cx. quinquefasciatus* (Tawatsin et al. 2001). Das et al. (2003) also reported that plant extracts combined with mustard oil could provide longer protection time against *Aedes albopictus*.

Additional research is needed on the behavioral responses of different mosquito species to hairy basil oil and other potentially useful essential oils exposed to differing test conditions (formulations, additives, test designs, abiotic conditions, etc.) when applied to human skin. For example, assessing the influence of body heat and odor, sweat, other skin-derived compounds or emanations, and other factors that might impact repellency performance is needed. The possibility exists that excito-repellency responses from experimental devices, exclusive of host attraction, might differ significantly from findings derived from repellents applied in direct skin tests; therefore more than one assay system may be necessary to advance product development.

Acknowledgments

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SPECIES DIVERSITY AND SEASONALITY OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) IN SATUN PROVINCE, THAILAND

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Abstract. Leishmaniasis is prevalent mainly in the southern provinces of Thailand where sand flies are considered to be an important vector. Sand flies were collected using Centers for Disease Control (CDC) light traps in Satun province from June 2013 to July 2014. A total of 1,982 sand flies (1,228 females and 754 males) were collected. Only female sand flies were identified to the species level and were tested for *Leishmania* infection using polymerase chain reaction (PCR). Morphological identification revealed 2 genera and 9 species: *Phlebotomus stantoni*, *P. argentipes*, *Sergentomyia gemmea, S. indica*, *S. barraudi*, *S. iyengari*, *S. bailyi*, *S. perturbans*, and *S. silvatica*. *S. gemmea* (57.2%) was the most abundant species. The diversity of sand flies was highest in Thung Wa district. The sand flies were most abundant late in the hot season and early in the rain season (April to June). The highest number of sand flies was collected in June. Significant correlations between the number of female sand flies and

rainfall and between *S. gemmea* and rainfall were found. Of the female sand flies tested, none were found to *Leishmania* infection.

Keywords: seasonality, diversity, sand flies, *Leishmania*, Satun province

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INTRODUCTION

Leishmaniasis is a zoonotic disease caused by parasitic protozoans of the genus *Leishmania* that are transmitted by the bite of female sand flies belonging to the subfamily Phlebotominae in the family Psychodidae (Desjeux, 1996; Herwaldt, 1999; Kobets *et al*, 2012). While leishmaniasis is non-endemic in Thailand, it is sporadic. Most imported cases of cutaneous and visceral leishmaniasis have been reported in Thai workers returning from the Middle East since 1960 (Laohapaibol and Siampakdi, 1960; Suttinont *et al*, 1987; Viriyavejakul *et al*, 1997). During 1996 to 2010, 58 leishmaniasis cases were reported from 10 provinces in Thailand. Most of these cases were in southern Thailand (Sukmee *et al*, 2011; Sukmee *et al*, 2008; Kongkaew *et al*, 2007; Thisyakorn *et al*, 1999). Until recently, 6 cases of autochthonous disseminated dermal and visceral leishmaniasis have been reported from different provinces in Thailand (Sukmee *et al*, 2008; Bualert *et al*, 2012; Polseela, 2012). Species of sand flies such as *Phlebotomus*

argentipes, a natural vector of *Leishmania donovani* in India, Nepal, and Bangladesh have been identified in Thailand (Sukra *et al*, 2013). *P. major major*, the proven vector of *L. infantum* in the Mediterranean region was mostly found in caves in Thailand (Apiwathnasorn *et al*, 1989). In addition, *Sergentomyia gemmea* and *S. barraudi* might be potential vectors of *L. siamensis*, the causative agent of autochthonous visceral leishmaniasis in southern Thailand (Kanjanopas *et al*, 2013; Chusri *et al*, 2014). The distribution and diversity of sand fly species can play roles in the maintenance of the transmission cycles of *Leishmania* spp. (Polseela *et al*, 2011a). However, little is known about the distribution of sand fly species and potential sand fly vectors of leishmaniasis in the southern region of Thailand. The aims of this study were to investigate the seasonal abundance and diversity of sand flies and the detection of *Leishmania* DNA within sand flies in Satun province, one of the affected areas of leishmaniasis in Thailand.

MATERIALS AND METHODS

Study areas

Sand flies were collected between June 2013 and July 2014 in 7 districts of Satun province; Muang, Tha Phae, Khuan Don, Khuan Kalong, Thung Wa, Manang, and La-ngu. The collection sites included peridomestic areas, domestic animal shelters for dogs, cats, cattle, and goats, and rubber and/or palm plantations. The weather in the collection areas is marked by 3 seasons: a hot season from mid-February to mid-May, a rainy season from mid-May to mid-October, and a colder season from mid-October to

mid-February. The average annual rainfall is 179 mm. The temperature range is 27-29 ºC with 65-85% relative humidity. These data were obtained from the Thai Meteorological Department weather station in Satun province (station: 570201) (Figure 1).

Sand fly collection

Sand flies were collected using Center for Disease Control (CDC) light traps and directly preserved in 70% ethanol. Traps were hung from trees approximately 50 cm above the ground between 6.00 PM and 6.00 AM. Sand flies were collected between June 2013 and July 2014 in seven districts of Satun Province. In Mueang District, collections were made monthly from June 2013 to May 2014 except during October using 2 CDC light traps for 2 consecutive nights. For the remaining 6 districts, sand flies were collected for at least 2 consecutive nights (4 traps per night) in one month: Tha Phae (June 2013), Khuan Don (July 2013), Khuan Kalong (August 2013), Thung Wa (October 2013), Manang, (November 2013), and La-ngu (December 2013). In four districts, the numbers of flies collected were low, so additional samples were taken using 3 traps per night for 2 nights: Khuan Kalong and Manang (June 2014) and Thung Wa and La-ngu (July 2014). Thus there was a total of 116 trap nights in the seven districts: Mueang (44 nights), Tha Phae (8 nights), Khuan Don (8 nights), Khuan Kalong, Thung Wa, Manang, and La-ngu (14 nights each).

Sand fly identification

Sand flies were examined using a stereomicroscope, separated from other insects and identified according to sex. For species identification, the head and the abdominal segments of 8-11 females were dissected and mounted on slides using Hoyer’s medium.

Sand flies were identified based on the morphology of cibarium teeth and spermatheca following the taxonomic key of Lewis (1978). The remaining body parts were kept in 70% ethanol until used for the detection of *Leishmania* DNA.

Detection of *Leishmania* DNA in sand flies

One to 10 specimens of female sand flies belonging to the same species, date, and site of capture were pooled. One hundred sixty-six pools were tested for *Leishmania* parasites. Extraction of genomic DNA from each pool was performed using the E.Z.N.A.® tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA). Detection of *Leishmania* DNA was performed using PCR analysis. The internal transcribed spacer 1 (ITS1) region of genomic DNA was amplified with LITSR and L5.8S primers (El Tai *et al*, 2001). The PCR analyses were performed in 20 µl volumes of reaction mix containing 2 µl of genomic DNA, 1x buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% (v/v) Triton X-100), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA Polymerase, and 1 pmol of each primer. The PCR reactions were processed on a MyCyclerTM Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The thermal cycling conditions were initial denaturation at 95 ºC for 2 min followed by 45 amplification cycles of denaturation at 95 ºC for 20 sec, annealing at 54 ºC for 30 sec, and extension at 72 ºC for 1 min, and a final extension step at 72 ºC for 6 min. PCR products were checked using electrophoresis in 1% agarose gel (SeaKem ME: FMC, USA).

Data analysis

The Shannon-Wiener species diversity index (Krebs, 1999) was calculated using the formula $H = -\sum_{i=1}^{S} (p_i)\,(\ln p_i)$, where H is the species diversity index, s is the number of

species and p_i is the proportion of the total sample belonging to i^{th} species. The correlations between the numbers of sand flies and temperature, relative humidity, and rainfall were assessed using the Pearson product-moment correlation coefficient. The SYSTAT® computer package version 8.0 (SYSTAT Software, 1998) was used for statistical analysis. A value of $p<0.05$ was considered significant.

RESULTS

A total of 1,982 sand flies (61.96% females and 38.04% males) were collected during the study period (Table 1). The ratio of females to males was 1.6: 1. The 1,228 female sand flies were identified as belonging to 2 genera and 9 species, with 2 species of *Phlebotomus* (*P. stantoni* and *P. argentipes*) and 7 species of *Sergentomyia* (*S. gemmea, S. indica*, *S. barraudi*, *S. iyengari*, *S. bailyi*, *S. perturbans,* and *S. silvatica*). *Sergentomyia gemmea* was the most abundant species (57.2%) followed by *S. indica* (26.9%), *S. barraudi* (6.3%), *P. stantoni* (4.1%), and *S. iyengari* (3.5%), respectively. The remaining species were present but less abundant: *S. bailyi* (0.9%), *S. perturbans* (0.6%), *S. silvatica* (0.4%), and *P. argentipes* (0.1%) (Table 2). *S. gemmea* and *S. indica* were found at all collection sites. The former of these 2 species was the most abundant at all collection sites. The highest values of diversity and species richness were found in Thung Wa (1.74 and 8, respectively) followed by Khuan Kalong (1.26 and 6, respectively) and Manang (1.18 and 4, respectively). The highest dominance level was 0.97 in Khuna Don followed by Tha Phae (0.91) whereas the lowest value was in Thung Wa (0.31).

The sand flies were most abundant late in the hot season and early in the rainy season (April to June), with a marked population increase between 2 rainfall peaks (Table 3 and Figure 1). The highest number of sand flies was collected in June. *S. gemmea* occurred almost throughout the year with a peak in June. *S. indica* was sampled throughout the year except in August and December, with a peak in May. *P. stantoni* was collected only in April, May, June, July, and September with fewer than 5 specimens collected each month. *S. barraudi* was found only in January, April, June, August, and December. *S. iyengari* and *S. perturbans* were collected in April and June. *P. argentipes* was only collected in April, but this month provided the highest number of species (7). The latter 4 species were represented by single specimens collected each month. The data analysis showed a significant correlation between the number of female sand flies and rainfall ($r = 0.722$, $p = 0.014$) and a significant correlation between *S. gemmea* and rainfall ($r = 0.736$, $p = 0.012$) (Table 4). None of the 1,228 females tested were positive for *Leishmania* spp.

Table 1 Numbers of male and female sand flies collected in 7 districts of Satun province, Thailand.

Sex	Collection sites (district)							
	Tha Phae	Khuan Don	Khuan Kalong	Thung Wa	Manang	La-ngu	Muang	Total (%)
Female	253	60	87	275	34	40	479	1,228
(%)	(74.63)	(52.17)	(58.39)	(47.83)	(55.74)	(54.05)	(71.60)	(61.96)
Male	86	55	62	300	27	34	190	754
(%)	(25.37)	(47.83)	(41.61)	(52.17)	(44.26)	(45.95)	(28.40)	(38.04)

Table 2 Species and distribution of female sand flies collected in 7 districts of Satun province, Thailand.

Genus	Species	Tha Phae		Khuan Don		Khuan Kalong		Thung Wa	
		Individuals	%	Individuals	%	Individuals	%	Individuals	%
Phlebotomus	*stantoni*	3	1.2	-	-	8	9.2	24	8.7
	argentipes	-	-	-	-	-	-	-	-
Sergentomyia	*gemmea*	**229**	**90.5**	**58**	**96.7**	**38**	**43.7**	**85**	**30.9**
	indica	11	4.3	2	3.3	33	37.9	55	20.0
	iyengari	1	0.4	-	-	3	3.4	32	11.6
	barraudi	9	3.6	-	-	4	4.6	59	21.5
	perturbans	-	-	-	-	1	1.2	4	1.5
	bailyi	-	-	-	-	-	-	11	4.0
	silvatica	-	-	-	-	-	-	5	1.8
Total number of individuals (N)		253	100	60	100	87	100	275	100
Total number of samples (n)		8		8		14		14	
Mean number of individuals (N/n)		31.63		7.50		6.21		19.64	
Total number of species		5		2		6		8	
Diversity (*H*)		0.42		0.14		1.26		1.74	
Dominance (Nmax/N)		0.91		0.97		0.44		0.31	

Nmax = Number of individuals in the dominant species, bold type

H = Shannon-Wiener species diversity index

Table 2 (Continued)

Genus	Species	Manang		La-ngu		Muang		Total	%
		Individuals	%	Individuals	%	Individuals	%		
Phlebotomus	*stantoni*	2	5.9	2	5.0	11	2.3	50	4.1
	argentipes	-	-	-	-	1	0.2	1	0.1
Sergentomyia	*gemmea*	**15**	**44.1**	**22**	**55.0**	**256**	**53.5**	**703**	**57.2**
	indica	12	35.3	16	40.0	202	42.2	331	26.9
	iyengari	5	14.7	-	-	2	0.4	43	3.5
	barraudi	-	-	-	-	5	1.0	77	6.3
	perturbans	-	-	-	-	2	0.4	7	0.6
	bailyi	-	-	-	-	-	-	11	0.9
	silvatica	-	-	-	-	-	-	5	0.4
Total number of individuals (N)		34	100	40	100	479	100	1228	100
Total number of samples (n)		14		14		44		116	
Mean number of individuals (N/n)		2.43		2.86		10.89		10.59	
Total number of species		4		3		7		9	
Diversity (*H*)		1.18		0.85		0.89		1.20	
Dominance (Nmax/N)		0.44		0.55		0.53		0.57	

Nmax = Number of individuals in the dominant species, bold type

H = Shannon-Wiener species diversity index

Table 3 Total numbers of female sand flies collected each month in Muang district, Satun province, during June 2013 to May 2014.

Species	Month											Total	%
	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Feb	Mar	Apr	May		
P. stantoni	4	1	0	1	0	0	0	0	0	3	2	11	2.4
P. argentipes	0	0	0	0	0	0	0	0	0	1	0	1	0.2
S. gemmea	115	29	8	2	13	5	1	2	6	30	45	256	53.4
S. indica	27	4	0	1	3	0	1	1	14	61	90	202	42.2
S. iyengari	1	0	0	0	0	0	0	0	0	1	0	2	0.4
S. barraudi	1	0	1	0	0	1	1	0	0	1	0	5	1
S. perturbans	1	0	0	0	0	0	0	0	0	1	0	2	0.4
Total	149	34	9	4	16	6	3	3	20	98	137	479	100
Total number of species	6	3	2	3	2	2	3	2	2	7	3		

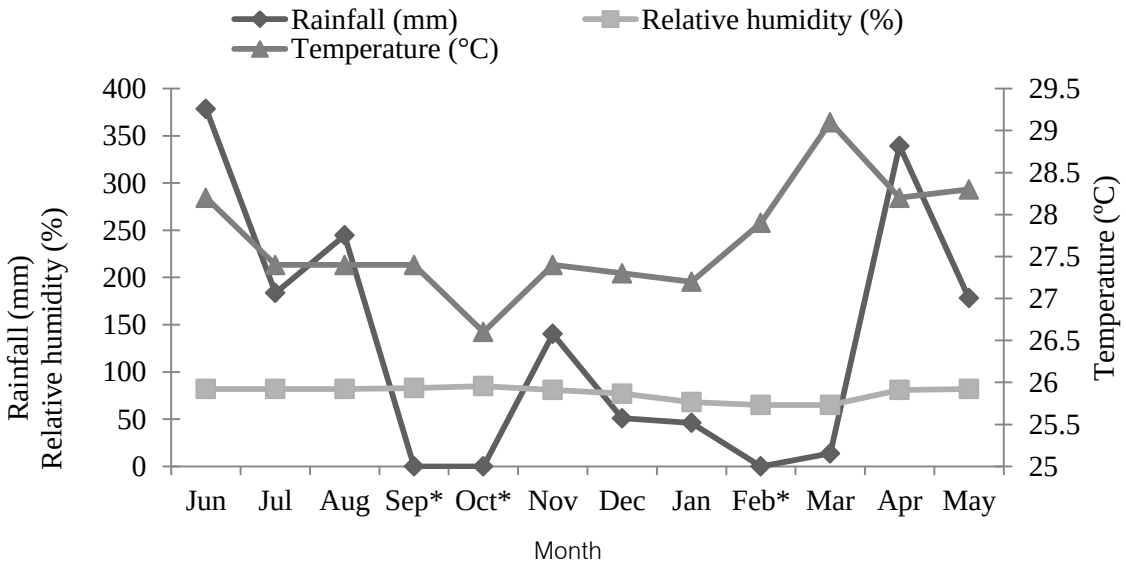


Figure 1 Mean monthly rainfall, relative humidity and temperature in the study area during June 2013 to May 2014.

* No rainfall data available

Table 4 Pearson product-moment correlation coefficient values (r) between total numbers of individuals and main species of sand flies captured and climatological data of Satun province from June 2013 to May 2014.

Weather conditions	Number of individuals	*Sergentomyia gemmea*	*Sergentomyia indica*
Rainfall (mm)	0.722*	0.736*	0.402
Relative humidity (%)	0.448	0.464	0.309
Temperature (°C)	0.523	0.307	0.53

* Significant at $p<0.05$

DISCUSSION

A total of 1,982 sand flies were collected in the study with a higher proportion of females (1,228) than males (754). Similar findings were reported by Polseela *et al* (2011a, 2011b) and Sukra *et al* (2013). Both sexes are generally considered to be crepuscular and nocturnal in their activity (Lewis, 1971; El-Badry *et al*, 2008). Only female sand flies feed on the blood of various vertebrate animals (Lane, 1987). After feeding, females fly to mates and to search for oviposition sites. Therefore, the females were more likely to be caught than males in all traps (Eiko *et al*, 2004).

During 1938-2011, at least 27 species of sand flies were recorded by many investigators in different areas of Thailand (Causey, 1938; Quate, 1962; Apiwathnasorn *et al*, 1993; Polseela *et al*, 2007; Nilsang *et al*, 2010; Apiwathnasorn *et al*, 2011; Sukmee *et al*, 2011; Polseela *et al*, 2011a). A previous survey of sand flies in Satun

province found 10 species: *P. stantoni*, *P. papatasi*, *P. major major*, *P. argentipes*, *S. gemmea*, *S. barraudi*, *S. iyengari*, *S. perturbans*, *S. indica*, and *S. anodotis*. Of these 10 species, *S. barraudi* was the most abundant (Nilsang *et al*, 2010). In the present study, 9 species of sand fly were trapped. Additionally, *S. bailyi* and *S. silvatica* were identified in this area. *S. gemmea* was the most abundant sand fly species. This result was consistent with previous findings that *S. gemmea* was the most predominant species in southern Thailand, the affected area of leishmaniasis caused by *L. siamensis* (Sukmee *et al*, 2008; Kanjanopas *et al*, 2013; Sukra *et al*, 2013). *Sergentomyia* species feed mainly on reptiles and some species of this genus bite humans, but rarely (Lane, 1993; Polseela *et al*, 2011b; Sadlova *et al*, 2013). Therefore, they were suspected as the potential vector of this disease in southern Thailand (Kanjanopas *et al*, 2013; Chusri *et al*, 2014). *P. argentipes*, a known vector of Kala-aza in India was found in the present study but in low numbers. This finding was similar to previous survey in Phang-nga and Suratthani provinces in Thailand (Sukra *et al*, 2013). This species was found mainly in caves (Apiwathnasorn *et al*, 1989). However, *P. argentipes* is widely distributed in Thailand (Apiwathnasorn *et al*, 1993).

The highest values of diversity and species richness of sand flies were observed in Thung Wa district which might indicate that this area contains suitable breeding and resting sites and feeding sources for sand flies. In addition, the degree of conservation in the region, the use of agroecosystems, the forest cover and human population density may affect sand fly diversity and abundance (Pinto *et al*, 2010; Nieves *et al*, 2014; Ramos *et al*, 2014).

Monthly collection of sand flies in Muang district showed sand fly numbers occurred throughout the year with peaks in June 2013 and April and May 2014. This finding was consistent with the results reported by Polseela *et al.* (2007) that sand flies were more prominent during the early rainy season (June-August) in Saraburi province, Thailand. The results of this study showed that high rainfall increased the number of sand flies caught. In addition, the temperature and the relative humidity may influence the density of sand fly caught (Rafatbakhsh-Iran *et al,* 2015). Sand flies were abundant in the warm and humid months when the average temperature was 28.2-28.3 ºC and the relative humidity was 81-82%. *P. argentipes* was active throughout the year in Saraburi province (Polseela *et al,* 2011a). In the present study, this species was caught only in April and *S. gemmea* was the most frequently identified species throughout the year followed by *S. indica*.

Although no *Leishmania* species among sand flies collected were detected in this study, people are at risk from infection if they live in a region where *Leishmania* species are found. In 2008, a case of visceral leishmaniasis caused by *L. siamensis* was reported in a 5-year old girl living in Satun province (Osatakul *et al*, 2014). *S. gemmea* and *S. barraudi* (potential vectors for *L. siamensis*) and *P. argentipes* (a natural vector for *L. donovani*) were identified in this study. Further studies are needed to prove the potential sand flies vectors of leishmaniasis in Thailand.

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A Comparison of Attractants for Sampling *Stomoxys calcitrans* (Diptera: Muscidae) on Dairy Farms in Saraburi Province, Thailand

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Abstract

The efficacy of different stable fly attractants was evaluated at four dairy cattle farms in Muak Lek district, Saraburi province, Thailand. Dry ice, octenol, a mixture of cow dung and urine, a combination of dry ice plus octenol, and no attractants (control) were tested with Vavoua traps. In total, 7,000 individuals of *Stomoxys* species were collected between July 2013 to September 2014, of which 1,058, 867, 1,274, and 3,801 were trapped on farms 1–4, respectively. Four species of *Stomoxys* were identified: *Stomoxys bengalensis* Picard, 1908, *Stomoxys calcitrans* (L., 1758), *Stomoxys indicus* Picard, 1908, and *Stomoxys sitiens* Rondani, 1873. *S. calcitrans* was the predominant species, comprising 99% of all the samples collected. The number of male and female *S. calcitrans* collected differed significantly by attractant type. Significantly more *S. calcitrans* were attracted to dry ice or a combination of dry ice plus octenol-baited traps than to unbaited or octenol-baited traps. The Vavoua traps baited with dry ice alone or a combination of dry ice plus octenol were effective attractants for *S. calcitrans.*

Key words: attractant, dry ice, octenol, *Stomoxys calcitrans*, Thailand

Stable flies (*Stomoxys* spp.) belong to the subfamily Stomoxyinae in the family Muscidae (Diptera). Among the 18 *Stomoxys* species described, six have been recorded in Thailand, with one synanthrope species, *Stomoxys calcitrans* (L.) (Zumpt 1973, Tumrasvin and Shinonaga 1978). Stable flies are an important and widely distributed insect pests of livestock, wildlife, and sometimes humans. Both sexes of adult stable flies are obligate blood feeder that cause painful bites and significant blood loss in some animals. Heavy burdens of biting activity can reduce animal productivity by reducing weight gain and milk production (Bruce and Decker 1958, Campbell et al. 1987, 2001, Wieman et al. 1992, Catangui et al. 1997). Stable flies may act as both mechanical and biological vectors of disease agents, including viruses, bacteria, protozoa, and helminthes (Baldacchino et al. 2013). Examples include equine infectious anemia virus (EIAV; Foil et al. 1983), trypanosomes *Trypanosoma evansi* and *Trypanosoma congolense* (Sumba et al. 1998), and the nematodes *Habronema microstoma* and *Habronema muscae* (Traversa et al. 2008).

Many types of traps have been developed for the survey and control of stable flies (Taylor and Berkebile 2006, Gilles et al. 2007). Two types of Alsynite sticky traps (Williams trap and Broce trap) have been used for sampling stable flies (Williams 1973, Broce 1988). However, one disadvantage of these traps is that they also catch many nontarget insects (Gilles et al. 2007). The Vavoua trap (Laveissière and Grebaut 1990) is designed for tsetse flies and is used to sample *Stomoxys* spp. in many African countries and is considered the best option for stable fly control (Gilles et al. 2007).

Additionally, attractants can be used in traps to reduce pest insect populations. The use of octenol (a component of cow breath) and CO_2 as attractants increases flight activity and trapping efficacy (Mihok et al. 1995, Schofield and Brady 1997, Schofield et al. 1997). Both traps and attractants do not injure animals or humans or leave harmful residues. They are relatively safe to use, inexpensive, and effective.

Little is known about the efficacy of different attractants for collecting stable flies in Thailand. The present study compared attractants for *S. calcitrans* on dairy farms in Saraburi province, Thailand. The information obtained in this study will benefit fly control programs by improving their effectiveness.

Materials and Methods

Study Sites

Four dairy farms were randomly selected in the Muak Lek district, Saraburi province. Saraburi is located in the central highlands of

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Table 1. Total numbers of *Stomoxys* spp. collected for each attractant treatment from four dairy farms in the Muak Lek district, Saraburi province

Species	Farm	Control	Dry ice	Octenol	Cow dung and urine	Dry ice + Octenol	Total
S. bengalensis Picard, 1908	1	0	0	0	0	1	1
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
S. calcitrans (L., 1758)	1	107	390	133	90	305	1,025
	2	83	289	100	88	279	839
	3	183	374	222	282	198	1,259
	4	516	1,011	549	358	1,356	3,790
S. indicus Picard, 1908	1	0	10	3	1	6	20
	2	0	1	1	1	2	5
	3	1	1	2	0	2	6
	4	0	0	1	0	0	1
S. sitiens Rondani, 1873	1	1	7	3	0	1	12
	2	3	9	1	0	10	23
	3	1	4	3	1	0	9
	4	2	2	3	2	1	10

Thailand, an area with relatively high plains and plateaus, about 140 km northeast of Bangkok. Muak Lek is located in the east of the province and is the largest district, which also has the largest number of dairy farms. Farm 1 (14° 45.084′ N, 101° 14.592′ E; 219 m above sea level [a.s.l]) had 113 cows in milk production, farm 2 (14° 45.156′ N, 101° 14.336′ E; 202 m a.s.l) had 30 cows, farm 3 (14° 47.319′ N, 101° 17.290′ E; 473 m a.s.l) had 77 cows, and farm 4 (14° 47.487′ N, 101° 17.278′ E; 467 m a.s.l) had 39 cows.

Specimen Collection

At each farm, stable flies were collected using five Vavoua traps. The traps were made from blue and black materials and white insect netting in Bangkok, Thailand. Thailand has three seasons: the rainy season from June to October, the dry cool season from November to February, and the dry hot season from March to May. The study was carried out over 2 yr (2013–2014) during two rainy seasons. In 2013, the experiment was conducted from July to October on farms 1 and 2 and in 2014 from June to September on farms 3 and 4. All trapping experiments were conducted for 5 wk. Each trapping week was performed over two consecutive days. The daily trapping period was between 10 a.m. and 4 p.m. The five traps were installed at random locations about 1 m from the cattle pen and at least 10 m from each other. The traps were placed at 30 cm above the ground (Thomas et al. 1989, Berkebile et al. 1994). After trapping, flies in the Vavoua traps were killed using ethanol spray. All collected flies were preserved in 80% ethanol. The specimens were brought back to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Thailand, for species identification using the key of Tumrasvin and Shinonaga (1978).

Attractants

The five treatments consisted of: control (no attractant), 10 kg of dry ice (CO_2 source) only, octenol only, a mixture of cow dung and urine, and a combination of 10 kg of dry ice plus octenol. The octenol lures (Envirosafe Technologies NZ Ltd., Ottawa, ON, New Zealand) contained octenol (1-octen-3-ol, 3.74 g per strip). The octenol strips were hung and left on the traps for two consecutive days during each collection period. The mixture of cow dung and urine was collected during the morning from cattle pens at the farm before traps were baited. The mixture of cow dung and urine was placed inside a black plastic container on the ground under the traps and replaced daily. Dry ice was placed inside a poly styrofoam box on the ground under the traps for two consecutive days. At each farm, the experiment was conducted over 10 d using a 5×5 Latin square design. To eliminate any position-specific effect, all attractant treatments were rotated to the next trap position each week.

Data Analysis

Pearson chi-square analysis was used to evaluate the differences in numbers of males and females attracted to each attractant type at each farm. The data were analyzed by univariate analysis of variance (ANOVA) with trap treatment as the fixed factor, and week and position as the random factors. Data on the number of stable flies collected were square root transformed before analysis. When the ANOVA was significant, variations in the numbers of each sex among treatment types were separated using the least significant difference (LSD) post hoc analysis. The data were analyzed using SPSS (Version 17, SPSS Inc., Chicago, IL). All statistical significance levels were set at $P < 0.05$.

Results

In total, 7,000 individuals in the *Stomoxys* species group were collected between July 2013 to September 2014, of which 1,058 were trapped on farm 1, farm 2 with 867, farm 3 with 1,274, and farm 4 with 3,801 (Table 1). The following four species of *Stomoxys* were captured: *Stomoxys bengalensis Picard*, *S. calcitrans*, *Stomoxys indicus* Picard, and *Stomoxys sitiens Rondani*. The most abundant species were, respectively, *S. calcitrans* (98.76%) followed by *S. sitiens* (0.77%), *S. indicus* (0.46%), and *S. bengalensis* (0.01%).

Pearson chi-square analysis showed that differences between the number of male and female *S. calcitrans* collected were influenced by the attractant type (farm 1: $\chi^2 = 22.75$, df = 4, $P < 0.05$; farm 2: $\chi^2 = 63.19$, df = 4, $P < 0.05$; farm 3: $\chi^2 = 15.89$, df = 4, $P < 0.05$; farm 4: $\chi^2 = 50.02$, df = 4, $P < 0.05$). All attractants captured more males than females on farms 2, 3, and 4. However, the number of captures between males and females varied with different attractants on farm 1, with dry ice, a mixture of cow dung and urine, and a combination of dry ice plus octenol capturing more females, while the control and octenol captured more males (Fig. 1).

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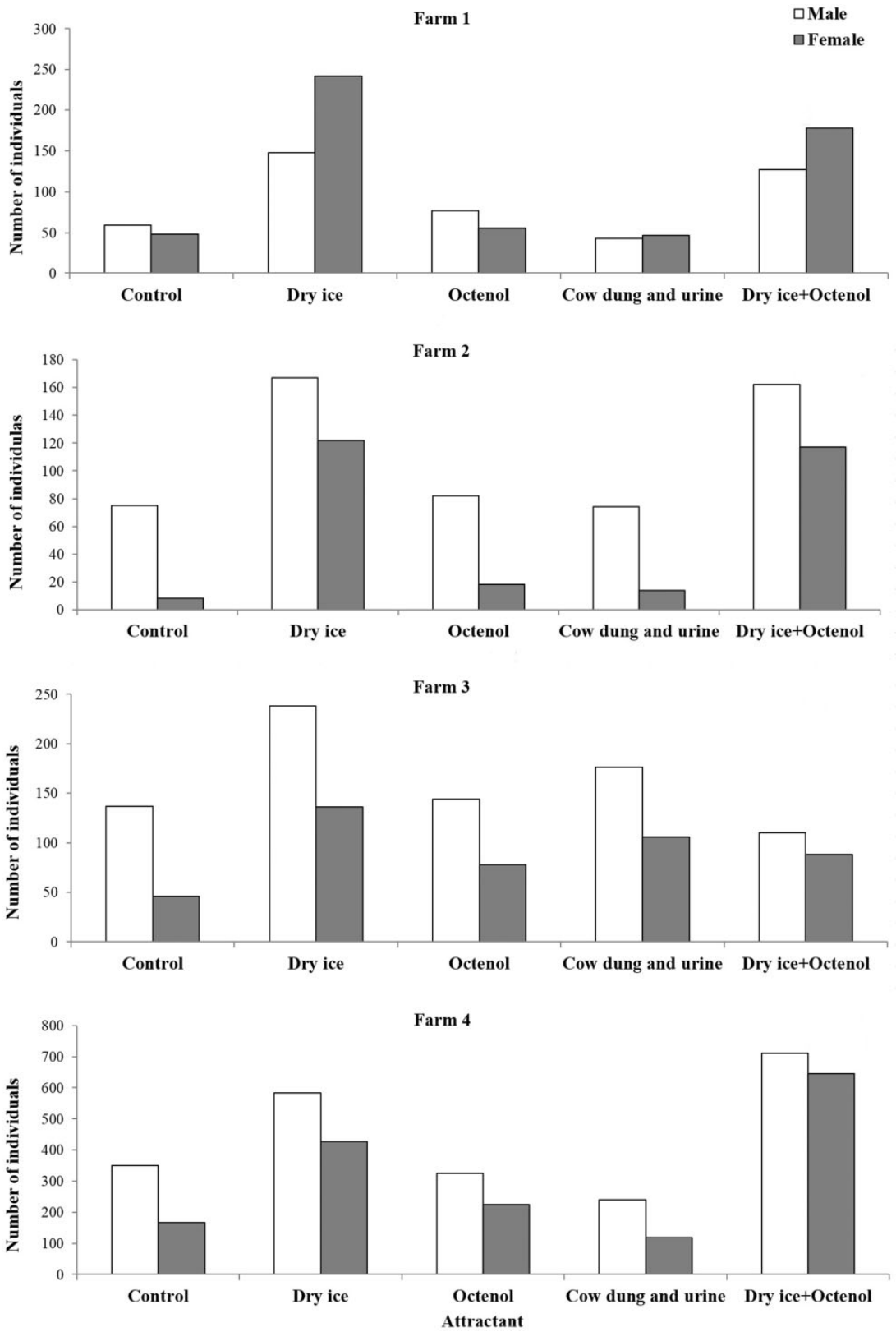


Fig. 1. Total number of *S. calcitrans* males and females collected by Vavoua traps baited with different attractants on four dairy farms.

On farm 1, the number of *S. calcitrans* males was significantly different among treatments ($F = 3.01$; df = 4, 37; $P = 0.030$). Traps baited with only dry ice captured significantly more males when compared with a mixture of cow dung and urine or the control, but traps baited with a combination of dry ice plus octenol did not significantly capture more males than either the dry ice, octenol, or the control. The number of females was significantly different among treatments ($F = 8.32$; df = 4, 37; $P = 0.000$). Traps baited with dry

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Table 2. Mean number (± SE) of *S. calcitrans* males and females collected by each attractant treatment in the Muak Lek district, Saraburi province

Attractant	Farm 1		Farm 2		Farm 3[a]		Farm 4[b]	
	Male	Female	Male	Female	Male	Female	Male	Female
Control	5.9 ± 2.8bc	4.8 ± 1.9b	7.5 ± 3.4a	0.8 ± 0.3c	13.7 ± 5.4a	4.6 ± 1.2a	38.89 ± 10.4bc	18.44 ± 4.2c
Dry ice	14.8 ± 5.1a	24.2 ± 9.7a	16.7 ± 7.4a	12.2 ± 6.9a	23.8 ± 8.6a	13.6 ± 4.1a	58.4 ± 11.0ab	42.7 ± 7.4b
Octenol	7.7 ± 2.6abc	5.6 ± 2.1b	8.2 ± 4.3a	1.8 ± 0.5abc	14.4 ± 4.4a	7.8 ± 1.9a	32.5 ± 5.7c	22.4 ± 5.9c
Cow dung and urine	4.3 ± 1.4cd	4.7 ± 1.8b	7.4 ± 3.3a	1.4 ± 0.6c	19.56 ± 8.5a	11.78 ± 4.2a	24 ± 2.6c	11.8 ± 1.2c
Dry ice + Octenol	12.7 ± 5.2ab	17.8 ± 7.3a	16.2 ± 7.8a	11.7 ± 6.5ab	13.75 ± 3.2a	11 ± 2.1a	79 ± 16.0a	71.67 ± 11.6a
ANOVA df = 4, 37	$F = 3.01$	$F = 8.32$	$F = 1.87$	$F = 3.45$	$F = 1.21$	$F = 2.24$	$F = 8.24$	$F = 17.38$
ANOVA df = 4, 35[b]	$P < 0.05$	$P < 0.05$	$P = 0.137$	$P < 0.05$	$P = 0.330$	$P = 0.085$	$P < 0.05$	$P < 0.05$
ANOVA df = 4, 34[a]								

Column means followed by the same letter in each column are not significantly different, LSD test, $P < 0.05$. Untransformed data shown.

[a, b] There were missing data during the experiment on farm 4 resulting in the following sample number: control ($n = 9$) and dry ice + octenol ($n = 9$) and on farm 3: cow dung and urine ($n = 9$) and dry ice + octenol ($n = 8$), respectively.

ice or a combination of dry ice plus octenol were more effective than those baited with octenol, a mixture of cow dung and urine, or the control (Table 2).

On farm 2, the number of males was not significantly different among treatments ($F = 1.87$; df = 4, 37; $P = 0.137$). However, the highest number of males captured was in traps baited with dry ice alone. The number of females was significantly different among treatments ($F = 3.45$; df = 4, 37; $P = 0.017$). Traps baited with dry ice or a combination of dry ice plus octenol captured significantly more females when compared with a mixture of cow dung and urine or the control (Table 2).

On farm 3, there were no significant differences between the numbers of both males and females among treatments ($F = 1.21$; df = 4, 34; $P = 0.326$, $F = 2.24$; df = 4, 34; $P = 0.085$), respectively. However, the highest numbers of both males and females captured were in traps baited with dry ice alone (Table 2).

On farm 4, there was a significant difference between the number of both males and females among treatments ($F = 8.24$; df = 4, 35; $P = 0.000$, $F = 17.38$; df = 4, 35; $P = 0.000$), respectively. A higher number of males were captured in traps baited with a combination of dry ice plus octenol, in comparison to octenol, a mixture of cow dung and urine, or the control; however, the number of males captured using traps baited with dry ice was not significantly different with either a combination of dry ice plus octenol or the control. There were no differences in the number of males captured in traps baited with octenol, a mixture of cow dung and urine, or the control. A higher number of females were captured in traps baited with a combination of dry ice plus octenol than those baited with dry ice, octenol, a mixture of cow dung and urine, or the control. However, traps baited with dry ice captured significantly more females when compared with octenol, a mixture of cow dung and urine, or the control, but there were no differences in the numbers of females captured in traps baited with octenol, a mixture of cow dung and urine, or the control. The highest numbers of both males and females were captured in traps baited with dry ice plus octenol (Table 2).

Discussion

Of the four species of *Stomoxys* collected, *S. calcitrans* was the most abundant species on the dairy cattle farms. This finding is in agreement with previous reports (Masmeatathip et al. 2006, Muenworn et al. 2010, Phasuk et al. 2013). Moreover, this species was present in large numbers at different zoos in Thailand (Tanasak et al. 2012).

In the current study, there was a significant difference in the numbers of captures between *S. calcitrans* males and females with different attractants. Males were reported to be more attracted to Vavoua traps than females (Masmeatathip et al. 2006, Muenworn et al. 2010, Phasuk et al. 2013). Those findings were similar to the results from the present study. Males responded to the baited traps as both assembly sites and waiting stations for mating (Beresford and Sutcliffe 2008). However, the number of captures between males and females varied with different attractants on farm 1. Further studies are needed to evaluate whether these differences are associated with the environment around the trap or other factors that influence the number of males and females captured.

The Vavoua traps baited with CO_2 alone or a combination of CO_2 plus octenol, collected significantly more *S. calcitrans* compared with octenol alone or no attractant. For example, CO_2 from dry ice has been reported as an effective attractant for increasing adult stable fly abundance on various traps (DeFoliart and Morris 1967, Hoy 1970, Cilek 1999). Generally, in the present study, there were no significant differences among trap collections when the combination of CO_2 plus octenol was compared with CO_2 alone. Cilek (1999) reported similar results from Alsynite cylinder traps. The present study showed traps baited with octenol caught more *S. calcitrans* than control traps, but the difference was not significant. Similar findings were reported from *S. calcitrans* collections with Alsynite cylinders baited with octenol compared with no odor (control) in Northern Florida (Cilek 1999) and in California, United States, although cylindrical blue cloth targets were used (Mullens et al. 1995). In Ontario, Canada, octenol increased Nzi trap catches of *S. calcitrans* at a residence in the absence of livestock, but did not increase catches at a dairy farm with cattle present at or near trap sites (Mihok et al. 2007). Broce and Haas (1999) reported that aged cattle manure was shown to attract stable flies, especially gravid females searching for oviposition sites. The abundance of stable flies increased significantly in grasslands and crop fields when the odor associated with cattle manure slurry was applied (Tangtrakulwanich et al. 2015). In contrast to these results, traps baited with a mixture of cow dung and urine produced no significant difference in capturing *S. calcitrans* when compared with no attractant. These baited traps were located in close proximity to cattle pens. This result suggested that the effects of manure odors from cattle pens may have masked the attractiveness of the traps baited with a mixture of cow dung and urine. The efficacy of the natural attractants is still very poorly known for collecting stable flies in Thailand; further studies are needed.

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In conclusion, baited traps should be placed in sunny locations away from bushes, trees, and cattle. The Vavoua traps baited with only CO_2 from the dry ice or a combination of CO_2 plus octenol were effective attractants for collecting *S. calcitrans*. However, both dry ice and octenol can be costly and inconvenient to use in remote areas where dry ice is unavailable. Additionally, octenol is difficult to obtain and so it may not be justified for use in combination with dry ice for routine stable fly sampling or large-scale trap out programs (Cilek 1999) .

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Establishment of Diagnostic Doses of Five Pyrethroids for Monitoring Physiological Resistance in *Aedes Albopictus* in Thailand

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ESTABLISHMENT OF DIAGNOSTIC DOSES OF FIVE PYRETHROIDS FOR MONITORING PHYSIOLOGICAL RESISTANCE IN *AEDES ALBOPICTUS* IN THAILAND

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ABSTRACT. Monitoring insecticide resistance of *Aedes albopictus* is required for implementing effective dengue and chikungunya vector control in Thailand. The World Health Organization standard susceptibility test for adult mosquitoes was used to determine the baseline susceptibility of a pyrethroid-susceptible laboratory strain of *Ae. albopictus* to 5 different pyrethroids (deltamethrin, permethrin, bifenthrin, cypermethrin, and α-cypermethrin). Subsequently, the respective established diagnostic doses (0.026% deltamethrin, 1.024% permethrin, 0.570% bifenthrin, 0.237% cypermethrin, and 0.035% α-cypermethrin) were used to test field-collected *Ae. albopictus* from Rayong, Koh Chang, and Pong Nom Ron. As expected, the laboratory strain was completely susceptible to all pyrethroid insecticides at the established concentrations. Rayong mosquitoes were found to be highly susceptible to bifenthrin, cypermethrin, and α-cypermethrin. Koh Chang mosquitoes were susceptible to only deltamethrin and permethrin. Pong Nom Ron mosquitoes were resistant to all pyrethroids tested. Routine assessment of these baseline results should guide future resistance monitoring to pyrethroid insecticides in *Ae. albopictus* in Thailand.

KEY WORDS *Aedes albopictus*, diagnostic dose, pyrethroid, susceptibility

INTRODUCTION

Aedes albopictus (Skuse) is the important vector of chikungunya (CHIK) virus in Thailand (Thavara et al. 2009) and regarded as an important secondary vector of dengue (DEN) virus (Srisawat et al. 2014). *Aedes albopictus* has been shown to be more competent for developing transmissible infection with CHIK virus than *Ae. aegypti* (L.) (Vega-Rua et al. 2014). In Thailand, this species is commonly found in rubber and coconut plantations (Thavara et al. 2001) and has been associated with areas of high numbers of human CHIK cases in southern Thailand (Chareonviriyaphap et al. 2003).

The first confirmed case of CHIK in Thailand was seen in Bangkok in 1958 followed by epidemics in eastern, northern, and southern parts of Thailand (Hammon et al. 1960). Decades later a large outbreak occurred in 2009, affecting >46,000 people from central, northeastern, northern, and southern provinces of Thailand (Pulmanausahakul et al. 2011). Annually, reports of human CHIK infections occur, primarily in the southern part (Narathiwat, Phuket, Suratthani, and Chumphon provinces) of the country, generally raising concern of new outbreaks of disease. In DEN- and CHIK-endemic areas, space spraying of insecticides plays an important role in preventing or stopping the spread of these viruses. Pyrethroids have been widely used for controlling adult *Ae. albopictus* in Thailand, although pyrethroid-resistant populations of *Ae. albopictus* have been documented (Chuaycharoensuk et al. 2011, Chareonviriyaphap et al. 2013). Similarly, Ponlawat et al. (2005) reported that *Ae. albopictus* larvae collected from Mae Sot (Tak Province) and Phatthalung Province were resistant to permethrin and slightly resistant to temephos (organophosphate used as a larvicide).

Insecticide resistance could become one of the major obstacles for the control of *Aedes* vectors; however, the extent and degree of resistance is unknown. The resistance status of adult mosquitoes, including *Ae. albopictus*, typically involves the use of the World Health Organization (WHO) tube assay with insecticide-treated papers (WHO 1998). This is a relatively simple technique for the routine evaluation of chemical susceptibility in mosquitoes and provides important data to support mosquito management strategies. The discriminating diagnostic concentrations of insecticides are typically those recommended by WHO from information gathered from WHO collaborating centers, and determined by consensus from expert committees and the WHO Pesticide Evaluation Scheme (Macoris et al. 2005). Currently, WHO recommended diagnostic concentrations are provided only for *Ae. aegypti*, *Culex quinquefasciatus* (Say), and *Anopheles*

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mosquitoes (WHO 1992). Therefore, the recommended diagnostic concentrations for *Ae. aegypti* have been commonly used for *Ae. albopictus*; however, there are only 2 chemicals currently indicated, lambda-cyhalothrin and permethrin (IRAC 2011). Therefore, the present study set out to determine the appropriate baseline lethal concentrations (LC_{50} and LC_{99}) for 5 different pyrethroids that are used for *Aedes* control in Thailand, beginning with a susceptible laboratory strain of *Ae. albopictus* established at Kasetsart University. Double the concentration of the LC_{99} of each active ingredient was used as the final diagnostic concentration for testing wild-caught field populations.

MATERIALS AND METHODS

Mosquitoes

The 3 populations of *Ae. albopictus* were collected as immature stages from Rayong Province (12°47′5.75″N, 101°38′53.94″E), Koh Chang (Trat Province; 12°02′22.4″N, 102°23′23.6″E), and Pong Nom Ron (Chanthaburi Province; 12°53′05.9″N, 102°26′00.4″E). Each was tested using the diagnostic doses for deltamethrin, permethrin, bifenthrin, cypermethrin, and α-cypermethrin. An insecticide-susceptible laboratory strain of *Ae. albopictus* (KU strain originally from Satun Province, southern Thailand) was obtained from the entomology laboratory at Kasetsart University, Bangkok. The KU strain has been maintained continuously in the laboratory for nearly 10 years. All field populations of *Ae. albopictus* were reared under standard laboratory conditions at 25 ± 2°C and 70–80% RH with a photoperiod of 12:12 L:D. Larvae were fed daily with fish food (TetraMin®, TetraGmbH, Melle, Germany). Pupae were collected and transferred into wire-mesh cages for adult emergence. Free-mating adults were provided 10% sugar solution as food. For subsequent test population propagation in the laboratory, female adults were fed on a live guinea pig for blood for egg production under animal use protocol number ACKU-01956 approved by the Committee on Animal Use, Kasetsart University. Only F_1- to F_3-generation adults were used in the tests.

Insecticides

Five active ingredient pyrethroids were used to determine the baseline diagnostic dose of each using the KU strain. Technical-grade pyrethroid chemicals were obtained from Sherwood Chemicals Public Company Ltd. (Bankok, Thailand) as deltamethrin (98.46% purity), permethrin (97.6%), bifenthrin (95.12%), cypermethrin (92.8%), and α-cypermethrin (97.05%).

Insecticide-impregnated papers

Insecticide solutions were prepared with a mixture of acetone as organic solvent and silicone as carrier in a ratio of 1.66:1.34. Each pyrethroid was diluted serially into 5 concentrations. Whatman (GE Healthcare UK Limited, Buckinghamshire, UK) No. 1 filter paper rectangular strips (12 cm × 15 cm) were impregnated with 2 ml of each dilution of pyrethroid solution. Untreated control papers were made with acetone and carrier alone. All impregnated papers were air-dried for at least 24 h before used in assays.

Establishing baseline diagnostic lethal concentrations

The *Ae. albopictus* KU strain was exposed to a series of concentrations of each test chemical (Table 1) to obtain baseline susceptibility data using the WHO adult susceptibility tube method (WHO 2013). Final dilution rates were determined from an initial series of susceptibility tests on the KU strain (data not shown) based on WHO (2013) recommended diagnostic concentrations for each chemical against anopheline species (except cypermethrin, which was based on the current recommendation for permethrin). Using findings from 4 susceptibility tests (minimum of 100 mosquitoes for each chemical), the highest and lowest percent concentration producing complete and the least mortality, respectively, were established. Subsequently, a final 5 serial test dilutions of AI concentrations were made at a 5-fold rate for deltamethrin, permethrin, and cypermethrin, at a 4-fold rate for bifenthrin, and a 3-fold rate for α-cypermethrin. Twenty-five nonbloodfed females (3–5 days old) were introduced into each holding tube lined with clean (untreated) paper. Each trial consisted of 4 treatment tubes and 4 control tubes. Each plastic holding tube was connected either to another identical tube containing the insecticide-impregnated papers or to a control tube lined with solvent plus carrier-impregnated papers. Mosquitoes were held in the holding tube for 60 min before transferring them to the respective test tubes and exposed for 1 h with tubes held in the upright position. Afterwards, mosquitoes were transferred back to the clean holding tubes by gently blowing and provided with 10% sugar solution on cotton wool. Knockdown was recorded after 1 h and the final mortality of mosquitoes was recorded after 24 h. The mortality data were analyzed using a Pearson chi-square statistic to measure goodness of fit based on comparisons of observed and expected distributions. The 50% and 99% lethal concentrations (LC_{50} and LC_{99}) with associated 95% fiducial limits were calculated from the baseline data using maximum likelihood estimates of parame-

Table 1. Knockdown and mortality of susceptible *Aedes albopictus* (KU strain) exposure to 5 serial concentrations of 5 different pyrethroids.

Insecticide	Concentration (%)	No.	Knockdown rate at 1-h postexposure	Mortality rate at 24-h postexposure
Deltamethrin	0.00004	100	0	0
	0.0002	108	1.9	1.9
	0.001	105	19.0	48.6
	0.005	107	79.4	88.8
	0.025	108	99.1	100
Permethrin	0.00112	106	0	0
	0.0056	113	0	1.8
	0.028	105	8.6	15.2
	0.14	116	65.5	82.8
	0.7	101	100	100
Bifenthrin	0.00391	103	1.0	2.9
	0.015625	106	2.8	18.8
	0.0625	105	5.7	74.3
	0.25	101	90.1	100
	1.0	104	100	100
Cypermethrin	0.0002	108	0	0
	0.001	107	5.6	3.7
	0.005	114	4.4	38.6
	0.025	108	85.2	83.3
	0.125	109	100	99.1
α-Cypermethrin	0.0008	99	9.1	25.3
	0.0024	100	33.0	71.0
	0.0072	100	96.0	90.0
	0.0216	98	100	100
	0.0648	100	99	100

ters and log-probit regression analysis (SAS/STAT version 8; SAS Institute Inc., Cary, NC). A doubling of the derived LC_{99} concentration was used as the final diagnostic dose for each insecticide (WHO 1981).

Diagnostic doses for pyrethroids

The diagnostic dose for each pyrethroid was prepared on the filter papers to evaluate the susceptibility status of 3 field populations of *Ae. albopictus*. The susceptibility test method was conducted in the same manner as described for the baseline determinations. The insecticide susceptibility status of 3 field strains of *Ae. albopictus* to deltamethrin, permethrin, bifenthrin, cypermethrin, and α-cypermethrin were defined according to the WHO criteria established for anophelines (WHO 2013); however, these criteria were considered valid for all mosquito genera. For final mortality between 98% and 100%, a test population was considered fully "susceptible"; mortality between 90% and 97% was treated as "suggestive of the existence of resistance" and requiring further investigation (i.e., additional bioassays or determination of mechanism(s); and mortality below 90% was an indication of outright "resistance" in the population. If at least 2 additional tests consistently showed mortality below 98%, then resistance was confirmed. Lastly, if mortality was <90%, confirmation of the existence of resistant genes in the test population with additional bioassays was not considered necessary, provided a minimum of 100 mosquitoes of each species (population) was tested.

RESULTS

The baseline measures for deltamethrin, permethrin, bifenthrin, cypermethrin, and α-cypermethrin were established using mortality data from a known pyrethroid-susceptible laboratory strain of *Ae. albopictus* using serial dilution concentrations of each test chemical. Based on the LC_{50} and LC_{99} values, the final diagnostic dose for each pyrethroid was calculated by doubling the estimated percent LC_{99} (Table 2). Permethrin produced the highest diagnostic dose, 1.024%, while deltamethrin had the lowest, 0.026%. The derived diagnostic dose for each pyrethroid was used to measure the susceptibility response of field-collected *Ae. albopictus* from Rayong, Koh Chang, and Pong Nom Ron compared with the susceptible KU strain. The 24-h mortality rates of all 4 populations to the diagnostic doses are presented in Table 3. Bifenthrin, cypermethrin, and α-cypermethrin were effective insecticides against *Ae. albopictus* from Rayong Province, with mortality rates between 99.1–100%, followed by deltamethrin with 95.3% mortality. Permethrin was much less effective against the Rayong population, causing only 50.1% mortality. Nearly all mosquitoes from

Table 2. Probit dose–mortality analysis of a laboratory pyrethroid-susceptible strain of *Aedes albopictus* (KU) exposed to 5 different pyrethroids using 5 different concentrations establishing percent lethal concentrations (LC) for each insecticide.

Insecticide	*N*	LC_{50} % (95% FL[1])	LC_{99} % (95% FL)	Slope ± SE	Diagnostic dose[2] (%)	χ^2 (df)[3]	*P*-value[3]
Deltamethrin	528	0.00122 (0.00101–0.00146)	0.01310 (0.00908–0.02140)	2.2566 ± 0.1861	0.0262	4.2049 (3)	0.2402
Permethrin	541	0.06140 (0.03975–0.09482)	0.51189 (0.25728–2.1867)	2.5258 ± 0.3101	1.02378	6.3835 (3)	0.0944
Bifenthrin	519	0.03233 (0.02745–0.03814)	0.28489 (0.20301–0.44825)	2.4614 ± 0.1993	0.56978	4.7620 (3)	0.1901
Cypermethrin	546	0.00755 (0.00622–0.00916)	0.11853 (0.07941–0.20072)	1.9455 ± 0.1486	0.23706	0.5606 (3)	0.9054
α-Cypermethrin	497	0.00153 (0.00126–0.00182)	0.0176 (0.0122–0.02956)	2.1924 ± 0.2085	0.0352	2.9669 (3)	0.3968

[1] FL = fiducial limits as 95% confidence level.
[2] Diagnostic dose = 2× LC_{99}.
[3] Goodness-of-fit test statistic.

Table 3. Laboratory and 3 field populations of *Aedes albopictus* exposed to established diagnostic doses of 5 pyrethroids.

			KU strain		Rayong		Koh Chang		Pong Nom Ron	
Insecticide	Diagnostic dose (%)	Exposure time (h)	No. tested	Dead (% ± SE)	No. tested	Dead (%[1] ± SE)	No. tested	Dead (% ± SE)	No. tested	Dead (% ± SE)
Deltamethrin	0.0262	1	110	110 (100)	109	104 (95.28 ± 2.78)	99	98 (98.96 ± 1.0)	106	71 (66.67 ± 5.88)
Permethrin	1.02378	1	110	110 (100)	107	56 (50.97 ± 7.79)	100	98 (97.94 ± 1.21)	108	64 (58.87 ± 4.38)
Bifenthrin	0.56978	1	106	106 (100)	108	108 (100)	107	96 (89.42 ± 1.29)	100	62 (61.64 ± 2.81)
Cypermethrin	0.23706	1	107	107 (100)	108	107 (99.05 ± 0.93)	98	93 (94.73 ± 2.02)	106	73 (68.57 ± 4.30)
α-Cypermethrin	0.0352	1	99	99 (100)	108	108 (100)	101	96 (94.89 ± 2.10)	97	34 (34.44 ± 4.66)

[1] Abbott's formula value.

Koh Chang were killed by deltamethrin and permethrin; whereas final mortality was less (89.4–95.0%) for the other 3 pyrethroids (Table 3). By comparison, the Pong Nom Ron population was the most resistant of those tested, with between 69% and 34.4% mortality among all insecticides. Following the WHO (2013) criteria for interpretation of the susceptibility status to insecticides by anopheline mosquitoes, the Pong Nom Ron population was defined as resistant to all test pyrethroids, while the Rayong mosquitoes appeared completely susceptible to bifenthrin, cypermethrin, and α-cypermethrin, with suggestive resistance to deltamethrin and frank resistance to permethrin. The Koh Chang population showed clear susceptibility to deltamethrin and permethrin, with suggestive resistance to the other 3 pyrethroids.

DISCUSSION

In this study, diagnostic doses of 5 pyrethroids were established using an insecticide-susceptible *Ae. albopictus* laboratory colony (KU strain) originating from Thailand. These doses were subsequently tested against recently collected field populations established in an insectary. Rayong and Kho Chang populations of *Ae. albopictus* demonstrated resistance to deltamethrin, bifenthrin, cypermethrin, and α-cypermethrin, while the population from Pong Num Ron was more strongly resistant to all 5 pyrethroids. This is the first published information on the determination of the diagnostic doses of pyrethroids against *Ae. albopictus* in Thailand. The establishment of diagnostic doses of insecticides are needed for more accurate monitoring of resistance patterns in *Ae. albopictus* populations throughout the country. This information is critical for guiding site-specific insecticide selection and use.

The source of selection pressure for development of resistance in the 3 field populations is unclear, but might have been influenced by the presence of exogenous agrochemicals used in each location. In Rayong and Kho Chang, where relatively less resistance was recorded, specimens were collected in rubber plantations where insecticides are infrequently used, whereas the Pong Num Ron population with high pyrethroid resistance was collected in a fruit orchard where agrochemicals were more frequency used to suppress pests. It has been suggested that *Ae. albopictus* might have developed resistance due to exposure to agrochemicals, thus leading to possible cross-resistance mechanisms between other insecticides, including those used in public health (Khan et al. 2011, Nkya et al. 2013). Alternatively, it is possible that insecticides used to control other pests or other mosquitoes as personal household use or public applications might inadvertently exert selection pressure on the peridomestic *Aedes* populations. For example, indoor residual spraying of deltamethrin for malaria control in Thailand could promote the selection of resistance in nontarget mosquitoes exposed to sublethal insecticide concentrations.

Aedes albopictus has the capacity to transmit DEN and CHIK viruses to humans (Thavara et al. 2009). It has become a species of greater medical importance due to rapid and extensive geographical expansion outside of its native Southeast Asian habitats (Kraemer et al. 2015). This invasive species can become relatively abundant once it becomes established, and typically exhibits an aggressive bloodfeeding behavior toward humans (Hawley 1988, Turell et al. 2001). Ecologically, *Ae. albopictus* is more commonly found in rural areas, but has the capacity to invade more urban and suburban areas, a particular concern as urbanization in the country expands (Preechaporn et al. 2006, Li et al. 2014). *Aedes albopictus* is generally regarded as a less efficient vector of DEN virus than *Ae. aegypti*; however, it is considered the most important vector of CHIK virus in Thailand. Unfortunately, few data are available regarding the chemical resistance of *Ae. albopictus*. Ponlawat et al. (2005) has reported *Ae. albopictus* from Mae Sot district in Tak Province and Phatthalung Province as resistant to permethrin. Field population of *Ae. albopictus* in Sadao district, Songkhla Province has also been found resistant to permethrin (<80% susceptible), but mosquitoes collected from Chumphon, Prachuap Khiri Khan, Songkhla (Namom and Sadao districts), and Surat Thani provinces were completely susceptible to deltamethrin and lambda-cyhalothrin (Chuaycharoensuk et al. 2011).

The detection of insecticide resistance in *Ae. albopictus* in Thailand has been based on the WHO-established diagnostic doses of insecticides against either *Ae. aegypti*, or when lacking information for this species, the recommended doses for *Anopheles* mosquitoes. The susceptibility-level baseline and diagnostic doses of some insecticides used to control *Ae. aegypti* in Thailand have been reported (Jirakanjanakit et al. 2007, Juntarajumnong et al. 2012). Jirakanjanakit et al. (2007) established diagnostic doses for deltamethrin (0.06%) and permethrin (0.9%) using an *Ae. aegypti* strain from Bora Bora (French Polynesia). In their study, nearly all *Ae. aegypti* collected from different parts of Thailand were deemed resistant to deltamethrin and permethrin, while most of *Ae. albopictus* tested were susceptible to the 2 insecticides. Juntarajumnong et al. (2012) established discriminating lethal concentrations for 6 different pyrethroids using *Ae. aegypti* (US Department of Agriculture susceptible strain), followed by testing 3 field populations of *Ae. aegypti*. Their study found the Khon Kaen population was highly resistant to

deltamethrin, bifenthrin, and lambda-cyhalothrin, while the Nong Khai population was resistant to nearly all pyrethroids tested and the population from Kanchanaburi showed a low-grade resistance to all 6 chemicals. The established diagnostic doses from both studies were different from the WHO-recommended 0.25% permethrin for *Ae. aegypti*. Jirakanjanakit et al. (2007) used 0.9%, and Juntarajumnong et al. (2012) reported 0.15% permethrin as diagnostic in their study. The difference in testing concentrations is likely the result of differing responses between the insecticide-susceptible strains of *Ae. aegypti*.

Various chemical methods have been used to control *Aedes* mosquitoes in both the immature stages (e.g., organophosphates and insect growth regulators) and adults (primarily pyrethroids and organophosphates) (Chareonviriyaphap et al. 2013). Pyrethroids (deltamethrin, permethrin, cyfluthrin, and cypermethrin) are the primary adulticides used in the national mosquito control program in Thailand (Chareonviriyaphap et al. 2013). In Thailand, the development of resistance in adult *Ae. aegypti* to pyrethroids, organophosphates, and carbamates and in larvae to organophosphates (temephos) has been documented throughout the country (Ponlawat et al. 2005, Paeporn et al. 2006, Thanispong et al. 2008, Komalamisra et al. 2011). *Aedes aegypti* generally inhabit more densely populated urban areas and frequently inside human habitations, whereas *Ae. albopictus* is more commonly found in rural areas (villages, plantations, forest areas) and more commonly in outdoor and peridomestic locations. Due to the closer connection to humans, *Ae. aegypti* is believed to generally have a higher exposure to insecticides than *Ae. albopictus*. Paeporn et al. (1996) and Chareonviriyaphap et al. (2013) reported that many commercially available household products containing pyrethroid insecticides are widely used to control mosquitoes in and around the home. It is little surprise that there have been increasing reports of pyrethroid resistance in *Ae. aegypti* across Thailand as opposed to *Ae. albopictus* (Jirakanjanakit et al. 2007, Chuaycharoensuk et al. 2011, Chareonviriyaphap et al. 2013).

Establishing standard diagnostic doses of insecticides for monitoring susceptibility of *Ae. albopictus* is required to accurately assess the minimum concentration that will kill wild-type adult mosquitoes. Routine monitoring can also be useful for comparison of response between different populations (locations), and as an important measure of changes in susceptibility over time so that adjustments can be made operationally to control this species. For *Ae. aegypti*, WHO has published a diagnostic dosage for only 2 insecticides, permethrin (0.25%) and lambda-cyhalothrin (0.03%) (WHO 1992). To date, no recommended concentrations have been published for *Ae. albopictus*, necessitating that baseline diagnostic lethal concentrations be established independently.

Due to the lack of diagnostic doses of insecticides for susceptibility testing on *Ae. albopictus*, insecticide resistance in this species has been neglected compared with other species of public health importance. The limited data on insecticide susceptibility patterns in different populations may result in less effective vector control responses to prevent or abate DEN and CHIK transmission. Because *Ae. albopictus* can be found across many rural and urban areas of Thailand, this species poses a high risk for disease transmission. Therefore, the diagnostic doses obtained from the present study will assist others to detect and characterize the current and future status of insecticide resistance in adult *Ae. albopictus* in the country. Further work, possibly using a combination of different bioassay systems, is needed to better quantify the intensity (or strength) of resistance and its potential impact on control of this species (and others) linked to epidemiological outcomes (Bagi et al. 2015). Standardized, continuous insecticide susceptibility surveillance throughout Thailand is required to ensure the use of only effective insecticides and to mitigate the development of resistance in target mosquito populations.

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Plants traditionally used as mosquito repellents and the implication for their use in vector control

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ABSTRACT

Numerous plants with insect repelling properties are native to the tropics where they are produced for a wide range of medicinal purposes. In Thailand, these native plant species have a history of use for personal protection against biting insects. From our investigation we identified 37 plant species within 14 plant families that showed some mosquito repellent properties. Of these, 9 plant species were characterized using an excito-repellency test system against several Thai mosquito species. Results from these studies revealed that five essential oils extracted from plants demonstrated promising insect repellent activity. These active ingredients show promise for further development into formulations that may serve as alternatives to DEET or possibly be used as natural bio-pesticides to kill mosquitoes.

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1. Background

Repellents play an important role in vector control and prevention, particularly in those areas where the biology and feeding behavior or mosquito vectors are less favorable to existing or available methods. Prior to the arrival of synthetic chemicals, people used plant-derived materials to repel or kill medically important arthropods, including mosquitoes (Curtis et al., 1990). Local knowledge and practices of indigenous peoples demonstrated the extensive information available associated with native plants with such potential usages. Traditionally, these plants are used by bruising freshly picked sprigs that are then hung inside houses or burned outdoors on the evening fire. Such methods are extensively used but unfairly dismissed or overlooked. Natural plants have also been used in the form of crude fumigants where plants are burned to drive away blood sucking arthropods or as essential oil formulations applied tropically onto the skin or clothes. This latter application was first reported by ancient Greek (Herodotus,

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1996), Roman (Owen, 1805), and Indian (Johnson, 1998) scholars. Even today, repellent plant species are used in traditional practices by local communities throughout the tropics and subtropics due to their safety, effectiveness and relative low cost. Because of these reasons, the popularity of the natural products from several plants increased for indoor and outdoor domestic using to the local consumer. Several native plant species have been the focus of recent repellent work and have proved particularly effective against several mosquito species as well as other medically important arthropods (Tawatsin et al., 2001; Phasomkusolsil and Soonwera, 2010). Chemicals from these botanical plants exhibit in both of behavioral and physiological effects, including repellents, attractants, feeding deterrents, toxins, insect growth regulators and hormones (Isman, 2006; Moore, 2014).

One location with high concentrations of botanical based insect repellents is in tropical Asia and Southeast Asia. Over 2300 plant species were identified as having potential mosquito repellent properties from throughout Southeast Asia (de Boer et al., 2010; Maia and Moore, 2011; Vongsombath, 2011; Obico and Ragragio, 2014). Recently, over 90 plant species were evaluated as potential repellents or toxicants against blood-feeding invertebrates and pestiferous flies in Loa PDR (de Boer et al., 2010). In the Philippines, a list of 54 species of plants encompasing 49 genera and 26 families were evaluated as repellents. From this group the family Fabaceae contains the greatest number of species with insect repellent activity (Obico and Ragragio, 2014). In Malaysia, six Malaysian plants, including turmeric (*Curcuma longa*), ginger (*Zingiber officinale*), pandan (*Pandanus odorus*), cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*) and lemongrass (*Cymbopogon citratus*) had been tested against the cockroach species, *Periplaneta americana* (Ahmad et al., 1995). Extracts of cinnamon (*C. zeylanicum*), black pepper (*Piper nigrum*), turmeric (*C. longa*) and the essential oil of ginger (*Z. officinale*) were observed to repel the adult rice weevil, *Sitophilus zeamais* (Ishii et al., 2010). In Indonesia, there are numerous plant species that have been used in the form of essential oil. Around 40 kinds of essential oils have been produced in Indonesia and are currently in use as medicinal therapies. In traditional medicine, patchouli (*Pogostemon cablin*), cananga (*Canangium odoratum*), lemongrass (*C. citratus*) and citronella (*Cymbopogon nardus*) are often used as insect repellents (TRECYDA, 2011). Javanese incense consists of ingredients from several kinds of plants and have also been found to be mosquito repellents (Sangat-Roemantyo, 1990).

There is also sporadic information about insect repellent plants from Cambodia, Myanmar, Vietnam and Singapore. In Cambodia, *Capsicum* peppers were used as an insect repellent by storing pieces of rice malt in a bamboo container with *bhlech* in Ratanakiri, northern Cambodia (Yamamoto et al., 2011). Fifteen plants have been used in traditional medicine. In addition, *Hydnocarpus anthelminticus* was found to be resistant to the harmful effects of insects (Ali et al., 2010). In Myanmar, the cajuput tree (*Melaleuca cajuputi*) has insect repellent properties and can be found throughout the country. This plant provides insecticidal activity against the maize weevil (*Sitophilus zeamais*) and the red flour beetle (*Tribolium castaneum*) (Ko et al., 2009). Several aromatic essential oils have been found from *Piper betle* and *Vitex negundo* (Ali et al., 2010). In Vietnam, the results showed that the number of psyllid infestation levels in citrus orchards planted with guava (*Psidium guajava*) lower when compared to citrus orchards lacking guava (Beattie et al., 2006). An increase in the protection time of a repellent was developed for female stable flies (*Stomoxys calcitrans)* by using essential oils with the addition of tamanu oil (clove bud oil + tamanu oil, clove leaf oil + tamanu oil, thyme white oil + tamanu oil, patchouli oil + tamanu oil, and savory oil + tamanu oil) (Hieu et al., 2010). *Abutilon indicum* known as Indian mallow, there have been used for healing human because this plant have medical properties and might also be used as an insect repellent due to finding geraniol (a constituent of insect repellents) in behavior modifying products (Ali et al., 2010; Saini et al., 2014). The other constiuents including α-pinene, caryophyllene, caryophyllene oxide, endesmol, farnesol, borenol, geraniol, geranyl acetate, elemene and α-cineole, phytoconstituents like β-Sitosterol, caffeic acid, fumaric acid, vanillin, *p*-coumaric acid, *p*-hydroxybenzoic acid, sesquiterpene including lactones, alantolactone and isoalanto-lactone have been reported from this plant.

Records show that limonene can be used as a botanical insecticide and that it is most active as an insecticide to a number of ectoparasites to include insects, mites, and can also use to against microorganisms (Ibrahim et al., 2001). The monoterpene (limonene) has been fund in the essential oils of two plants in Vietnam (*Adenosma glutinosum* and *Illicium verum*) and in that of one plant in Myanmar (*Elettaria cardamomum*) (Ali et al., 2010). In Singapore, the repellent activity of essential oils of pandan (*Pandanus amaryllifolius*) reported to repel German cockroaches (*Blattella germanica*) (Li and Ho, 2003). Essential oils of garlic and nutmeg are toxic to the red flour beetle (*T. castaneum)* and the maize weevil (*S. zeamais*) (Ho et al., 1996; Huang et al., 1997). The essential oil of edible fruits of *Dennettia tripetala* was found to be toxic to nymphal and adult stages of the American cockroach, *P. americana*, and the grasshopper, *Zonocerus variegatus* (Iwuala et al., 1981)

2. Plants with mosquito repellent activity in Thailand

For centuries, plants have been available for use as medicines to combat certain diseases worldwide. Relatively few plants, however, have gained significant traction for use to control mosquitoes and other arthropod pests. This could be due to a lack of scientific data rather than a lack of plant activity. Selection by plants to produce repellents can be readily explained by their protective role against phytophagus insects (Maia and Moore, 2011). The use of plants as mosquito repellents also has a high level of consumer acceptance due to the perception that natural repellents are safer than synthetics and easier to find for people who lives in the local area (Nerio et al., 2010). More importantly, mosquito repellents derived from plants can be an inexpensive, sustainable method of preventing disease in high-risk areas.

In Thailand, numerous essential oils extracted from wild and domestic plants have been investigated for mosquito repellent activity (Table 1). The most common native plant families that produce essential oils used as repellents include Araliaceae (Tawatsin et al., 2006), Compositae or Asteraceae (Trongtokit et al., 2005b), Labiatae (Tawatsin et al., 2006), Magnoliaceae (Tawatsin et al., 2006), Meliaceae (Tawatsin et al., 2006), Myristicaceae (Trongtokit et al., 2005b), Piperaceae (Tawatsin et al., 2006), Rutaceae (Trongtokit et al., 2005b), Saururaceae (Tawatsin et al., 2006), Umbelliferae (Trongtokit et al., 2005b), and Zingiberaceae (Trongtokit et al., 2005b). The most effective plant species that have been used against mosquitoes include catnip (Lamiaceae), hairy basil (Lamiaceae), citronella (Poaceae), vetiver (Poaceae) and clove (Myrtaceae) (Polsomboon et al., 2008; Suwansirisilp et al., 2012; Sathantriphop et al., 2014a). These five plants produced comparative yields of essential oils. The yield from catnip (4.60–5.70%) was higher than other plant species followed by vetiver (2.10–2.90%), clove (1.28%), citronella (0.68%) and hairy basil oil (0.05%) respectively (Chungsamarnyart and Jiwajinda, 1992; Dethier et al., 1997; Louey et al., 2001; Kerdchoechuen et al., 2010). These five essential oils resulted in significant repellent responses against several species of mosquitoes at very low concentration (Suwansirisilp et al., 2012; Boonyuan et al., 2014; Sathantriphop et al., 2014a). In addition, there are several other plant species that have lower yields of essential oils but that could be potentially useful as

Table 1
An overview of repellent plant efficacy against mosquito in Thailand.

Plant	Common name	Family	% Repellency (protection time)	Mosquito species	Assay Type	Sources
Aglaia odorata	Prayong	Meliaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Alpinia galanga	Galanga	Zingiberaceae	(7.8 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Boesenbergia rotunda	Finger root	Zingiberaceae		Mosquitoes	Arm-in-cage test	Phukerd and Soonwera (2014)
Chromolaena odorata	Bitter bush, Siam weed	Asteraceae (Compositae)	(7.5 h)	*A. albopictus*	Arm-in-cage test	Bansidhi et al. (2005)
			(7.2 h)	*C. quinquefasciatus*	Arm-in-cage test	Bansidhi et al. (2005)
			(8 h)	*A. dirus*	Arm-in-cage test	Bansidhi et al. (2005)
Cymbopogon citratus	Lemon grass	Poaceae	98.67% (116 min)	*A. aegypti*	Arm-in-cage test	Soonwera and Phasomkusolsil (2014)
			99.75% (128 min)	*C. quinquefasciatus*	Arm-in-cage test	Soonwera and Phasomkusolsil (2014)
Curcuma aromatica	Aromatic turmeric	Zingiberaceae	(0.5 h)	*A. aegypti*	Arm-in-cage test	Choochote et al. (2005)
			(240 min)	*C. quinquefasciatus*	Arm-in-cage test	Choochote et al. (2005)
			(210 min)	*A. dirus*	Arm-in-cage test	Choochote et al. (2005)
				Mosquitoes	Arm-in-cage test	Choochote et al. (2005)
Curcuma longa	Turmeric	Zingiberaceae	(7.7 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*C. quinquefasciatus*	Arm-in-cage test	Tawatsin et al. (2006)
				Mosquitoes	Arm and leg tests	Tawatsin et al., 2001, Choochote et al. (2007)
Cymbopogon citratus	Ta kai	Poaceae		Mosquitoes	Arm-in-cage test	Trongtokit et al. (2005b)
Cymbopogon nardus	citronella	Poaceae	(2 h)	*A. aegypti*	Arm-in-cage test	Trongtokit et al. (2005b)
			(100 mins)	*C. quinquefasciatus*	Arm-in-cage test	Trongtokit et al. (2005b)
			(130 min)	*A. minimus*	Arm-in-cage test	Phasomkusolsil and Soonwera (2010)
			(140 min)	*C. quinquefasciatus*	Arm-in-cage test	Phasomkusolsil and Soonwera (2010)
			(115 min)	*A. aegypti*	Arm-in-cage test	Phasomkusolsil and Soonwera (2010)
				Mosquitoes	Arm or lower leg tests	Trongtokit et al. (2005a)
Cymbopogon winterianus	Kaffir lime	Poaceae		Mosquitoes	Arm and leg tests	Tawatsin et al. (2001)
Eleutherococcus trifoliatus	Phakpaem	Araliaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
Eryngium foetidum	Sawtooth coriander/spiny coriander	Apiaceae	(5 h)	*A. albopictus*	Arm-in-cage test	Bansidhi et al. (2005)
			(7.8 h)	*C. quinquefasciatus*	Arm-in-cage test	Bansidhi et al. (2005)
			(8 h)	*A. dirus*	Arm-in-cage test	Bansidhi et al. (2005)

Table 1 (*Continued*)

Plant	Common name	Family	% Repellency (protection time)	Mosquito species	Assay Type	Sources
Eucalyptus globulus	Eucalyptus	Myrtaceae		Mosquitoes	Arm-in-cage test	Sritabutra et al. (2011)
Hedychium coronarium	White ginger	Zingiberaceae	(7.5 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(7.7 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Houttuynia cordata	Fishwort	Saururaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Litsea cubeba	Ta khrai ton	Lauraceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Litsea petiolata	Litsea tree	Lauraceae		Mosquitoes	Arm-in-cage test	Phukerd and Soonwera (2014)
Melaleuca cajuputi	Cajuput tree	Myrtaceae	(7.9 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Myristica fragrans	Nutmeg tree	Myristicaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Ocimum americanum	Hairy basil	Lamiaceae		Mosquitoes	Arm and leg tests	Tawatsin et al. (2001)
Ocimum basilicum	Sweet basil	Lamiaceae		Mosquitoes	Arm-in-cage test	Phasomkusolsil and Soonwera (2010), Sritabutra et al. (2011)
Ocimum sanctum	Holy basil	Lamiaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Piper betle	Betel pepper	Piperaceae	(7.6 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
			(6.7 h)	*C. quinquefasciatus*	Arm-in-cage test	Tawatsin et al. (2006)
Piper nigrum	Black pepper	Piperaceae	(8 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
			(7.8 h)	*C. quinquefasciatus*	Arm-in-cage test	Tawatsin et al. (2006)
Pogostemon cablin	patchuli	Lamiaceae	(2 h)	*A. aegypti*	Arm-in-cage test	Trongtokit et al. (2005b)
			(170 min)	*A. dirus*	Arm-in-cage test	Trongtokit et al. (2005b)
			(150 min)	*C. quinquefasciatus*	Arm-in-cage test	Trongtokit et al. (2005b)
				Mosquitoes	Arm or lower leg tests	Trongtokit et al. (2005a)
Psidium guajava	Guava	Myrtaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Schefflera leucantha	Hanuman prasankal	Araliaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*C. quinquefasciatus*	Arm-in-cage test	Tawatsin et al. (2006)
Syzygium aromaticum	clove	Myrtaceae	32.4%	*A. aegypti*	Arm-in-cage test	Trongtokit et al. (2004)
			89.0%	*C. tritaeniorhynchus* and *C. gelidus*	Arm-in-cage test	Trongtokit et al. (2004)
			(2 h)	*A. aegypti*	Arm or lower leg tests	Trongtokit et al. (2005a)
Uraria crinita	Hang ma chok	Fabaceae		Fly larvae	–	Chuakul et al. (2002)
Vitex trifolia	Khonthi so	Lamiaceae	(8 h)	*A. albopictus*	Arm-in-cage test	Tawatsin et al. (2006)
			(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Zanthoxylum limonella	makaen	Rutaceae	(190 min)	*A. dirus*	Arm-in-cage test	Trongtokit et al. (2005b)
			(170 min)	*C. quinquefasciatus*	Arm-in-cage test	Trongtokit et al. (2005b)
Zingiber cassumunar	Cassumunar ginger	Zingiberaceae		Mosquitoes	Arm-in-cage test	Phukerd and Soonwera (2014)
Zingiber officinale	Ginger	Zingiberaceae	(8 h)	*A. dirus*	Arm-in-cage test	Tawatsin et al. (2006)
Zingiber zerumbet	Wild ginger	Zingiberaceae		Mosquitoes	Arm-in-cage test	Phukerd and Soonwera (2014)

mosquito repellents. These include makaen (Rutaceae), patchouli (Apiaceae), cedar (Pinaceae), verbena (Verbenaceae), geranium (Geraniaceae), lavender (Lamiaceae), pine (Pinaceae), cinnamon (Lauraceae), rosemary (Lamiaceae), thyme (Lamiaceae), garlic (Alliaceae), and peppermint (Apiaceae) (Sharma et al., 1993; Trongtokit et al., 2005a).

3. Botanical essential oils with natural repellent activity against a range of arthropods

Essential oils derived from a variety of plant species have shown considerable efficacy as repellents against various hematophagous arthropods, especially mosquitoes, and are regarded as safer than conventional synthetic chemicals that also perform as repellents, e.g., pyrethroids (Harrewijn et al., 1995; Maia and Moore, 2011; Tawatsin et al., 2001, 2006; Sathantriphop et al., 2014a,b). For example, catnip (*Nepeta cataria)*, which contains two monoterpenes (*Z*, *E*- and *E*, *Z*-nepetalactone) as its major active components have been used to control termite (*Coptotermes formosanus*) infestations (Chauhan and Raina, 2006) and the German cockroach (*B. germanica*) (Peterson et al., 2002). Shadia et al. (2007) documented that hairy basil (*Ocimum americanum*) essential oil was effective in killing the cutworm, *Agrotis ipsilon*. In addition, Tunón et al. (2006) reported that phenylethyl alcohol, β-citronellol, cinnamyl alcohol, geraniol, and α-pinene isolated from carnations (*Dianthus caryophyllum*) demonstrated promising repellent activities against ticks (*Ixodes ricinus*). Repellent properties by the essential oil from the sticky purple cleome (*Cleome hirta*) and its three active constituents (phytol, (+)-cedrol, and *n*-octacosane) were effective against the livestock tick, *Rhipicephalus appendiculatus* (Ndungu et al., 1999). Vongsombath (2011) found 15 plant species (oils) useful in to repelling mites from chicken roosts. Especially in developing countries, the use of readily available local plants showing significant repellent activity against arthropods provides a possible alternative and sustainable strategy for the control of disease transmission by potential vectors or harm (e.g., predation, parasitism) caused by economic pests.

4. Excito-repellent properties of essential oils against mosquito populations in Thailand

Behavioral responses of mosquitoes to candidate chemicals provide useful insights of components of their efficacy in vector control strategies. Attempts have been made using various types of excito-repellency test boxes to assess the behavioral responses of mosquitoes to chemicals. However, no single test system has been fully accepted. The test system described in Chareonviriyaphap et al. (2002) provides modifications that expand the capability of the assay to monitor behavioral responses, with and without the mosquitoes making physical contact with the chemically treated substrate. The stainless steel outer chamber device measures $23.5 \times 23.5 \times 23.5\,cm^3$ is made up of several components numbered from 1 to 7; (1) a rear door cover, (2) an inner Plexiglas glass panel with a rubber latex-sealed door, (3) a Plexiglas holding frame, (4) a screened inner chamber, (5) an outer chamber, (6) a front door, and (7) an exit portal slot (Fig. 1). A behavioral response resulting after contact is defined as irritancy and a response resulting in the absence of contact is termed repellency (Roberts et al., 1997; Polsomboon et al., 2008; Tisgratog et al., 2011). The system has been used to study the excito-repellenct responses of several mosquito vector species. Recently, a novel modular assay system was developed for mass screening of chemical actions; including contact irritancy, spatial repellency, and toxicity in adult mosquitoes (Grieco et al., 2005). This modular system is of a reduced size compared to the previous excito-repellency box and minimizes the treated surface area thereby reducing the amount of chemical required for testing.

Using the excito-repellency test system, it is now possible to observe and quantify both irritant and repellent responses of mosquitoes exposed to test compounds. In Thailand, steam-distillates of several plants including catnip (*N. cataria*) (Polsomboon et al., 2008), citronella (*C. nardus*), hairy basil (*Ocimum americanum*), vetiver (*Vetiveria zizanioides*) (Tawatsin et al., 2001; Sathantriphop et al., 2014a), lemongrass oil (*C. citratus*) (Phasomkusolsil and Soonwera, 2010), citronella grass (*C. nardus*) (Phasomkusolsil and Soonwera, 2010; Suwansirisilp et al., 2012), plai (*Zingiber cassumunar*) (Phasomkusolsil and Soonwera, 2010), ginger (*Z. officinale*) (Tawatsin et al., 2006), Makaen (Thai name) (*Zanthoxylum limonella*) and clove (*S. aromaticum*) (Trongtokit et al., 2005b) were explored and tested as insect repellents against several species of mosquitoes (Tables 2–4). While a great number of essential oils from native plants are known to repel mosquitoes, little data exists to expand our understanding of the magnitude of the response to varying concentrations of the essential oils.

Further investigations using the excito-repellency assay have been conducted to evaluate the responses of promising plants on different mosquito species. Other studies on the behavioral modifying properties of catnip oil (*N. cataria*) were investigated for behavior modifying properties against field collected *Aedes aegypti* and *Anopheles harrisoni* using the excito-repellency test system. *A. aegypti* showed significantly higher escape rates from the contact chamber at 5% catnip oil whereas *A. harrisoni* displayed a high escape response at 2.5% catnip oil. Alternate studies of the behavioral modifying properties of catnip oil were also evaluated on two field caught mosquito species, *A. minimus* and *A. aegypti* and both irritant and repellent actions were documented. In addition, catnip oil at 2.5% showed a high escape response with *A. albopictus* (Sathantriphop et al., 2014a). The essential oil from catnip has proved to be a safe and promising insect repellent. This oil contains 2 stereoisomers of nepetalactone (*E*,*Z* and *Z*,*E*-isomer). The 2 stereoisomers have been reported to function as insect repellents against 13 families of insects (Eisner, 1964). The *E*,*Z*-nepetalactone form showed to be a stronger repellent against the German cockroach (*Blattella gemaniga* L.) than the *Z*,*E*-nepetalactone one (Peterson et al., 2002). Catnip oil was also reported to be a good repellent for short-term action against house flies and American cockroaches (Schultz et al., 2004). Additionally, catnip oil was found to be a good spatial repellent compound in protecting humans from mosquito bites for up to 6 h post application (Bernier et al., 2005; Zhu et al., 2006).

In addition, citronella (*C. nardus*) oil is widely used and has been shown to effectively repel *Anopheles minimus*, *Culex quinquefasciatus*, and *Aedes aegypti* (Thomas et al., 2009; Phasomkusolsil and Soonwera, 2010). Concentration of citronella oil at 2.5% and 10% produced a strong behavioral avoidance response in *A. aegypti* (Sathantriphop et al., 2014b) and *C. quinquefasicatus* (Suwansirisilp et al., 2013; Sathantriphop et al., 2014b). The results from these studies showed that higher concentration of citronella oil gave more toxicity to both *A. aegypti* and *C. quinquefasicatus* mosquitoes when compared with lower concentration (Suwansirisilp et al., 2012). Besides, hairy basil at 2.5% showed the highest behavior response (73.3% irritant and 70.69% repellent) when exposure to *A. aegypti* from Kanchanaburi Province (Boonyuan et al., 2014) whereas a lower behavioral response (47.3% irritant) in *A. aegyti* was observed in the laboratory when exposed at the same concentration of hairy basil (Sathantriphop et al., 2014a). Another potential plant derivative, vetiver oil, used at 2.5%, showed a high escape response in *A. albopictus* (Sathantriphop et al., 2014a).

Essential oils and extracts belonging to plants in the citronella genus (Poaceae) are commonly used as ingredients of plant-based mosquito repellents, mainly *C. nardus* that is sold in Europe and

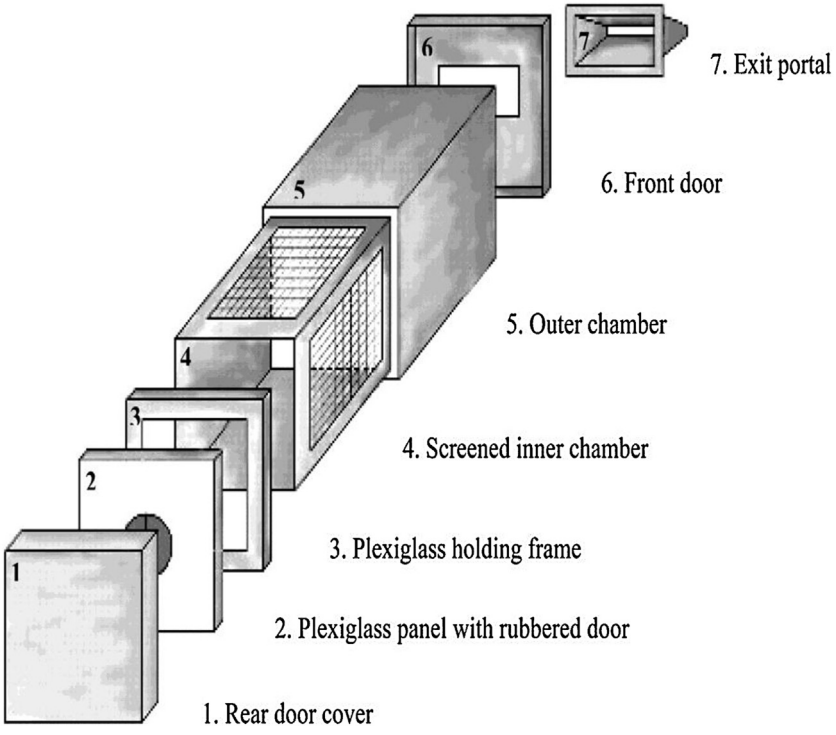


Fig. 1. Excito-repellency test chamber used to observe behavioral responses.

Table 2
Essential oils tested on mosquitoes using an excito-repellency test system to *Aedes* mosquitoes.

Plant species	Common name	Part used (%)	Mosquito species	Type of action			Sources
				Irritant	Repellant	Toxic	
Cymbopogon citratus	Lemongrass	Dried leave (5)	*A. aegypti* (Field)	70.18	–	–	Boonyuan et al. (2014)
Cymbopogon nardus	Citronella	Grass (2.5)	*A. aegypti* (Field)	49.1	–	–	Sathantriphop et al. (2014b)
		Grass (5)	*A. aegypti* (Field)	67.86	–	–	Boonyuan et al. (2014)
		Grass (2.5)	*A. aegypti* (Field)	84.4	51.6	–	Sathantriphop et al. (2014b)
Nepeta cataria	Catnip	Grass (10)	*A. aegypti* (Field)	59.6	53.57	–	Polsomboon et al. (2008)
		Grass (2.5)	*A. aegypti* (Lab)	32.8	–	–	Sathantriphop et al. (2014a)
		Grass (2.5)	*A. albopictus* (Lab)	61.1	60.0	–	Sathantriphop et al. (2014a)
Ocimum americanum	Hairy basil	Leave (2.5)	*A. aegypti* (Field)	73.3	70.69	–	Boonyuan et al. (2014)
		Leave (2.5)	*A. aegypti* (Lab)	47.3	–	–	Sathantriphop et al. (2014a)
Syzygium aromaticum	Clove	Dried flower (10)	*A. aegypti* (Field)	–	–	46.5	Suwansirisilp et al. (2012)
Vetiveria zizanoides	Vetiver	Root (2.5)	*A. albopictus* (Lab)	–	63.7		Sathantriphop et al. (2014a)
Zingiber cassumunar	Plai	Root (10)	*A. aegypti* (Field)	56.9	–	–	Boonyuan et al. (2014)
Zingiber officinale	Ginger	Root (5)	*A. aegypti* (Field)	64.41	–	–	Boonyuan et al. (2014)

North America in commercial formulations. The repellent components in citronella oil are citronellal, citronellol and geraniol (Duke, 2000). Citronella is the main constituent of citronella oil (Trongtokit et al., 2005a). Citronella oil is all-natural and consists of distilled oils from several varieties of grass. Citronella is approved by the Food and Drug Administration and is a common ingredient in various foods and beverages. Aside from causing a mild allergic reaction in certain individuals, citronella oil is considered safe and non-toxic to humans and animals as it can be broken down and secreted through the urine if ingested.

Citronella has found its way into many commercial preparations through its familiarity, rather than its efficacy. Today, citronella

Table 3
Essential oils tested on mosquitoes using an excito-repellency test system to *Anopheles* mosquitoes.

Plant species	Common name	Part used (%)	Mosquito species	Type of action			Sources
				Irritant	Repellant	Toxic	
Cymbopogon nardus	Citronella	Grass (2.5)	*A. minimus* (Lab)	86.3	–	–	Sathantriphop et al. (2014a)
Ocimum americanum	Hairy basil	Leave (2.5)	*A. minimus* (Lab)	90.8	78.1	–	Sathantriphop et al. (2014a)
Nepeta cataria	Catnip	Grass (2.5)	*A. harrisoni* (Field)	71.19	63.16	–	Polsomboon et al. (2008)
		Grass (5)	*A. harrisoni* (Field)	58.62	67.86	–	Polsomboon et al., (2008)
Syzygium aromaticum	Clove	Grass (2.5)	*A. minimus* (Lab)	87.9	94.4	76.7	Sathantriphop et al. (2014a)
Vetiveria zizanoides	Vetiver	Root (2.5)	*A. minimus* (Lab)	96.5	66	–	Sathantriphop et al. (2014a)

Table 4
Essential oils tested on mosquitoes using an excito-repellency test system to *Culex* mosquitoes.

Plant species	Common name	Part used (%)	Mosquito species	Type of action			Sources
				Irritant	Repellant	Toxic	
Cinnamomum verum	Cinnamom	Leave & Twig (2.5)	*C. quinquefasciatus* (Field)	59.7	–	–	Suwansirisilp et al. (2012)
		Leave & Twig (5)	*C. quinquefasciatus* (Field)	65.5	49.2	–	Suwansirisilp et al. (2012)
		Leave & Twig (10)	*C. quinquefasciatus* (Field)	60.3	47.5	–	Suwansirisilp et al. (2012)
Cymbopogon nardus	Citronella	Grass (5)	*C. quinquefasciatus* (Field)	75.0	73.3	–	Suwansirisilp et al. (2012)
		Grass (10)	*C. quinquefasciatus* (Field)	87.7	83	–	Suwansirisilp et al. (2012)
		Grass (2.5)	*C. quinquefasciatus* (Lab)	80.8	85.9		Sathantriphop et al. (2014a)
Nepeta cataria	Catnip	Grass (2.5)	*C. quinquefasciatus* (Lab)	80.7	72.4	–	Sathantriphop et al. (2014a)
Syzygium aromaticum	Clove	Dried flower (2.5)	*C. quinquefasciatus* (Field)	76.3	57.9	–	Suwansirisilp et al. (2012)
		Dried flower (5)	*C. quinquefasciatus* (Field)	76.8	69	–	Suwansirisilp et al., (2012)
		Dried flower (10)	*C. quinquefasciatus* (Field)	75.4	70.7	–	Suwansirisilp et al., (2012)
Vetiveria zizanoides	Vetiver	Root (2.5)	*C. quinquefasciatus* (Lab)	96.6	–	–	Sathantriphop et al., (2014a)

is one of the most widely used natural repellents on the market. However, there are relatively few studies that have been carried out to determine the efficacy of citronella as an arthropod repellent. Citronella-based repellents only protect from host-seeking mosquitoes for about two hours, although formulating the repellent is very important in this regard (Trongtokit et al., 2005a; Goodyer et al., 2010). Some studies have shown citronella to be as effective, dose for dose, as DEET (Curtis et al., 1987). Citronella has been used as a mosquito repellent and is a common ingredient in plants-based mosquito repellents (Cox, 2005). In addition to repelling mosquitoes, citronella oil also works well to treat fleas on cats and dogs as well as having antibacterial properties and is used as a herbal remedy for viruses and infections.

One of the other viable plant species, hairy basil, gave over 50% knockdowns in *A. albopictus* (Boonyuan et al., 2014; Sathantriphop et al., 2014a,b). In a previous study, they demonstrated that 10% concentration of hairy basil resulted in almost 90% mortality in *A. aegypti* in a contact assay and 69% mortality in a non-contact assay when using the excito-repellency test system (Sathantriphop et al., 2014a). These results demonstrate the utility of hairy basil as a candidate for development as a natural insecticide. Hairy basil also shows strong repellency against three mosquito species (*Aedes aegypti*, *Anopheles dirus* and *Culex quinquefasciatus*) when applied to human skin.

Essential oils from vetiver are not usually extracted as chemically pure substances but consist of mixtures of many compounds. Vetiver oil has been found to be the most effective repellent against termites due to its long residual activity (Zhu et al., 2001). Nuchuchua et al. (2009) reported that a significant increase in protection time was achieved in the presence of 10% vetiver oil against *A. aegypti* and a longer protection time was obtained when 5% hairy basil oil and 5% vetiver oil were combined. Vetiver oil is also used to repel mosquitoes (*A. aegypti*, *A. dirus* and *C. quinquefasciatus*) (Zhu et al., 2001; Trongtokit et al., 2005a). Vetiver oil consists of a complex mixture of more than 300 compounds, however, the major constituents include six compounds: α-, β-, vetivone, khusimone, zizanal, epizizanal and (+)-(1*S*,10*R*)-1,10-dimethylbicyclo[4.4.0]dec-6-en-3-one (Jain et al., 1982).

Clove oil is widely used for dental caries and as a food flavoring in Thailand (Trongtokit et al., 2004). Mixtures of clove and makaen oils have proven to be as effective as citronella oil when exposed to mosquito (Trongtokit et al., 2005b). Barnard (1999) reported that clove oil is the most effective repellent against mosquitoes and results in 1.5–3.5 h of protection time. Protection times for clove oil against three mosquito species, *A. aegypti*, *A. minimus* and *C. quinquefasciatus* were 80 min, 120 min and 90 min, respectively (Phasomkusolsil and Soonwera, 2010). The major constituents of clove oil are eugenol, eugenol-acetate and beta-caryophyllene (Leung and Foster, 1996; Kerdchoechuen et al., 2010). Eugenol resulted in long-lasing repellency action against tick (Thorsell et al., 2006). There has been found 96–100% mortality in the maize weevil after exposure to clove oil (Kerdchoechuen et al., 2010). Even the lowest volume of clove oil (30 μl) can cause a toxic response resulting in 100% mortality within 2 h. Combining clove oil (50%) with either geranium oil (50%) or thyme oil (50%) resulted in a greater efficacy as a mosquito repellent but also caused cutaneous irritation (Barnard, 1999). The main chemical constituents of essential oils have been shown in Table 5.

5. Conclusion

Plant essential oils such as citronella have become popular for use as safe mosquito repellents (Fradin, 1998). Besides citronella oil, numerous other plant extracts have been available as insect

Table 5
Chemical Compositions of essential oils.

Plants	Components	References
Catnip (*Nepeta cataria*)	*Z,E*nepetalactone, *E,Z*-nepetalactone, *Z,E*-nepetalic acid, *E,Z*-nepetalic acid	Chauhan and Raina (2006)
Citronella (*Cymbopogon nardus*)	Citral, citronellal, limonene, myrcene, geraniol, *trans*-citral, *cis*-citral, geranyl acetate, citronellol, γ-terpineol, *cis*-sabinene hydrate, linalool, β-caryophyllene	Hsu et al. (2013), Nakahara et al. (2003)
Clove (*Syzygium aromaticum*)	Eugenol, β-caryophyllene	Park and Shin (2005), Prashar et al. (2006)
Hairy basil (*Ocimum americanum*)	Methyl cinnamate, d-camphor, *E*-citral, *Z*citral, eugenol, methyl-chavicol, terpneol, farnesene, myrcene, α-pinene, β-pinene, 1,8-cineol, camphene, β-bisabolene, α-pinene, β-pinene, α-terpinene, limonene, linalool, linalyl acetate, methyl-eugenol, iso-eugenol, farnesol, Ocimene, terpinolene, geraniol, β-caryophyelene, germacrene D, geranyl iso-butyrate, α-cadinol	TCFF, Khalid (2006)
Vetiver (*Vetiveria zizanioides*)	terpinen-4-ol, 5-epiprezizane, khusimene, α-muurolene, khusimone, calacorene, β-humulene, α-longipinene, γ-selinene, δ-selinene, δ-cadinene, valencene, calarene,-gurjunene, α-amorphene, epizizanal, 3-epizizanol, khusimol, iso-khusimol, Valerenol, α-vetivone, β-vetivone	Jain et al. (1982), Thubthimthed et al. (2003)

repellents for protection against mosquitoes. Understanding how alternative natural products function to prevent mosquito human contact is vital in optimizing tools for personal protection. Insecticide resistance phenotypes in many important vector species will continue to pressure the scientific community to develop new and improved vector control strategies. Combination approaches are a good way to ensure the beneficial qualities of the various tools. Essential oils are one such area for exploration. As a result of the current review, clove, citronella, hairy basil, catnip, and vetiver essential oils could serve as potential mosquito repellent products against *C. quinquefasciatus* and *A. minimus*. Further testing with higher concentrations of all essential oils should be performed to find an effective repellent dose against *A. aegypti*. According to Phasomkusolsil and Soonwera (2010), increasing concentrations of essential oils can increase their repellent activity. However, development of a natural repellent formulation with long-lasting protection is necessary to warrant the replacement of the "gold standard" topical repellent DEET.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

All the authors have contributed significantly to this study. Manuscript drafted and revised by R.T., U.S., J.G., R.N. and T.C. All authors read and approved the final manuscript.

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AVOIDANCE BEHAVIOR TO ESSENTIAL OILS BY *ANOPHELES MINIMUS*, A MALARIA VECTOR IN THAILAND

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ABSTRACT. Essential oils extracted from 4 different plant species—citronella (*Cymbopogon nardus*), hairy basil (*Ocimum americanum*), sweet basil (*Ocimum basilicum*), and vetiver (*Vetiveria zizanioides*)—were investigated for their irritant and repellent activities against *Anopheles minimus*, using an excito-repellency test system. Pure essential oils were used in absolute ethanol at the concentrations of 0.5%, 1%, 2.5%, and 5% (v/v) compared with deet. At the lowest concentration of 0.5%, hairy basil displayed the best irritant and repellent effects against *An. minimus*. Citronella and vetiver at 1–5% showed strong irritant effects with >80% escape, while repellent effects of both oils were observed at 1% and 2.5% citronella (73–89% escape) and at 5% vetiver (83.9% escape). Sweet basil had only moderate irritant action at 5% concentration (69.6% escape) and sightly repellent on test mosquitoes (<50% escape). The results found that hairy basil, citronella, and vetiver are promising potential mosquito repellent products for protection against *An. minimus*.

KEY WORDS *Anopheles minimus,* deet, essential oils, excito-repellency

INTRODUCTION

Anopheles minimus Theobald is commonly found in high-density populations and also is the most important vector of malaria in Thailand (Tainchum et al. 2014). There are approximately 32 million people living along the Thailand borders, around 50% of the total population, who are at risk for contracting malaria. The undeveloped borders between Thailand–Myanmar remain the most heavily impacted area (BVBD 2013). Despite years of public health efforts and research progress, no effective vaccine for malaria has been made commercially available. Therefore, malaria prevention remains dependent on vector management and control strategies to reduce the risk of transmission (BVBD 2013). However, controlling malaria vectors like *An. minimus* has proven difficult due to their high abundance throughout the year and close proximity to humans (Chareonviriyaphap et al. 2003). In addition, this species is often in association with various human activities in agricultural systems such as stream pools, stream margins, irrigation ditches, and rice fields (Suwonkerd et al. 2013).

Chemical insecticides have been used as the primary mosquito control weapon. A clear understanding behavioral responses of mosquitoes to chemical compounds is of paramount importance for launching control program activities. Theoretically, chemical compounds can protect humans from mosquito bites in at least 2 different ways, by causing mortality or noncontact responses that modify mosquito behavior by inhibiting bloodfeeding (Roberts and Andre 1994, Thanispong et al. 2009). Most work on how chemicals function has been centered on toxicity, whereas less focus has been on the 2 types of behavioral avoidance responses: irritability and repellency (Grieco et al. 2007). The outcome of these 2 different forms of behavioral avoidance can be quantified using a specially designed excito-repellency test system (Chareonviriyaphap et al. 2004). Most excito-repellency studies have concentrated on synthetic chemicals, especially pyrethroids, which may result in environmental contamination (Corbel et al. 2013). Furthermore, due to the widespread use patterns spanning several decades, pesticides from 4 main chemical classes (e.g., organochlorines, organophosphates, carbamates, and pyrethroids) have been found to be ineffective due to the development of physiological resistance in several mosquito species (Chareonviriyaphap et al. 2013). For this reason, developing alternative compounds or new mosquito control methods is important. One potential alternative may be utilizing botanical compounds for insect repellents. These available sources can be safe and have become increasingly popular for domestic use against indoor and outdoor biting mosquitoes.

One of the most common, widely commercially available insect repellents is deet (diethyl methyl benzamide) (Fradin and Day 2002). As

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reviewed by Brown and Hebert (1997) and Qiu et al. (1998), research has shown that deet had irritant effect to human skin, especially when misapplied at high concentrations. In addition, deet dissolves some plastics and synthetic materials (Odalo et al. 2005). For this reason, there is interest in developing insect repellent products from natural plant extracts. Numerous essential oils extracted from plants have been evaluated for insect repellent activity on human skin against mosquito vectors, such as citronella grass (*Cymbopogon nardus* Rendle), hairy basil (*Ocimum americanum* L.), sweet basil (*Ocimum basilicum* L.), turmeric (*Curcuma ionga* L.), phlai (*Zingiber cassumunar* Roxb), clove (*Syzygium aromaticum* L.), kaffir lime (*Citrus hystrix* DC.), and eucalyptus (*Corymbia citriodora* Hook) (Tawatsin et al. 2001, Phasomkusolsil and Soonwera 2010).

Investigating mosquito avoidance behavior to plant essential oils has been progressively studied using an excito-repellency test system. The excito-repellency action has been evaluated with oils extracted from *Melaleuca leucadendron* (L.), *Litsea cubeba* (Lour.) Persoon, *Litsea salicifolia* (Roxburgh ex Nees) Hook. f., catnip leaf (*Nepeta cataria* L.), orange peel (*Citrus aurantium* L.), cinnamon leaf (*Cinnamomum verum* J. Presl), citronella grass, clove flower, hairy basil leaf, and vetiver (*Vetiveria zizanioides* L.) against laboratory-colonized *Aedes aegypti* (L.) (Polsomboon et al. 2008, Suwansirisilp et al. 2012, Noosidum et al. 2014, Sathantriphop et al. 2014). In addition, investigations of oils from hairy basil, ginger (*Zingiber officina* Roscoe), lemon grass (*Cymbopogon citratus* Stapf), citronella grass, and plai against *Ae. aegypti* field populations have been studied by Boonyuan et al. (2014).

However, little has been done to evaluate irritancy and repellency behavioral responses using essential oils at different concentrations against *An. minimus*. In our study 4 essential oils—citronella, hairy basil, sweet basil, and vetiver—and deet as a standard repellent were evaluated at different concentrations for contact irritant and spatial repellent actions against *An. minimus*, using an excito-repellency test system.

MATERIALS AND METHODS

Mosquito population

The laboratory colony of *An. minimus* from the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, was used for testing. This colony has been maintained continuously in the insectary for >15 years, under the insectary conditions of 25 ± 5°C and 80 ± 10% RH and photoperiod 12:12 (L:D). Larvae were fed with finely ground powdered fish food (TetraMin®) 3 times per day. All adults were reared in 30 × 30 × 30-cm screened cages and provided with 10% sugar solution. Female mosquitoes aged 3–5 days were starved for sugar 24 h before testing.

Compounds

Four plant essential oils and the standard repellent deet were used in this study. These oils were purchased at the maximum extraction concentration from Thai-China Flavors and Fragrances Industry Co., Ltd. (TCFF; Phra Nakhon Si Ayutthaya Province, Thailand). The steam distillation–extracted oils comprised of citronella grass (product code: MK-40012), hairy basil leaf (product code: 201-40028), sweet basil leaf and flowering shrub (product code: MK-40002), and vetiver roots and rootlets oil (product code: 201-20033). Technical deet (99.13% purity; Lot 0326/2009) was obtained from Sherwood Chemicals Public Company Limited (Thailand).

Impregnated paper

The concentrations of each essential oil and deet were prepared at 0.5%, 1%, 2.5%, and 5% (v/v) with absolute ethanol. A volume of 2.8 ml of each test solution was impregnated on a 14.7 × 17.5-cm filter paper (Whatman™ GE Healthcare, Buckinghamshire, United Kingdom) using an automatic pipette to achieve uniform distribution on each filter paper (Sathantriphop et al. 2014). Control papers were treated in the same manner but with only ethanol. All papers were air-dried at room temperature for 1 h before use and papers only once per test (Licciardi et al. 2006).

Excito-repellency test system

An excito-repellency test system was used to perform behavioral assays that identify irritancy and repellency responses (Chareonviriyaphap et al. 2004). To prepare the excito-repellency system, all 4 test chambers were provided for a complete test system (contact and noncontact trials, with 2 paired treatment and control chambers per trial) (Fig. 1). A paper box with lid (21.5 × 24.5 × 21.0 cm) was connected to each test chamber for collecting the escaped mosquitoes. In the contact chamber, treated papers were attached in front of the screens, so that the exposed surfaces were available for mosquitoes to land on, while in the noncontact chamber, the treated papers were placed behind the screen to prevent the mosquitoes from having direct exposure.

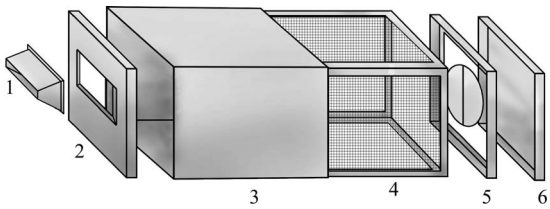


Fig. 1. The excito-repellency test chamber. The components: 1, exit door; 2, front door; 3, outer chamber; 4, screened inner chamber; 5, Plexiglas panel with rubber latex door; and 6, rear door cover (Sathantriphop et al. 2014).

Behavioral responses

Fifteen unfed female mosquitoes aged 3–5 days old were introduced into each test chamber and held for a 3-min "resting" period before the exit door of the chamber was opened to allow the mosquitoes to escape into the receiving box, then the observational period began. Tests were performed during 0900–1500 h, and each test was replicated 4 times. The number of mosquitoes escaping from the chamber was observed at 1-min intervals until the end of the test period at 30 min. The knockdown mosquitoes were recorded after 30 min of exposure time, and mortality was assessed after 24 h. The tested chambers were cleaned with alcohol and allowed to dry at least 24 h before starting the next experiment.

Statistical analysis

A log-rank test was conducted for paired comparisons of the differential escape patterns among the 5 compounds (4 essential oils and deet) and the 4 concentrations were analyzed and interpreted using the log-rank method (Mantel and Haenszel 1959). Statistical significance was accepted for all tests at $P < 0.05$. This experiment investigated the differences between survival curves (the death or escape rate) of one group is consistently higher than the corresponding rate in another group. With excito-repellency data, the log-rank test was used for observations of mosquito escape by 1-min intervals in excite repellency test (Polsomboon et al. 2008). Kaplan–Meier survival analysis was used to evaluate escaping mosquitoes from each test chamber of the excito-repellency test system (Roberts et al. 1997). An estimate of the escape time (ET) in minutes from the beginning of test required for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) was calculated. The mean percent escape was calculated per test chamber. Abbott's formula was applied to adjust escape responses based on paired control escape (Abbott 1925).

RESULTS

Behavioral response tests on *An. minimus* exposed to 4 essential oils compared with deet using an excito-repellency test system. Figure 2 demonstrated the proportions of mosquitoes remaining in the exposure chambers under different concentrations of essential oils and deet in contact and noncontact tests. In general, the escape rates were higher in contact than in noncontact assays when the same concentration was compared for all essential oils. Citronella and vetiver produced the greatest escape response at 5% in contact and noncontact assays. In contrast, hairy basil showed the highest escape rate at 2.5% for both test conditions. Delayed escape response was observed in sweet basil for all concentrations in both assays. With deet, the escape rate was increased with higher concentrations. At 5% deet was very effective against *An. minimus* (92.28% escape for contact and 72.59% escape for noncontact), whereas a concentration of 0.5% deet provided <50% of escaped mosquitoes.

Tables 1 and 2 show that contact with essential oils elicited stronger escape responses than with paired controls and noncontact trials. In contact and noncontact trials at 1%, citronella (94.43% contact and 88.68% noncontact) displayed the greatest irritant responses followed by vetiver (80.89% contact and 60.16% noncontact) and hairy basil (61.53% contact and 53.84% noncontact). Vetiver produced strongest escape response, with 96.48% contact and 66.05% noncontact at 2.5% and 97.95% contact and 83.93% noncontact at 5%. Sweet basil exhibited the lowest irritant and repellent response across all 4 concentrations compared with the other 3 oils. Among 4 concentrations of deet, 5% produced the strongest irritant responses (92.28%) and 2.5% displayed noncontact repellent response (77.69%).

For the toxicity data, percent knockdown and mortality of mosquitoes are summarized in Tables 1 and 2. The higher knockdown and mortality rates were recorded from nonescaped mosquitoes compared to mosquitoes that exited the treated chambers, and from exposure at higher versus lower concentrations. Specifically, no mortality or knockdown were counted from chambers treated with sweet basil and deet, regardless of the test concentration. With vetiver, comparatively low mortality occurred in escape (3.3%) and nonescaped (4.9%) mosquitoes at 2.5% and no knockdown data were recorded from either escape or nonescaped mosquitoes. A low percentage of knockdown escaped specimens were observed from contact and noncontact chambers treated with citronella (0–1.69%) and hairy basil (0–9.83%), depending upon chemical concentrations. The greatest percentage of knock-

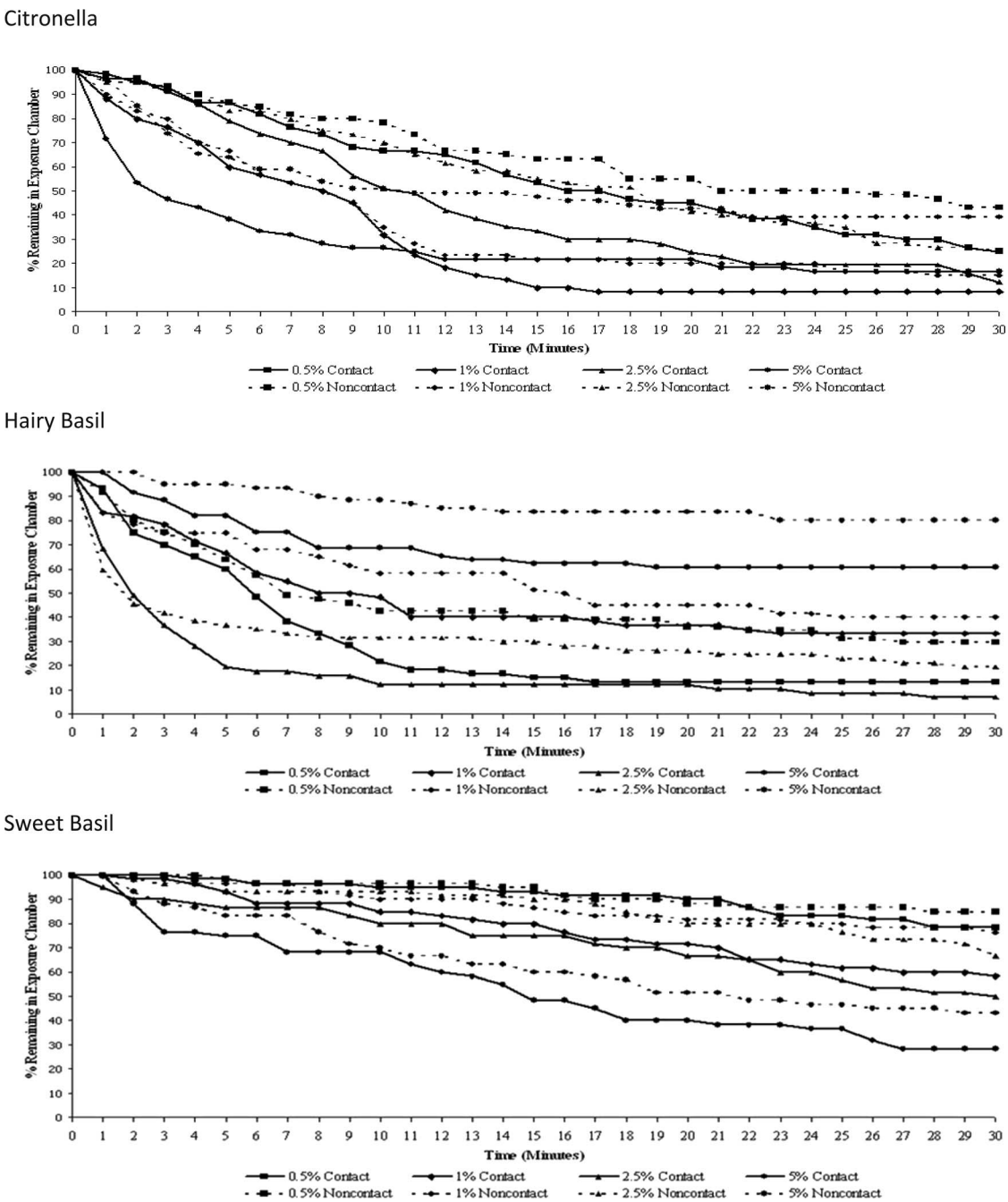
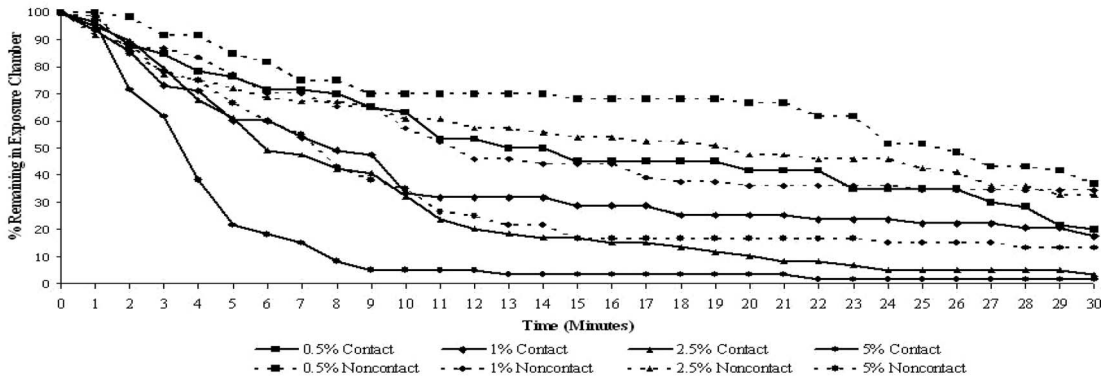


Fig. 2. Kaplan–Meier survival curves indicating the percentage of *Anopheles minimus* remaining inside the contact and noncontact chambers exposure to 4 concentrations of essential oils and deet (v/v) in ethanol during the 30-min exposure.

down was observed in nonescaped mosquitoes at 5% hairy basil in noncontact trial (80.32%), followed by 2.5% hairy basil in contact trial (57.37%). The highest mortality rate (73.77%) was found in nonescaped mosquitoes tested with 5% hairy basil.

Multiple log-rank comparisons were performed in a paired chamber and control trials for all compounds by concentration. Statistical differences in escape pattern responses were observed in all comparisons of contact and noncontact treatments ($P < 0.05$), except at 0.5% and 1%

Vetiver



Deet

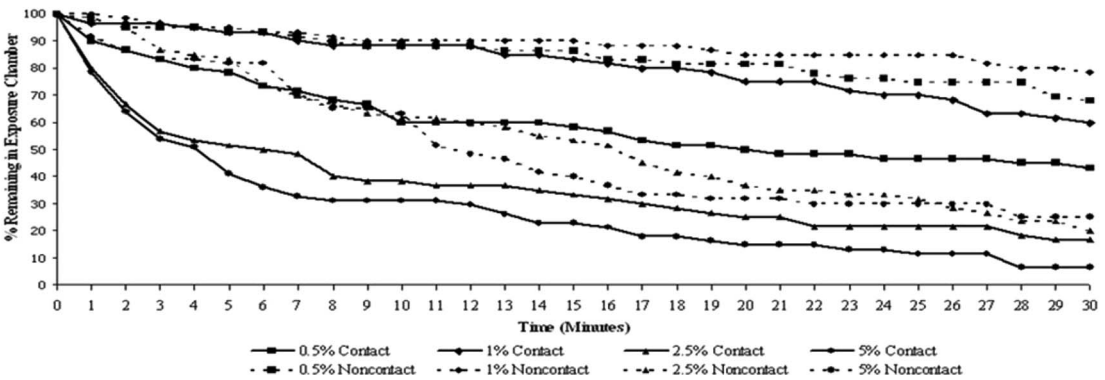


Fig. 2. Continued

citronella, 1% and 2.5% hairy basil, and 0.5% and 5% sweet basil oils (Table 3). Significant differences in the escape pattern responses were observed for all paired comparisons of vetiver and deet. Few significant differences were found at concentrations of essential oils between 0.5% and 1%. Comparisons between deet and each of 4 essential oils for contact and noncontact trials are shown in Table 4. Hairy basil was found to have greater escape response when compared with deet, and significant differences in escape responses were observed for both compounds at all concentrations. Statistical significance in escape responses was found when deet was compared with all concentrations of vetiver, except at 2.5%. In addition, paired-dose comparisons of escape responses of *An. minimus* are shown in Table 5. Few significant differences were found at concentrations of essential oils between 0.5% and 1%.

Escape time patterns calculated for (ET) 25%, 50%, and 75% during the 30-min exposure of treatment assays are given in Table 6. The lowest ET_{25}, ET_{50}, and ET_{75} values (between 1 and 10 min) were found with hairy basil oil at all concentrations compared with others except at 5%, only ET_{25} could be estimated (6 min). At 5% vetiver displayed the lowest ET values (between 2–5 min in contact and 4–10 min in noncontact). Deet displayed high ET values at the lower doses compared with higher ones. At 5% deet in contact trial produced the ET_{25} and ET_{50} values of 2 and 4 min, respectively, which was equal to vetiver values at 5%. Escape time values for controls could not be calculated due to $< 25\%$ of mosquitoes escaping within 1 h of exposure.

DISCUSSION

The potential insect repellents of citronella, hairy basil, sweet basil, and vetiver essential oils were screened against a laboratory strain of *An. minimus* based on the 2 nontoxic properties, contact irritability and noncontact repellency. The current study demonstrated the potential of citronella, hairy basil, and vetiver to be highly effective as repellents and/or irritants to *An. minimus*. Thus, hairy basil and vetiver could

Table 1. Percent escape response, knockdown, and mortality of *Anopheles minimus* exposed to 4 essential oils and deet in ethanol in contact trial.[1]

						% knockdown after 30-min exposure				% mortality at 24 h			
		Treatment[2]		Control		Treatment		Control		Treatment		Control	
Compound	Dose (%)	*N*	% Esp	*N*	% Esp	Esp	NE	Esp	NE	Esp	NE	Esp	NE
Citronella	0.5	60	70.90	60	8.33	—	—	—	—	—	—	—	—
	1.0	60	94.43	59	10.17	—	—	—	—	—	—	—	—
	2.5	59	85.66	59	10.17	—	—	—	—	—	3.4	—	—
	5.0	59	82.91	60	10	1.69	5.08	—	—	3.39	5.08	—	—
Hairy basil	0.5	60	79.85	58	17.24	—	—	—	—	—	—	—	—
	1.0	60	61.53	60	13.33	—	—	—	—	—	—	—	—
	2.5	58	90.75	59	6.78	—	6.89	—	—	—	3.4	—	—
	5.0	61	32.60	60	10	—	57.37	—	—	1.14	37.70	—	—
Sweet basil	0.5	60	17.53	60	5	—	—	—	—	—	—	—	—
	1.0	60	29.62	60	10	—	—	—	—	—	—	—	—
	2.5	60	43.40	60	11.67	—	—	—	—	—	—	—	—
	5.0	60	69.64	60	6.67	—	—	—	—	—	—	—	—
Vetiver	0.5	60	76	60	16.67	—	—	—	—	—	—	—	—
	1.0	63	80.89	58	8.62	—	—	—	—	—	—	—	—
	2.5	60	96.48	57	10.53	—	—	—	—	—	—	—	—
	5.0	60	97.95	60	18.33	—	—	—	—	—	—	—	—
Deet	0.5	60	46.73	59	18.64	—	—	—	—	—	—	—	—
	1.0	60	64.12	60	16.67	—	—	—	—	—	—	—	—
	2.5	61	80.67	60	6.67	—	—	—	—	—	—	—	—
	5.0	61	92.28	60	15	—	—	—	—	—	—	—	—

[1] Esp = escaped mosquitoes; NE = nonescaped mosquitoes.
[2] Escape rate corrected for paired control responses using Abbott's formula.

Table 2. Percent escape response, knockdown, and mortality of *Anopleles minimus* exposed to 4 essential oils and deet in ethanol in noncontact trial.[1]

						% knockdown after 30 min exposure				% mortality at 24 h			
		Treatment[2]		Control		Treatment		Control		Treatment		Control	
Compound	Dose (%)	*N*	% Esp	*N*	% Esp	Esp	NE	Esp	NE	Esp	NE	Esp	NE
Citronella	0.5	60	51.84	60	10	—	—	—	—	—	—	—	—
	1.0	60	88.68	60	11.67	—	—	—	—	—	—	—	—
	2.5	61	73.29	61	6.56	—	—	—	—	—	—	—	—
	5.0	61	57.94	60	5	—	24.59	—	—	1.04	11.47	—	—
Hairy basil	0.5	61	64.46	59	16.95	—	—	—	—	—	—	—	—
	1.0	60	53.84	60	13.33	—	—	—	—	—	—	—	—
	2.5	57	78.19	60	11.67	1.66	17.54	—	—	1.8	3.5	—	—
	5.0	61	12.09	58	8.62	9.83	80.32	—	—	4.92	73.77	—	—
Sweet basil	0.5	60	13.56	60	1.67	—	—	—	—	—	—	—	—
	1.0	60	11.53	60	13.33	—	—	—	—	—	—	—	—
	2.5	60	23.07	61	13.11	—	—	—	—	—	—	—	—
	5.0	60	48	60	16.67	—	—	—	—	—	—	—	—
Vetiver	0.5	60	57.69	60	13.33	—	—	—	—	—	—	—	—
	1.0	61	60.16	59	13.56	—	—	—	—	—	—	—	—
	2.5	60	66.05	59	3.39	—	—	—	—	3.3	4.9	—	—
	5.0	60	83.93	59	16.95	—	—	—	—	—	—	—	—
Deet	0.5	59	24.19	60	8.33	—	—	—	—	—	—	—	—
	1.0	60	55.76	60	13.33	—	—	—	—	—	—	—	—
	2.5	58	77.69	58	10.34	—	—	—	—	—	—	—	—
	5.0	60	72.59	57	8.77	—	—	—	—	—	—	—	—

[1] Esp = escaped mosquitoes; NE = nonescaped mosquitoes.
[2] Escape rate corrected for paired control responses using Abbott's formula.

Table 3. Multiple log-rank comparisons in paired contact, noncontact, and control trials for *Anopheles minimus* exposed to 4 essential oils and deet.[1]

		P-value[2]		
Compound	Dose (%)	NC vs. NT	CC vs. CT	NT vs. CT
Citronella	0.5	<0.0001	<0.0001	0.0574
	1	<0.0001	<0.0001	0.3459
	2.5	<0.0001	<0.0001	0.0293
	5	<0.0001	<0.0001	0.0005
Hairy basil	0.5	<0.0001	<0.0001	0.0296
	1	<0.0001	<0.0001	0.3288
	2.5	<0.0001	<0.0001	0.1230
	5	0.0894	0.0001	0.0117
Sweet basil	0.5	0.0083	0.0080	0.3777
	1	0.1305	<0.0001	0.0358
	2.5	0.0133	<0.0001	0.0379
	5	<0.0001	<0.0001	0.0965
Vetiver	0.5	<0.0001	<0.0001	<0.0001
	1	<0.0001	<0.0001	<0.0001
	2.5	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001
Deet	0.5	<0.0001	<0.0001	<0.0001
	1	<0.0001	<0.0001	<0.0001
	2.5	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001

[1] CC = contact control; NC = noncontact control; NT = noncontact treatment; CT = contact treatment.
[2] The significance level is set at $P < 0.05$.

be promoted as alternative natural mosquito repellents like citronella and also avoid using toxic and unpleasant odor chemical repellents such as deet. A similar study was conducted by Sathantriphop et al. (2014), hairy basil and vetiver at concentration of 2.5% v/v had strong irritant effects against *An. minimus* and *Culex quinquefasciatus* Say. Boonyuan et al. (2014) also reported that *Ae. aegypti* produced a good response to 2.5% hairy basil. Tawatsin et al. (2001) additionally revealed that hairy basil was effective against 3 mosquito species, *Ae. aegypti*, *An. dirus* Peyton and Harrison, and *Cx. quinquefasciatus* in tests on human skin. Nuchuchua et al. (2009) found 5% w/w hairy basil and 5% w/w vetiver in nanoemulsion had good repellent activities with long protection time (4.7 h) against *Ae. aegypti*.

Several chemical compounds were detected in hairy basil and vetiver essential oils. The oils that were used in this study were evaluated using mass spectroscopy/gas chromatography to get the constituents of each oil by the TCFF essential oil company. The main chemical components of 18
the hairy basil leaf oil were methyl cinnamate, *E*- and *Z*-citral, and D-camphor while vetiver roots and rootlets oil were vetiveryl acetate, vetiverol,

Table 4. Comparisons of escape responses between 4 essential oils and deet for *Anopheles minimus* exposed to contact and noncontact trials using the log-rank test.

	P-value[1]			
Dose (%)	Deet vs. citronella	Deet vs. hairy basil	Deet vs. sweet basil	Deet vs. vetiver
Contact				
0.5	0.1651	<0.0001	<0.0001	0.0440
1	<0.0001	0.0001	0.7479	<0.0001
2.5	0.3960	0.0059	<0.0001	0.2015
5	0.7131	<0.0001	<0.0001	0.0378
Noncontact				
0.5	0.0043	<0.0001	0.0294	0.0009
1	<0.0001	<0.0001	0.8163	<0.0001
2.5	0.4878	0.0281	0.0001	0.2619
5	0.4172	<0.0001	0.0244	0.0125

[1] Statistical differences in escape pattern responses between paired deet and each oil was set at $P < 0.05$.

Table 5. Comparison of escape responses between essential oil and deet concentrations (v/v of essential oil and deet in ethanol) in contact and noncontact trials for *Anopheles minimus* using the log-rank test. 17

		Dose (%) comparison (*P*-value)[1]					
Compound	Condition	0.5 vs. 1	0.5 vs. 2.5	0.5 vs. 5	1 vs. 2.5	1 vs. 5	2.5 vs. 5
Citronella	Contact	<0.0001	0.0310	0.0004	0.0147	0.5671	0.0389
	Noncontact	<0.0001	0.0588	0.1953	0.0031	0.0186	0.7403
Hairy basil	Contact	0.0124	0.0026	<0.0001	<0.0001	0.0023	<0.0001
	Noncontact	0.2036	0.0228	<0.0001	0.0019	<0.0001	<0.0001
Sweet basil	Contact	0.0126	0.0007	<0.0001	0.3747	0.0003	0.0074
	Noncontact	0.2350	0.0244	<0.0001	0.2911	0.0001	0.0024
Vetiver	Contact	0.1375	<0.0001	<0.0001	0.0567	<0.0001	<0.0001
	Noncontact	0.2483	0.2954	<0.0001	0.9169	0.0029	0.0022
Deet	Contact	0.0203	0.0005	<0.0001	<0.0001	<0.0001	0.1135
	Noncontact	0.2065	<0.0001	<0.0001	<0.0001	<0.0001	0.9621

[1] The significance level is set at $P < 0.05$.

vitivone, and terpenes. The chemicals found in plants and their characteristic odors could be effective as mosquito repellents (Maia and Moore 2011). Mosquitoes had olfactory (smell) receptors on their antennae or maxillary palps and gustatory (contact) receptors on the labella and tarsi that response to some chemicals in plant essential oils (Yang and Eilerman 1999, Das et al. 19 2014). Mosquitoes dislike smell and taste of some essential oils derived from plants, so these can repel or act as barriers against mosquitoes. Methyl cinnamate in *Ocimum forskolei* was proved to be effective in repelling *Ae. aegypti* 20 and *An. arabiensis* Patton (Dekker et al. 2011), whereas *E*- and *Z*-citral found in *Eucalyptus* spp. provided protection from mosquito bites for 2 h (Maia and Moore 2011). Other studies investigated the repellent effect of vetiver on termites. 22 21 Zhu et al. (2001) and Jayashree et al. (2014) showed the vetiver oil and some of its components were extremely repellent and toxic to Formosan subterranean termites. Furthermore, 23 vetiver oil was reported to be effective against ants, ticks, and cockroaches (Henderson et al. 2005). Previous and current studies have sup- 24 ported hairy basil and vetiver to be effective mosquito repellents and also repelled a great variety of other insect pests. However, plant essential oils are volatile, so they can evaporate quickly. There are many methods to slow the evaporation of essential oils. Tawatsin et al. (2001) and Kongkaew et al. (2011) reported adding vanillin to plant essential oils could prolong the protection time. Also, applying nanotechnology to make essential oil in slow-release microcapsule against mosquitoes could improve product quality (Nuchuchua et al. 2009). Moreover, suitable formulating forms of plant essential oils, such as gels, creams, and lotions,

Table 6. Escape time (ET) in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of *Anopheles minimus* to escape from the exposure chambers treated with 4 essential oils and deet in contact and noncontact trials.

	0.5%			1%			2.5%			5%		
Compound	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact												
Citronella	8	16	30	4	8	11	6	11	22	<1	3	6
Hairy basil	2	6	10	4	8	—	<1	2	5	6	—	—
Sweet basil	—[1]	—	—	17	—	—	13	30	—	5	15	—
Vetiver	6	13	29	3	8	15	4	6	11	2	4	5
Deet	6	20	—	20	—	—	2	6	20	2	4	13
Noncontact												
Citronella	11	21	—	4	8	12	8	19	30	3	3	—
Hairy basil	3	7	—	3	16	—	<1	2	27	—	—	—
Sweet basil	—	—	—	—	—	—	26	—	—	9	9	—
Vetiver	7	26	—	6	12	—	4	19	—	4	4	10
Deet	25	—	—	—	—	—	6	17	28	7	7	28

[1] Very few mosquitoes escaped from exposure chambers so that the ET values could not be calculated for a 30-min exposure period.

can extend the effectiveness and persistence of natural mosquito repellents.

Our study provides preliminary data on the repellent and/or irritant properties of citronella, hairy basil, sweet basil, and vetiver oils against laboratory strain of *An. minimus*. However, further study should be performed under realistic field conditions because heat, moisture, and air in the environment can affect the effectiveness of essential oils. Also, study on human skin is necessary to confirm their safety.

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Contributed Paper

Gut Bacterial Diversity in *Plasmodium*-infected and *Plasmodium*-uninfected *Anopheles minimus*

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ABSTRACT

Anopheles minimus is one of the main malaria vectors in Thailand. *Plasmodium* transmission depends primarily on the success of the parasite survival in the mosquito's gut. Several factors affect the development of *Plasmodium* in the mosquito, including the gut microbiota. Here, we used culture-independent method to identify microbiota and compared the bacterial communities in the gut of *Plasmodium*-infected and *Plasmodium*-uninfected mosquitoes. Fifty-three genera within four phyla were detected and 14 of them were discovered in malaria vectors for the first time. In addition, we found that the bacterial diversity and the profile of the gut bacterial communities between the *Plasmodium*-infected and those of the uninfected mosquitoes were quite different. The result showed that the bacterial diversity in the gut of the uninfected mosquitoes was also much higher than that of the infected counterpart. Gammaproteobacteria were prevalent in the infected *An. minimus* while betaproteobacteria were the most abundant in the uninfected mosquitoes. Three genera, *Acinetobacter* in gammaproteobacteria, *Alcaligenes* and *Burkholderia* in betaproteobacteria were the core set of bacteria found in the gut of the malaria vector.

Keywords: *Anopheles minimus*, gut microbiome, 454 sequencing, malaria vector, *Plasmodium*

1. INTRODUCTION

Malaria is one of the serious public health concerns in several countries. Approximately 3.4 billion people worldwide are at risk of being infected with malaria [1]. In 2012, there were estimated 207 million cases of malaria and 627,000 deaths [1]. Although the disease can be cured by anti-malarial drugs, the resistance of *Plasmodium* to the medicines has been found worldwide, especially in Asia [2,3]. Even though using of bed net and indoor

insecticides has been widely practiced, a number of people are still infected with *Plasmodium*. Hence, the alternative protection methods have been developed such as symbiotic control, which is the use of natural symbiotic microorganisms to control vector-borne diseases [4,5].

Plasmodium transmission depends primarily on the success of the parasite survival in the mosquito gut. The lumen is the first place *Plasmodium* will attach, grow and transform to the next developmental stages in the life cycle [6]. However, a number of the parasites were dramatically reduced in the gut phase [6]. Many factors that affect *Plasmodium* development in *Anopheles* mosquitoes including microbiota in the anopheline midgut have been reported [6,7]. For two decades, researchers have tried to isolate bacteria in the gut of *Anopheles* species and studied the relationship between these bacteria and *Plasmodium* development [5]. They also found that certain bacterial strains could inhibit the growth of *Plasmodium* [7,8]. Since only a small number of microorganisms could grow in synthetic media, culture-independent methods, which incorporate the use of next generation sequencing (NGS) technology, have been applied to investigate unculturable microbial communities. In the past few years, NGS has widely been used to study microbial diversities from various sources, including *Anopheles* mosquito gut, especially *An. gambiae* [9,10,11]. However, identification of gut microbiome in *An. minimus*, a malaria vector, has not been carried out using NGS technology.

An. minimus is one of the main malaria vectors in Thailand, found primarily in forest regions along the border. In our work, bacterial diversity in the gut of *An. minimus,* and bacterial communities of *Plasmodium*-infected and *Plasmodium*-uninfected mosquitoes were identified and compared using culture-independent method.

2. MATERIALS AND METHODS

2.1 Mosquito Collection and Ethics Statement

Mosquitoes were collected at Mae Sot district, Tak Province, Thailand using outdoor human-landing collections. This site is located close to a refugee camp on Thai-Myanmar border and it is one of the malaria-endemic regions in Thailand. All specimens were kept in plastic cups, which contained cotton soaked with 10% sterile sugar solution and stored at -80°C until use. The mosquitoes were surface rinsed with 70% ethanol. Head-thorax and gut sections were dissected and used for species identification and bacterial identification, respectively.

Formal animal/human use approval for this research was granted by the Ethic Review Committee for Research Involving Human Research Subject, Health Science Group, Chulalongkorn University (COA No. 167.2013).

2.2 Species Identification and Detection of Plasmodium Infection

DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instruction and then it was used for species identification in *An. minimus* complex according to the method of Sharpe *et al.* [12]. The result showed that the mosquitoes were *An. minimus*.

Detection of *Plasmodium* infection followed the method of Rougemont *et al.* [13]. PCRs were performed in a final volume of 20 µL consisting of 2 µL of DNA template, 1 U of GoTaq polymerase (Promega, USA), 1x *Taq* reaction buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 500 nM of each primer and sterile distilled water to make up the remainder of the 20–µL volume.

Conditions used for amplification in a thermocycler (Biometra, Germany) were as follows: pre-incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min and a final extension step at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel, purified using the QIAquick Gel Extraction kit (Qiagen, Germany) and sequenced by Macrogen (Korea). The local alignment results indicated that certain specimens were infected with *Plasmodium falciparum*. Six guts from three *Plasmodium*-infected and three *Plasmodium*-uninfected female mosquitoes were used for 16S rRNA amplicon survey study.

2.3 Gut Bacterial Species Identification

DNA isolated from the gut was used as a template in PCR reactions. Additional eight-nucleotide sequences [14] (Table 1) attached to two primers; 347F (5'-GGAG GCAGCAGTRR-GGAAT-3') and 803R (5'-CTACCRGGGTATCTAATCC-3') [15], were used to tag each mosquito's gut specimen. The partial 16S rRNA gene was amplified using HotStar Hifidelity Polymerase kit (Qiagen, Germany). The reaction contained 2 µL of DNA template, 1 U of HotStar Hifidelity Polymerase (Qiagen, Germany), 1x HotStar Hifidelity PCR buffer containing 1.5 mM $MgSO_4$ and 0.3 mM dNTPs, 500 nM of each primer and sterile distilled water to make up the remainder of the 20-µL volume. The amplification cycles were as followed: pre-incubation at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min and a final extension step at 72°C for 5 min. The PCR products were then purified with MinElute PCR Purification kit (Qiagen, Germany) and their concentrations were measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). The integrity of the DNA was verified by a Bioanalyzer (Agilent) prior to sequencing. Purified PCR products from each mosquito were diluted to the same concentration and pooled in equimolar amount. Approximately 200 ng of pooled DNA was sequenced using a GS-FLX Titanium platform (Roche Applied Science, Germany). The sequencing was carried out according to the manufacturer's protocol.

Table 1. Nucleotide sequences of primers and tags used in the present study. The sequence of each tag was underline.

Primer name	Sequence (52 ->32)
347F-01	TCTCTGTGGGAGGCAGCAGTRRGGAAT
803R-01	TCTCTGTGCTACCRGGGTATCTAATCC
347F-02	TCTACTCGGGAG GCAGCAGTRRGGAAT
803R-02	TCTACTCGCTACCRGGGTATCTAATCC
347F-03	TAGTAGCGGGAGGCAGCAGTRRGGAAT
803R-03	TAGTAGCGCTACCRGGGTATCTAATCC
347F-04	AGACGACGGGAGGCAGCAGTR RGGAAT
803R-04	AGACGACGCTACCRGGGTATCTAATCC
347F-05	ACTCGTAGGGAGGCAGCAGTRRGGAAT
803R-05	ACTCGTAGCTACCRGGGTATCTAATCC
347F-06	ACATCGAGGGAGGCAGCAGTRRGGAAT
803R-06	ACATCGAGCTACCRGGGTATCTAATCC

2.4 Sequence Cleaning

Sequences obtained from the GS-FLX Titanium sequencer were demultiplexed according to the tagged barcode sequences. The sequences were cleaned by trimming the 454 adapter and barcodes using the custom python script. Chimeric sequences were identified and removed using UCHIME [16] against referenced database from SILVA [17]. To improve the robustness of analyses, the clean reads were then filtered and size-selected: only high-quality reads that were at least 200 nucleotides in length were included in further analyses. The 16S rRNA libraries sequences of bacteria from the guts are available in the Sequence Read Archive (SRA) on NCBI with the accession number SRX481169.

2.5 Taxonomic Classification and Statistical Analysis of Pyrosequencing Data

The cleaned sequences from the previous step were assigned their phylotypes using Ribosomal Database Project (RDP) naïve Bayesian Classifier [18] with 80% confidence threshold. Operational taxonomic units (OTUs) were determined at sequence similarity levels of 85%, 90%, 95%, and 97% by MOTHUR [19] based on the furthest-neighbor method. The richness (sobs), the Chao1 richness estimator, the abundance-based coverage estimator (ACE) and the Shannon-Weaver diversity index were calculated using MOTHUR software in order to compare microbial diversity between the *Plasmodium*-infected and the *Plasmodium*-uninfected mosquitoes. Good's coverage was calculated as $G = 1 - n/N$, where n is the number of singleton phylotypes and N is the total number of sequences in the sample. The visualization and comparison of microbial communities were performed by STAMP (Statistical Analysis of Metagenomic Profiles) [20].

3. RESULTS

3.1 454 Sequencing and Statistical Analyses

We obtained a total of 42,599 raw reads with the average read length of 272 nucleotides. After the adapter and low-quality trimming, 25, 641 filtered reads (with the mean read length of 360 bases) were assigned the phylotypes using the RDP classifier (Table 2). We were able to designate 99.96% of the reads as originating from bacteria, and 92.30% as well as 83.32% of the trimmed reads were assigned to bacteria at the family and the genus levels, respectively (Table 2). To analyze whether the diversity of the gut microbiome is sufficiently covered by our sequence data, rarefaction and species richness calculations were performed. We carried out the rarefaction analyses at four different dissimilarity cutoffs. At sequence similarity levels of 85% and 90%, the rarefaction curves computed for three *Plasmodium*-infected and three uninfected specimens appeared to have leveled off, suggesting that our sequence data have covered almost all phylogenetic groups underlying the gut microbial communities at the family/class levels (Figure 1). At sequence similarity levels of 95% and 97%, the dataset did not seem to reach the plateau, however, the percentages of Good's coverage at all taxonomic levels were very high (Table 3). We also applied statistical models to assess the bacterial diversity of the *An. minimus* gut metagenomes (Table 3).

At all sequence similarity levels, the richness of the uninfected mosquitoes was 1.30-1.64 folds higher than that of the infected ones. The Chao1 and ACE values of the uninfected ones were higher than those of the infected mosquitoes at 1.62-1.85 and 1.60-2.12 fold, respectively. However, the values of Shannon index of the uninfected and the infected specimens were not different

(0.99-1.02). Altogether, the metrics suggested that the gut microbiome of the uninfected mosquitoes display a slightly higher degree of diversity compared to the *Plasmodium*-infected individuals.

Table 2. Summary of 16S rRNA tagged-pyrosequencing data from three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

Sample	Raw sequence		Cleaned sequence	
	Number of sequence	Average length (bp)	Number of sequence	Average length (bp)
PI1	5,041	284.21	3,350	356.59
PI2	1,629	222.03	694	387.34
PI3	10,106	323.68	7,851	370.82
PU1	12,867	229.05	5,833	347.40
PU2	6,355	272.57	3,881	353.76
PU3	6,601	275.94	4,032	359.88
Total	42,599	263.51	25,641	359.78

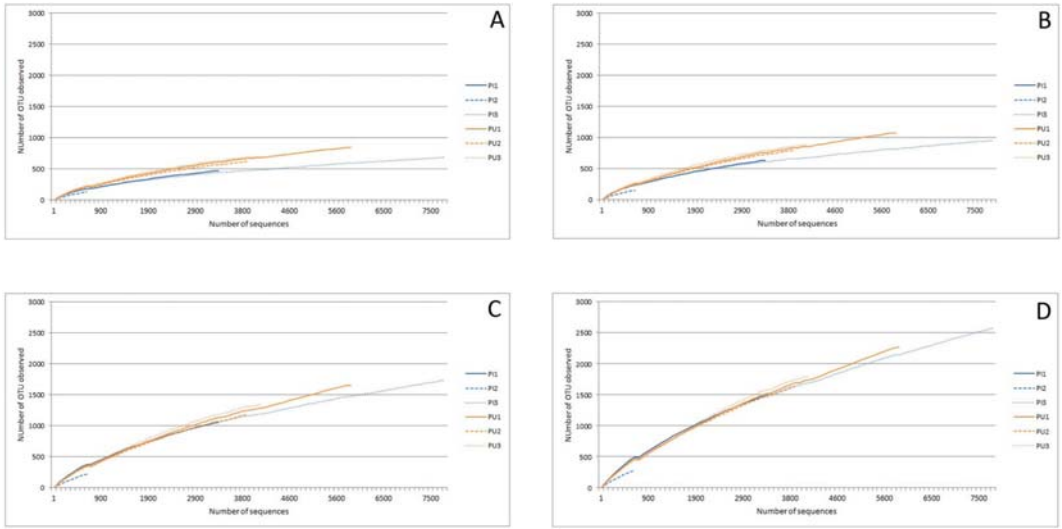


Figure 1. Rarefraction curves of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus* at four similarity levels; 85% (A), 90% (B), 95% (C) and 97% (D), respectively.

Table 3. Statistical analysis and biodiversity index of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus* at similarity levels of 85%, 90%, 95% and 97 %.

Sample	Similarity level	Richness	Chao1	ACE	Shannon	Good coverage (%)
PI	85%	1,004	1,806	2,472	4.7064	95.76
PI1		470	951	1,489	4.3999	92.24
PI2		130	241	355	2.9468	89.19
PI3		691	1,327	1,737	4.4769	95.54
PU		1,647	3,282	4,849	4.8233	93.37
PU1		849	1,763	2,476	4.3774	91.67
PU2		623	1,423	1,985	4.1966	90.60
PU3		703	1,386	2,008	4.6006	90.20
PI	90%	1,379	2,517	3,523	5.3125	94.05
PI1		634	1,232	2,085	4.9437	89.40
PI2		153	351	586	3.1219	85.88
PI3		955	1,773	2,388	5.1161	93.90
PU		2,150	4,649	7,473	5.3186	90.77
PU1		1,078	2,463	3,987	4.8561	88.69
PU2		798	1,929	2,981	4.6932	87.19
PU3		888	1,923	2,970	4.9427	86.83
PI	95%	2,472	5,255	7,912	6.4946	88.05
PI1		1,059	2,317	3,599	6.1181	80.99
PI2		222	661	911	3.7611	76.95
PI3		1,745	3,546	5,014	6.2664	87.62
PU		3,402	8,931	15,281	6.4340	83.66
PU1		1,657	4,430	7,822	5.8876	80.80
PU2		1,185	2,997	5,208	5.7522	79.82
PU3		1,346	3,689	6,059	5.8430	77.21
PI	97%	3,651	8,637	14,622	7.2968	80.82
PI1		1,485	3,544	6,182	6.8210	71.31
PI2		282	861	2,177	4.3715	69.31
PI3		2,588	6,010	9,3222	7.0339	79.51
PU		4,734	13,984	3,449	7.4156	76.27
PU1		2,269	7,355	13,729	6.7847	72.00
PU2		1,629	4,305	7,622	6.5854	71.09
PU3		1,798	5,225	9,032	6.7272	68.18

3.2 Gut Bacterial Classification

Taxonomic classification with the RDP Classifier detected the presence of 53 genera in four bacterial phyla in the gut of *An. minimus* (Table S1). Proteobacteria was far more abundant than the other groups, representing 95.02% of the OTUs assigned and containing the 43 distinct genera (Table 4). At the class level, the gut community was dominated by two taxonomic classes: gammaproteobacteria

(57.40%) and betaproteobacteria (35.12%; Table S1). Among members of the gammaproteobacteria, *Moraxellaceae* was associated with 28.79% of the sequences while other predominant OTUs were assigned to *Enterobacteriaceae* (16.50%). For betaproteobacteria, the major bacterial groups were *Alcaligenaceae* (16.26%) and *Burkholderiaceae* (15.79%). The variation of the gut bacterial communities among the specimens at phylum and class levels could be observed, however, composition patterns of gut microbiota of *An. minimus* in the same group were quite similar (Figure 2). At the genus level, only twenty six groups of bacteria represented more than 1% of the total OUTs in each specimen are shown in Figure 3. Most of them were detected in at least 50% of all specimens examined. The result showed that the most abundant genera (30% of all OTUs in at least one specimen) were *Alcaligenes, Burkhoderia*, *Thorsellia*, unclassified *Enterobacteriaceae* and *Acinetobacter* (Figure 3).

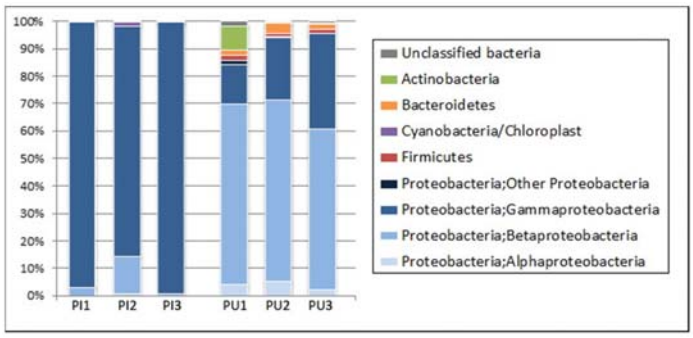


Figure 2. Taxonomic classification of microbiota in the gut of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.



Figure 3. Heat map of bacterial profiles in the gut of the *Plasmodium*-infected (PI) and the *Plasmodium*-uninfected *An. minimus*. Only genera represented more than 1% of the total OTUs in each specimen were included.

3.3 Bacterial Community in *Plasmodium*-infected and *Plasmodium*-uninfected Hosts

Out of 53 genera, 20 were found in both infected and uninfected mosquitoes while 7 and 26 genera were detected only in the *Plasmodium*-infected or the *Plasmodium*-uninfected individuals, respectively (Table 4). The majority of bacteria presented in the gut of the infected host were gammaproteobacteria (97.40%), followed by betaproteobacteria (2.19%; Figure 4). *Moraxellaceae* (69.50%) and *Enterobacteriaceae* (26.28%) families were the most abundance in the gammaproteobacteria (Figure 4). The scatter plot showed that *Acinetobacter* in the *Moraxellaceae*, *Thorsellia* in the *Enterobacteriaceae* and unclassified *Enterobacteriaceae* were abundant in the *Plasmodium*-infected hosts (Figure 5). In contrast, the gut of the uninfected mosquitoes was dominated with betaproteobacteria (63.62%; Figure 4). Moreover, Actinobacteria, alphaproteobacteria, Firmicutes and Bacteroidetes were almost exclusively found in the gut of the uninfected mosquitoes. Among them, *Burkholderia* in the family *Burkholderiaceae*, *Alcaligenes* in the family *Alcaligeneceae* and *Serratia* in the family *Enterobacteraceae* were the most prevalent (Figure 4).

Table 4. The 53 genera discovered in the gut of *Anopheles minimus*. Certain bacterial genera were detected in both *Plasmodium*-uninfected and infected mosquitoes (B) whereas some of them were presented only in the *Plasmodium*-uninfected mosquito (PU) or in the *Plasmodium*-infected mosquito (PI).

Phylum/class	Genus	Detection (B, PU, PI)	Percentage from total OTUs
Actinobacteria	Corynebacterium[a]	PU	0.07
	Brachybacterium[a,b]	B	0.47
	Kocuria[a]	PU	0.07
	Micrococcus[a]	PU	1.22
	Propionibacterium[a]	PU	0.16
Bacteroidetes	Elizabethkingia[a]	PU	1.05
	Flavobacterium[a]	B	0.02
	Flavisolibacter[a,b]	PU	0.12
Firmicutes	Bacillus[a]	PU	0.03
	Staphylococcus[a]	B	0.71
Proteobacteria/ Alphaproteobacteria	Brevundimonas[a]	PU	0.02
	Bradyrhizobium[a]	PU	0.01
	Nitrobacter[a,b]	PU	0.04
	Methylobacterium[a]	B	0.08
	Mesorhizobium[a,b]	PU	0.04
	Rhizobium[a,b]	PU	0.10
	Asaia[a]	PU	0.01
	Roseomonas[a]	PU	0.13
	Novosphingobium[a]	B	0.13
	Sphingomonas[a]	B	0.61

Table 4. Continued.

Phylum/class	Genus	Detection (B, PU, PI)	Percentage from total OTUs
Proteobacteria/ Betaproteobacteria	*Achromobacter*[a]	B	2.48
	Alcaligenes[a]	PU	14.69
	Burkholderia[a]	B	14.59
	Ralstonia[a]	PU	0.06
	Aquabacterium[a]	PU	0.04
	Acidovorax[a]	PU	0.08
	Comamonas[a]	B	0.01
	Delftia[a]	B	0.51
	Tepidicella[a,b]	PI	<0.01
	Variovorax[a,b]	PU	<0.01
	Massilia[a,b]	B	0.31
	Naxibacter[a,b]	PU	<0.01
	Methyloversatilisa[a,b]	PU	0.01
Proteobacteria/ Deltaproteobacteria	*Desulfohalobium*[a,b]	PU	0.23
Proteobacteria/ Gammaproteobacteria	*Aeromonas*	PI	<0.01
	Pseudoalteromonas[a,b]	PU	0.37
	Citrobacter	PI	<0.01
	Enterobacter	PI	<0.01
	Escherichia/Shigella	PI	0.02
	Klebsiella[a]	B	0.41
	Pantoea	PI	<0.01
	Salmonella[a]	B	0.16
	Serratia	B	3.94
	Thorsellia[a]	B	2.08
	Trabulsiella[a,b]	PI	<0.01
	Zymobacter[a]	PU	0.02
	Acinetobacter	B	32.37
	Enhydrobacter[a]	B	1.35
	Pseudomonas	B	1.20
	Lucibacterium[a,b]	PU	0.09
	Vibrio[a]	PU	0.02
	Stenotrophomonas[a]	B	2.32
	Xanthomonas[a,b]	B	1.37

Note: [a] the new genera firstly detected in *Anopheles minimus*.

[b] the new genera detected in *Anopheles* species for the first time.

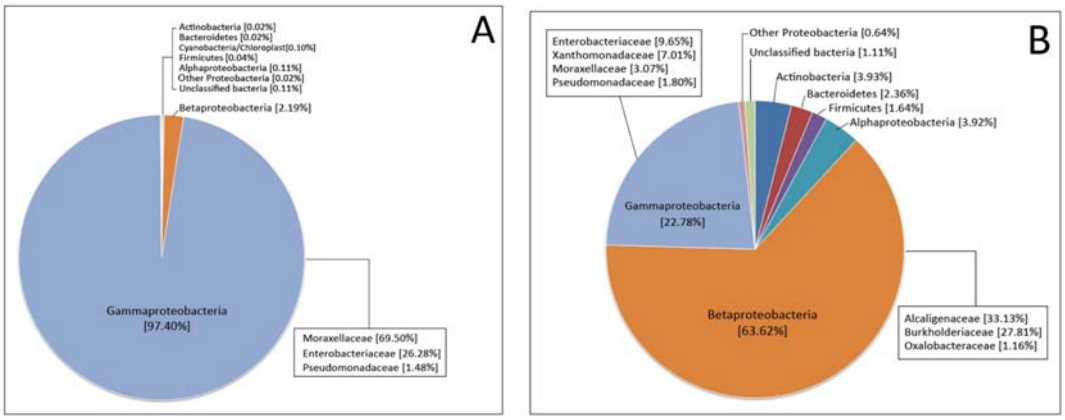


Figure 4. Percentages of relative abundance at taxonomic levels. (A) and (B) indicate the relative abundance (%) of bacteria in the gut of the *Plasmodium*-infected and the *Plasmodium*-uninfected *An. minimus*, respectively.

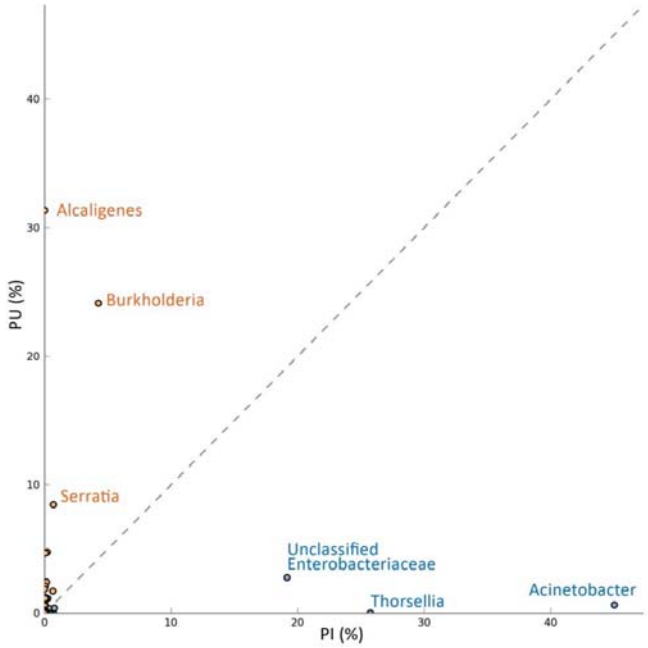


Figure 5. Scatter plot of prevalent bacterial genera comparing between the gut microbiota in the *Plasmodium*-infected (PI; PI1, PI2, PI3) and the *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

4. DISCUSSION

4.1 First Discovery of Certain Bacterial Genera in the Gut of *An. minimus*

In the present study, 45 of the total 53 genera were detected for the first time in *An. minimus*. Of the 45 genera, 31 were found in other malaria-vectors while one genus, *Enhydrobacter*, was present in a non-malaria vector, *An. barbumbrosus* [11, 21]. Six of ten midgut core microbiota, *Burkholderia, Serratia, Acinetobacter, Pseudomonas, Sphingomonas* and *Staphylococcus* detected in *An. gambiae* in a

previous study were also abundant in the current study [11]. Similarly to previous reports, a small proportion of gram-positive bacteria, Actinobacteria and Bacilli, was found in the gut of our specimens [10, 11]. However, 14 genera discovered in the present study have never been found in any *Anopheles* species. Most of them were present in low frequency with an exception of *Xanthomonas*, the abundance of which was higher than 1%. All 14 genera were isolated from plant tissues, soils, air and water resources [22]. These findings suggested that the mosquitoes might acquire these bacteria by feeding on plant sap or becoming in contact with the environments that contained the bacteria.

4.2 The Relationship between Gut Bacteria and *Plasmodium*

A large number of microorganisms are generally present within the body of mosquito, especially in the gut [6]. Several studies have reported the benefits of gut bacteria to the host, including parasite-development inhibition [23]. Antibiotic-treated *An. gambiae* and *An. stephensi* had lower bacteria contents and higher *P. falciparum* infection rates than the untreated mosquitoes [24]. In addition, intrathoracic inoculation of bacteria in *An. gambiae* induced antibacterial peptide production that inhibited *Plasmodium* development [25]. Moreover, Cirimotich *et al.* [7] revealed that a strain of *Enterobacter* isolated from wild-caught *An. arabiensis* could kill *P. falciparum* using reactive oxygen species . Recently, *Serratia marcescens* HB3 had been shown to inhibit *P. berghei* oocyst formation in *An. stephensi* [23]. These findings suggested that certain gram-negative bacteria play an important role to protect mosquitoes from *Plasmodium* infection.

The gram-negative bacteria, *Acinetobacter*, were the most abundant bacteria in the gut samples from our study. It was found that *Acinetobacter* species isolated from wild-caught *An. arabiensis* could reduce the number of oocysts in the midgut of *An. gambiae* via the activation of the immune deficiency (IMD) immune signaling pathway [26]. In addition, the infection of *Acinetobacter* in the mosquito midgut reduced longevity of *An. gambiae* [26]. *Elizabethkingia meningoseptica* and *Serratia marcescens* were also detected in our work (data not shown) and they previously exhibited anti-*Plasmodium* activity in *Anopheles* species [8, 23, 26]. *E. meningoseptica* was able to inhibit the development of *P. falciparum* at the gametocyte transmission stage [27]. Recently, Bahia *et al.* [26] reported that the anti-*Plasmodium* activity of *S. marcescens* derived from secreted factors. The findings demonstrated that *Acinetobacter*, *S. marcescens* and *E. meningoseptica* could be used for malarial control in *Anopheles* mosquitoes including *An. minimus*.

4.3 Core Gut Microbiota and Symbiotic Candidates for Malaria Control

Even with regular applications of insecticides and the availability of antimalarial drugs, over 200 million of malaria cases have been reported every year [28]. Unfortunately, malaria vectors have exhibited increased resistance to the insecticides and the antimalarial drugs has become less effective [28]. Novel preventive strategies including symbiotic control might be an alternative to other conventional approaches. The symbiotic control is a strategy to control insect borne diseases by reducing vector competence [4]. A symbiotic candidate should be selected from core gut microbiota, which is abundant and mainly found in a host species. In our study, there were 20 genera residing in the gut of the mosquitoes higher than one percent of all OTUs in each specimen; however only three genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* represented more than 10% of the

total OTUs. According to the abundance of the three genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* were core gut microbiota in *An. minimus* and were eligible symbiotic candidates for malaria control. *Acinetobacter* have the ability to reduce the number of *Plasmodium* oocysts in the midgut of *An. gambiae* [26], so we could use them directly to control the transmission of malaria in the malaria-vector. In contrast, the anti-*Plasmodium* activity in *Alcaligenes* and *Burkholderia* has not been reported. *Burkholderia* appeared to colonize in the gut of all mosquitoes examined. This genus is known as an insect symbiont, and it increases bacterial and fungal resistance of insects [29]. *Alcaligenes* was the most prevalent in the gut of the uninfected *An. minimus,* and it was detected in all of the uninfected samples. *Alcaligenes* has been used as a symbiont to control insect borne diseases in the plants [30]. However, the relationship between the two genera, *Burkholderia* as well as *Alcaligenes*, and *Plasmodium* is still unclear. Further studies are required to determine whether these bacteria possess any activities that lead to the inhibition of *Plasmodium* development in the mosquito gut. Certain species of bacterial flora were shown to trigger mosquito innate immune responses against *Plasmodium* infection [27].

5. CONCLUSION

Bacterial communities in the gut of the infected and the uninfected *Anopheles minimus* were different. In the uninfected mosquito, the gut bacteria were more diverse than those in the infected mosquito. Many bacterial genera in this study were detected for the first time in malaria vectors but they were present only in a small proportion, except *Xanthomonas*. Our study showed that three bacterial genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* were the most abundant. One of them, *Acinetobacter* has been reported that it had the ability to control the *Plasmodium* transmission by activating the IMD immune response of *Anopheles* mosquito whereas the relationship between the other two bacteria and *Plasmodium* are unclear.

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