รายงานวิจัยฉบับสมบูรณ์

ภาษาไทย ชีววิทยา นิเวศวิทยาและความสำคัญทางด้านระบาดวิทยาของยุงและแมลงวันคอกสัตว์ในประเทศไทย

ภาษาอังกฤษ BIOLOGY, ECOLOGY AND EPIDEMIC IMPORTANCE OF MOSQUITOES AND STABLE FLIES IN THAILAND

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สัญญาเลขที่ RTA5280007

รายงานวิจัยฉบับสมบูรณ์

ภาษาไทย **ชีววิทยา นิเวศวิทยาและความสำคัญทางด้านระบาดวิทยาของยุงและแมลงวันคอกสัตว์ในประเทศไทย**

ภาษาอังกฤษ **BIOLOGY, ECOLOGY AND EPIDEMIC IMPORTANCE OF MOSQUITOES AND STABLE FLIES IN THAILAND**

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Various important aspects on biology and ecology of two medically important insects, mosquitoes and stomoxyine flies, were charectized during a three-year period. For mosquitoes, several biological aspects of two important genus, *Anopheles* and *Aedes*, were studied. With Anopheline mosquitoes, 7 species of the three complexes were identified, including *Anopheles dirus, Anopheles baimai, Anopheles minimus, Anopheles harrisoni, Anopheles aconitus, Anopheles maculatus,* and *Anopheles sawadwongporn*i. Clear trophic behavior and host preference of four major vectors within the three complexes, *An. dirus, An. minimus, An. maculatus,* and *An. sawadwongporn*i were obtained. Pathogenic agents in the potential malaria/filarial vectors were demonstrated of which *An. dirus* and *An. minimus* are regarded as the potential vectors. Responses to several chemical compounds tested by the two different assays systems on several female Anopheline mosquito species were evaluated. For *Aedes albopictus,* information on biting patterns and flight range were investigated. In addition, behavioural responses of *Aedes aegypti* to various compounds were tested using the three standard systems, namely an excito repellency assay, a high throughput screening assay, and an experimental hut. Our study represents the first group to discover the three chemical actions, including contact irritant, sptial repellent, and toxicant on mosquito populations. In addition, we obtained various aspects on species diversity, biology and ecology of stable flies. Five species of stomoxyine fly were identified, including a cosmopolitan species, *Stomoxys calstrans*. We also obtained information on population dynamics, phylogeny and circadian activity of stable fles. A better understanding of both insect vectors' biology and ecology will facilitate and improve the efficiency of vector control measures in private and government sectors.

Keywords; Anopheles, Aedes, ecology, biology, species, insecticide, diversity, behavior, stable fly

บทคัดย่อ

ได้มีการศึกษาความสำคัญด้านต่างๆ ทางชีววิทยา นิเวศวิทยาของยุงและแมลงวันคอกสัตว์ซึ่งแมลงทั้งสองกลุ่มจัดเป็นแมลงที่มีความสำคัญทางการแพทย์และสัตวแพทย์ การศึกษาวิจัยในครั้งนี้ใช้เวลาทั้งสิ้นสามปี (2552-2555) เริ่มจากการศึกษายุงสองกลุ่มคือยุงก้นปล่องและยุงลาย นักวิจัยพบยุงก้นปล่องที่สำคัญจำนวนเจ็ดชนิดจากยุงชนิดซับซ้อนจำนวนสามกลุ่มโดยใช้เทคนิคทางโมเลกุล ยุงทั้งเจ็ดชนิดนี้ได้แก่ *Anopheles dirus, Anopheles baimai, Anopheles minimus, Anopheles harrisoni, Anopheles aconitus, Anopheles maculatus* และ *Anopheles sawadwongporn*i และได้มีการศึกษาพฤติกรรมการกินอาหารและการออกหาเหยื่อของยุงก้นปล่องสี่ชนิดคือ *An. dirus, An. minimus, An. maculatus,* และ *An. sawadwongporn*i ซึ่งเป็นสมาชิกในกลุ่มซับซ้อนทำให้ทราบพฤติกรรมเหล่านี้อย่างถูกต้องแม่นยำในระดับสปีชีร์จากเทคนิคทางโมเลกุลวิทยา ในจำนวนนี้พบเชื้อมาลาเรียในต่อมน้ำลาย จากยุง *Anopheles dirus* และ *Anopheles minimus* งานวิจัยครอบคลุมไปถึงการศึกษาการตอบสนองของสารเคมีในยุงก้นปล่องหลายชนิดโดยใช้เครื่องมือทดสอบที่ทีมงานได้พัฒนาขึ้นมาใช้ในงานวิจัยนี้ พบว่าสารเคมีหลายชนิดต้องมีการศึกษาวิจัยอย่างละเอียดก่อนนำไปใช้ในการควบคุมยุง สำหรับยุงลายสวนได้มีการศึกษาระยะทางการบินและรูปแบบการหากินในแต่ละช่วงเวลา ทำให้ทราบข้อมูลพื้นฐานของยุงลายสวนมากขึ้น งานวิจัยของเรายังใช้เครื่องมือสามชนิดเพื่อศึกษาฤทธิ์ของสารเคมีที่มีต่อยุงลาย และพบเป็นครั้งแรกว่า สารเคมีมีฤทธิ์ถึงสามรูปแบบ คือ สารรำคาญแมลง สารไล่แมลงและสารฆ่าแมลง นอกจากยุงแล้วทีมวิจัยได้ศึกษาชีววิทยาและนิเวศวิทยาด้านต่างๆ ของแมลงวันคอกสัตว์ ในการศึกษาพบว่าในประเทศไทยมีแมลงวันคอกสัตว์อย่างน้อยห้าชนิด โดยมี *Stomoxys calstrans* เป็นชนิดที่พบทั่วไป ในการศึกษาแมลงวันคอกสัตว์มีการศึกษาด้านพลวัตของประชากร ความสัมพันธ์ทางด้านวิวัฒนาการทางสายพันธุ์และกิจกรรมในแต่ละช่วงวันของแมลงวันคอกสัตว์ ความเข้าใจในรายละเอียดด้านต่างๆ ของแมลงที่มีความสำคัญทางการแพทย์และสัตวแพทย์ทั้งสองชนิดนี้เป็นพื้นฐานที่สำคัญในการควบคุมแมลงเหล่านี้ต่อไป

คำสำคัญ: ยุงก้นปล่อง, ยุงลาย, นิเวศวิทยา, ชีววิทยา, ชนิด, สารฆ่าแมลง, ความหลากหลาย, พฤติกรรม, แมลงวันคอกสัตว์

Executive Summary

1. Biting patterns and flight range of *Aedes albopictus*, vector of Chikungunya, in Mae Tang District, Chaingmai Province (Dr.Wannapa Suwonkerd)

2. Disease pathogens in field collected *Anopheles* mosquitoes and parasite transmission in Thailand (Dr.Waraporn Juntarajumnong)

3. Vectorial capacity of potential vectors in a rubber plantation forest of Kanchanaburi Province, the co-endemic area of filarial and malarial deseases in Thailand (Dr Jinrapa Phothikasikorn)

4. Molecular population genetics of *Anopheles minimus* complex in Thailand
(Dr. Uraiwan Arunyawat)

5. Trophic behavior and ecological characteristics of *Anopheles dirus* complex in man-made habitats (Dr.Sungsit Sungvornyothin)

6. Species diversity, population dynamics and phylogeny of stable fly in Thailand
(Dr.Suprada Sukhonthabhirom na Pathalung)

7. Circadian activity of *Stomoxys calcitrans* in Thailand
(Dr.Jumnongjit Phasuk)

Diseases transmitted by arthropods are referred to vector-borne diseases. These diseases are now increasing and emerging in several areas of the world, causing of death and suffering worldwide. There has been a significant growth in the human population combined with demographic movement to the urban residential settings and an increase in tourism-based facilities. These modifications influence the favor the proliferation of given arthropod vectors. More than a billion people, mainly in undeveloped and developing countries, are now at risk for serious diseases. Based on current prediction, it is estimated that the disease burden from malaria as well as dengue (arthropod borne virus: arboviruses) will become worse and worse in the near future, without any solutions near at hand, especially in African, Central and South American and Asian countries.

Despite decades of vector and public health activities, several vector borne diseases remain major health threats in Thailand, principally malaria, dengue fever and dengue hemorrhagic fever, lymphatic filarialsis and Japanese encephalitis. We have a lesson learnt from dengue disease that first epidemic occurred only in Bangkok metropolitan but later dengue spread through out Thailand. All dieases transmitted by various species of mosquitoes, some of which remain capable hosts of conveying more than one disease pathogen. For example, malaria is still one of the important

infectious diseases in Thailand. The disease remains most prevalent along the undeveloped borders of eastern Myanmar, western Cambodia and northern Malaysia. Malaria parasites are only transmitted by *Anopheles* mosquitoes. Of 73 species recognized in Thailand, five species are now considered to be potential malaria vectors. These include *Anopheles dirus*, *Anopheles baimaii*, *Anopheles minimus*, *Anopheles pseudowillmori* and *Anopheles aconitus*. Most of them line into the complex and are not easily recognize from one another. As a consequence, little has been known about knowledge on behavior, ecology and genetics on each individual species. Dengue is another arthropod-borne viral disease in the world and commonly occurs in Thailand. Only two species of *Aedes* mosquitoes, *Aedes aegypti* and *Aedes albopictus* are considered the dengue virus and chikungunya vectors. *Aedes aegypti* is more prevalent around human dwellings and is a principal vector in urban zone while *Ae albopictus* is known as a vector in the rural areas. Controlling dengue and Chikungunya is extremely difficult and completely needs community participation. The most effective proven methods for disease preventive has been by vector reduction using various chemical means. In addition, there is a lack of information about the potential vector status and clear transmission cycle of Chikungunya. Also, behavioral responses of *Ae aegypti* to potentially toxic chemicals are limited. Lymphatic filariasis is vector borne disease in which presents in Thailand in two different forms, *Wuchereria bancrofti* and *Brugia malayai*. Several mosquito species involve in filariasis transmission. *Culex quinquefasiatus* is the principal vector of urban *Wuchereria bancrofti* whereas several *Aedes* species have been implicated as either secondary vector of filariasis in Thailand. The main vector of *B. malayi* is *Mansonia* species. Detection of potential vectors of lymphatic filariasis in Thailand is completely needed.

In addition to mosquitoes, stable flies are also considered to be one of the most potential pests of human and animal diseases. Stable flies belong to subfamily Stomoxyinae in family Muscidae (Diptera). The genus *Stomoxys* contains 18 species. They are blood sucking insects and considered as pests of livestock and other warm-blooded animals in many parts of the world. Among these 18 species, 17 have an African and/or Asian distribution and only one is cosmopolitan: *Stomoxys calcitrans* (L. 1758). In addition to *Stomoxys calcitrans*, several other stomoxyine flies can also attack animals, including *Stomoxys niger, Stomoxys sitiens* and *Stomoxys indica*. Both male and female stable flies feed on blood, generally once a day per fly. These insects are aggressive and sometimes bite human in extreme conditions. Although they are quite active at the livestock farms, they are invariably a nuisance insect on beaches and in residential areas used for agricultural purposes. Flight range is more or less one mile. 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.

In Thailand, little has been known about the stable fly, particularly on the biology, ecology and genetic aspects. Basic knowledge on certain potential vectors of diseases is absolutely needed, especially information on behavior, ecology and genetics. This information is of paramount importance to understand the epidemiology of disease transmission and vector prevention and control. In addition, such information assists to define a species capacity to acquire and transmit parasites, its contribution to the relative risk for disease transmission in the animal and human populations, and facilitate the active planning, design and implementation of appropriate vector prevention and control strategies.

Subproject-1 (Dr.Wannapa Suwonkerd)

Comparative on a seasonal trend of susceptibility of *Aedes aegypti* against temephos in Chiang Mai province, northern Thailand

Summary

Despite decades of vector borne diseases, principally malaria, dengue fever and dengue hemorrhagic fever, causes a public health threatening in Thailand. More recently chikungunya, the mosquito borne disease caused by Alphavirus and transmitted by some species of Aedes, has been now reemerging and caused big epidemic in especially in southern Thailand. Later disease expanding northward to some particular provinces in Thailand. Beyond the infrastructure changing additional with the vector surveillance system has not been well developed a chickungunya might be possible epidemic at all time. We have a lesson learnt from dengue disease that first epidemic in only Bangkok but later dengue spread through out Thailand. *Aedes aegypti* and *Aedes albopictus* are the vectors transmitted dengue fever and chikungunya, prior to availableness of effective dengue vaccine, appropriate vector control still recommendation, however, some knowledge about the vector biology need an up to dated following global changing as well as Thailand become an destination in several issues, business, tourist etc. This subject aims to investigate the dynamic of vector biology of *Aedes aegypti* in general and and *Ae albopictus* in particular, including biting pattern, flight range, insecticide susceptibility level and its relation to disease by mean of Pupa Index, studies were undertaken in municipality and rural areas, the results were further reported separately each topic.

The work was financial supported by the TRF-Senior Research Scholar Program (RTA5280007) to Theeraphap Chareonviriyaphap, and partly by the regular budget from Department of Disease Control, Ministry of Public Health. The results from this subprojects will be submitted for publication in the international journals that have contributed to the following manuscripts:

1. Wannapa Suwonkerd, Nantawan Suwannachote and Theeraphap Charoenviriyaphap Mark- Release-Recapture study to measure dispersal of *Aedes albopictus* (Skuse) vector of dengue fever and Chikungunya in Chiang Mai province, northern Thailand. (manuscript in preparation).
2. Wannapa Suwonkerd, Nantawan Suwannachoteand and Theeraphap Charoenviriyaphap. Assessment of pupal Index in high and low endemic dengue fever areas in northern Thailand, prospective of effective control of dengue vectors (manuscript in preparation).
3. Wannapa Suwonkerd, Nantawan Suwannachote and Theeraphap Charoenviriyaphap Seasonal pattern of *Aedes albopictus (Skuse)* vector of Dengue fever and Chikungunya in municipality and rural areas of Chiang Mai Province, northern Thailand
4. Wannapa Suwonkerd, Nantawan Suwannachoteand Theeraphap Charoenviriyaphap. Comparative on a seasonal trend of susceptibility of *Aedes aegypti* against temephos in Chiang Mai province, northern Thailand

OUTPUT

Manuscripts for international journals

1. Wannapa Suwonkerd, Nantawan Suwannachote and Theeraphap Charoenviriyaphap. Mark-Release-Recapture study to measure dispersal of *Aedes albopictus* (Skuse) vector of dengue fever and Chikungunya in Chiang Mai province, northern Thailand. (manuscript in preparation).

2 Wannapa Suwonkerd, Nantawan Suwannachoteand and Theeraphap Charoenviriyaphap. Assessment of pupal Index in high and low endemic dengue fever areas in northern Thailand, prospective of effective control of dengue vectors (manuscript in preparation).

3. Wannapa Suwonkerd, Nantawan Suwannachote and Theeraphap Charoenviriyaphap Seasonal pattern of *Aedes albopictus (Skuse)* vector of Dengue fever and Chikungunya in municipality and rural areas of Chiang Mai Province, northern Thailand

4. Wannapa Suwonkerd, Nantawan Suwannachoteand Theeraphap Charoenviriyaphap. Comparative on a seasonal trend of susceptibility of *Aedes aegypti* against temephos in Chiang Mai province, northern Thailand

International conferences/workshop

1 Wannapa Suwonkerd, Nantawan Suwannachote, Theeraphap Chareonviriyaphap. Late Biting of *Aedes albopictus* in Chiang Mai province, northern Thailand, chance for prevention and mosquito control. ASTMH 2009 (poster)
2. Wannapa Suwonkerd, Nantawan Suwannachote, Thum Boonti, Theeraphap Chareonviriyaphap. Biting pattern of a dengue vectors, *Aedes aegypti* and *Aedes albopictus* in urban and rural gradient in Chiang Mai province, northern Thailand ASTMH 2010 (poster)
3. Wannapa Suwonkerd, Nantawan Suwannachote, Thum Boonti, Theeraphap Chareonviriyaphap. Mark- Release-Recapture study to measure dispersal of *Aedes albopictus* (Skuse) vector of dengue fever and Chikungunya in Chiang Mai province, northern Thailand. ASTMH 2011 (poster)
4. Wannapa Suwonkerd, Nantawan Suwannachote, Thum Boonti, Theeraphap Chareonviriyaphap Seasonal variation and nocturnal biting pattern of *Aedes aegypti (L) and albopictus (Skuse) in urban* and rural areas of Chaing Mai Province, northern Thailand. AMCA 2012 Texas. (oral presentation).

Subproject-2 (Dr.Waraporn Juntarajumnong)

Disease pathogens in field collected *Anopheles* mosquitoes and parasite transmission in Thailand (Dr.Waraporn Juntarajumnong)

Abstract A vector-borne disease is one in which the pathogenic microorganism is transmitted from an infected individual to another by an arthropod. Malaria is a common vector-borne disease worldwide caused by parasites belonging to the *Plasmodium* species especially, *P. falciparum* and *P. vivax* and transmitted by *Anopheles* species. These diseases are also found in many developing countries including Cambodia. Therefore, the samples of *Anopheles* mosquitoes were collected from Ratanakiri and Mondulkiri province in Cambodia considered to be malaria-endemic area by human-bait and cattle-bait. The mosquitoes were collected from June 2010 to May 2011 in every three months. After collection, the species *of Anopheles* mosquitoes were identified by

morphological characteristic. The head-thoracic part dissected from whole body of each mosquito was later extracted to obtain genomic DNA. The genomic DNA was used as template of the species-specific PCR to confirm the mosquito species. Of the 557 collected *Anopheles* mosquitoes, there are still some misidentified specimens in the morphological method. Furthermore, the mitochondrial DNA from both *P*. *falciparum*, *P*. *vivax* sporozoites containing in DNA extracted from head-thoracic parts of mosquitoes were then detected by PCR amplification simultaneously. The results show that *P. falciparum* and *P. vivax* were observed in some head-thoracic of mosquitoes especially *An. minimus* collected in this time period except in June exhibiting that these infected mosquitoes can be the vector which transmit the malaria to people in Cambodia.

KEY WORDS *Anopheles* mosquitoes, *Plasmidoum vivax*, *Plasmodium falciparum*, Polymerase Chain Reaction

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 for rapid, easy and reliable identification (Garros et al. 2004).

Anopheles species are involved in malaria transmission (Manguin et al. 2009). 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 parasite is typically detected by conventional microscopy in stained blood smear. This method is often time-consuming and requires experience. 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. DNA-based methods such as

polymerase Chain Reaction are sensitive to detect Plasmodium. Small subunit ribosomal RNA (ssRNA) or mitochondrial DNA can be used to detect *Plasmodium* (Chansiri et al. 2001). In this study, malaria parasites including *Plasmodium vivax* and *Plasmodium falciparum* infected in *Anophleles* mosquitoes collected from Cambodia were simultaneously detected by amplification of mitochondrial DNA. Since this gene presents a high copy number of the genome in the infected cells making more likely to amplify target genes by single-round PCR (Cunha et al. 2009).

Materials and Methods

Mosquito collection. Female *Anopheles* mosquitoes were collected from June 2010 to May 2011 in every three months at Ratanakiri and Mondulkiri, Cambodia based on endemicity of malaria using human-bait and cattle-bait.

Morphological identification of mosquitoes. The mosquito samples used in this study were identified based on their morphology according to Rattanarithikul (1996). After identification, each individual specimen was dissected into two parts; the head-thoracic part was kept in one microcentrifuge tube and the rest of the body in another. The samples were kept in -80 $^{\circ}$C for DNA isolations

DNA extraction. The two parts of mosquito samples were homogenized in 50 µl extraction buffer (0.2M sucrose, 0.1M Tris-HCl at pH 8.0, 50mM EDTA and 0.5% SDS). The tubes were incubated at 65 $^{\circ}$C for 30 min, 11 µl of 5mM KOAc (pH 9.0) added and the tubes placed on ice for another 30 min. The homogenate was centrifuged at 12,000 rpm for 20 min. Supernatant was removed to a clean tube and 100 µl of absolute ethanol was added and kept in 4 $^{\circ}$C for 10 min before spinning at 12,000 rpm for 20 min. The pellet was washed by 150 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 min. The DNA pellet was washed again by 100 µl of absolute ethanol before spinning at 12,000 rpm for 5 min. The pellet was dried at room temperature and re-suspended in ultrapure water.

PCR amplification for species specific. For species identification of *Anopheles minimus* group mosquitoes (Garros et al. 2004), the ITS2A forward 5'-TGT GAA CTG CAG GAC ACA T-3', MIA reverse 5' CCC GTG CGA CTT GAC GA 3', MIC 5'-GTT CAT TCA GCA ACA TCA GT-3', ACO 5'-ACA GCG TGT ACG TCC AGT-3', PAM 5'-TGT ACA TCG GCC GGG GTA-3', VAR 5'-TTG ACC ACT TTC GAC GCA-3' were used to amplify ITS2A region. The PCR was performed in 25 µl PCR reaction mix using 2 µl of DNA template. The samples were heated at 94 $^{\circ}$C for 2 min before 40

cycles of amplification at 94 °C 30 sec, 50 °C 30 sec and 72 °C 40 sec followed by a final extension step at 72 °C for 5 min. For species identification of *Anopheles dirus* group mosquitoes (Walton et al. 1999), the D-U 5' CGC CGG GGC CGA GGT GG 3', D-AC 5' CAC AGC GAC TCC ACA CG 3' and

D-D 5' GCG CGG GAC CGT CCG TT 3' were used to amplify ITS2 region. The PCR was performed in 25 µl PCR reaction mix using 2 µl of DNA template. The samples were heated at 94 °C for 5 min before 32 cycles of amplification at 94 °C 15 sec, 55 °C 15 sec and 72 °C 30 sec followed by a final extension step at 72 °C for 10 min. For species identification of *Anopheles maculates* group mosquitoes (Walton et al. 2007), the 5,8F 5' ATC ACT CGG CTC GTG GAT CG 3', Mac 5' GAC GGT CAG TCT GGT AAA GT 3', Drav 5' GCC TAC TTT GAG CGA GAC CA 3', K 5' TTC ATC GCT CGC CCT TAC AA 3', Saw 5' ACG GTC CCG CAT CAG GTG C 3' and Pseu 5' GCC CCC GGG TGT CAA ACA G 3' were used to amplify ITS2 region. The PCR was performed in 25 µl PCR reaction mix using 2 µl of DNA template. The samples were heated at 94 °C for 5 min before 35 cycles of amplification at 94 °C 60 sec, 61 °C 30 sec and 72 °C 30 sec followed by a final extension step at 72 °C for 5 min. For species identification of *Anopheles annularis* group mosquitoes (Walton et al. 2007), the 5.8F 5' TGT GAA CTG CAG GAC ACA TG 3', PHI 5' GCA CGC CAT TAT GCG ACA AAC 3' and NIV 5' CAT GTA CCT CAC GAT ACA TGT A 3' were used to amplify ITS2 region. The PCR was performed in 25 µl PCR reaction mix using 2 µl of DNA template. The samples were heated at 94 °C for 5 min before 35 cycles of amplification at 94 °C 60 sec, 61 °C 30 sec and 72 °C 30 sec followed by a final extension step at 72 °C for 5 min. Ten µl of PCR product was loaded to electrophoresis in 2% agarose gel with TAE buffer and stain with Gel Star.

PCR amplification for Plasmodium detection. *Plasmodium* detection (Cunha et al. 2009), the 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 from *Anopheles minimus* group mosquitoes. 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.

Results

The 563 of *Anopheles* mosquitoes were collected from Ratanakiri and Mondulkiri, Cambodia from June 2010 to May 2011. The samples were collected by human-bait and cattle-bait every three months in June, September, December and May. The morphological character-based identification demonstrated that all mosquitoes are 59 of *An*. *minimus*, 2 of *An*. *harrisoni*, 37 of *An*. *maculatus*, 39 of *An*. *dirus*, 1 of *An. pampani*, 71 of *An. phillippinensis*, 137 of *An. nivipes*, 12 of *An. aconitus*, 30 of *An. sawadwongporni*, 174 of *An. splendidus* and 1 of *An. tessellatus*. However, *An*. *harrisoni*, *An. pampani* were found only in June. *Anopheles aconitus* was found only in December. *Anopheles splendidus* and *An. tessellates* were found only in May. *Anopheles phillippinensis* and *An. nivipes* were found in September and December while *An. sawadwongporni* were found in December and May. The rest were found in June, September, December and May. The species-specific PCR of these mosquitoes using genomic DNA extracted from head-thoracic part exhibited that there are still some misidentification using morphological characteristic. The different species demonstrated by the different size of PCR product of each mosquito compared to PCR product size of the mosquitoes which species was already identified by both morphology and molecular method (Fig. 1). Unfortunately, the PCR product can not be observed in the some samples so these samples are non-identify.

. For *Plasmodium* detection using *P. vivax* and *P. falciparum* primers to perform simultaneously PCR amplification, the PCR product of *P. vivax* and *P. falciparum* were obtained in pooled head-thoracic of mosquitoes collected in September, December and March. The PCR product of *P. falciparum* and *P. vivax* were illustrated in Figure 2. The *P. vivax* and P. *falciparum* PCR products cannot be observed in the mosquitoes collected in June. Therefore the individual mosquito from pooled head-thoracic mosquitoes was performed PCR amplification to detect *P. vivax* and *P. falciparum* simultaneously. The results exhibited that the PCR product of *P. vivax* and *P. falciparum* were obtained from some of *An. dirus* and *An. minimus*.

Discussion

Previously, molecular techniques for *Plasmodium* detection are nested-PCR (Snounou et al. 1993), real-time PCR (Men 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 simultaneously detect *P. vivax* and *P. falciparum* in field-collected mosquito samples, especially in cases of mixed species infection (Ochola et al. 2006). This method is less laborious and time-consuming when compared to nested PCR. Therefore, the *Anopheles* mosquitoes were collected in Ratanakiri and Mondulkiri, Cambodia to detect *P. vivax* and *P. falciparum*. The results showed that the amplified mitochondrial DNA fragment of *P. vivax* using both *P.vivax* and *P.falciparum* primers were obtained in mosquitoes collected in September, December and March which are dry season. However, both Plasmodium species were not detected in the mosquitoes collected in June which is wet season. It might cause from the number of mosquitoes collected in each month is difference. The number of mosquitoes collected in June is only thirty four which is much lower than in September, December and March. Therefore, the opportunity to detect plasmodium species is also quite low in June.

Plasmodium falciparum and *P. vivax* can be then detected in *An. dirus* and *An. minimus*. It can be concluded that *An. dirus* and *An. minimus* mosquitoes collected in September from Moldulkiri in Cambodia are malaria vector because it can transmit *P. vivax* sporozoite in salivary gland in head-thoracic part of these samples. Normally, parasites ingested with the bloodmeal decrease in number before coming into contact with host tissues. Many factors could be responsible for this reduction in parasite number especially microorganisms in midgut of mosquitoes (Azambuja et al. 2005). However, real time PCR should be performed to confirm the results since PCR sometimes gave the false positive.

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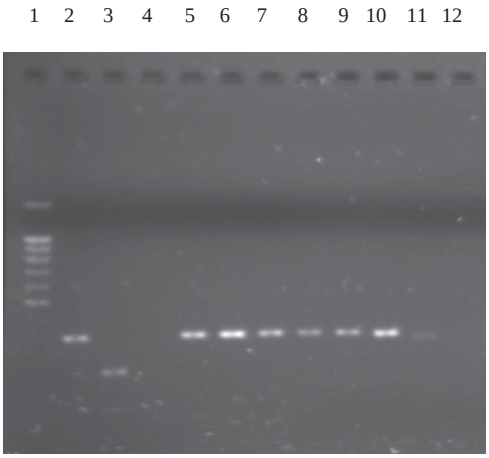

Figure 1

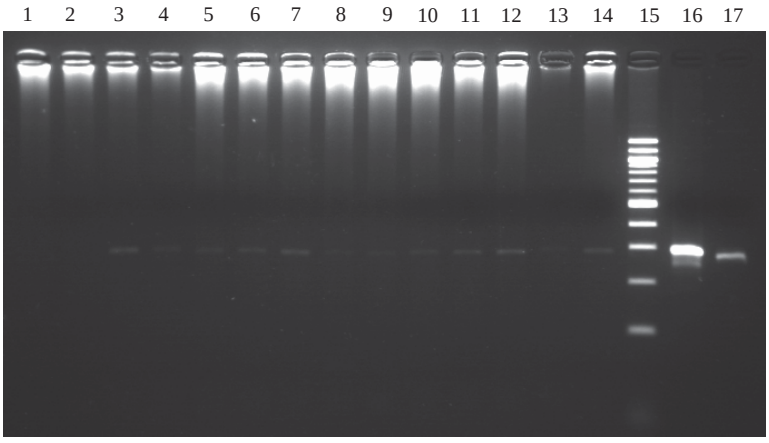

Figure 2

Figure legends

Figure 1. Amplified fragments using the species-specific polymerase chain reaction assay for identifying members of the *An. minimus* groups. Lane 1: molecular marker (1.5 kb+100 bp ladder); lane 2-4: *A.minimus*, *A.harrisoni* and *A. aconitus* (positive control); lane 5-11: some samples of mosquitoes collected from Cambodia; lane 12: negative control.

Figure 2. Amplification of mitochondrial DNA of *P. falciparum* and *P. vivax*. Lane 1-14: amplified fragments of *P. falciparum* or *P. vivax* obtained from head-thoracic of *Anopheles* mosquito samples collected in Cambodia; Lane 15: molecular marker (1.5 kb+100 bp ladder); Lane 16-17: PCR product amplified by PCR with specific primers from DNA obtained from *P. vivax* and *P. falciparum*, respectively.

Subproject-3 (Dr. Jinrapa Phothikasikorn)

Vectorial capacity of various mosquitoes in a rubber plantation forest of Kanchanaburi province, the co-endemic area of filarial and malarial diseases, to transmit lymphatic filariasis and malaria in Thailand

Abstract The research was aimed to survey the diversity of mosquitoes which are vectors of filarial and malarial parasites of human in sub-urban and forest areas at Thong Pha Phum district, Kanchanaburi province. The result in this period is representing on the distribution of mosquitoes between December 2009 to August 2011 and the data is shown in Table 1. During this period representing 45 species of various mosquitoes were found, among the total number of wild caught mosquitoes of 8,316 specimens. 40 species have yet to be identified and had to be change the name almost of them follow the new key of Rattanarithikul et al 2006. For the filarial detected and identification, there is still had only 17 mosquitoes of 9 species compost with *Stegomyia* (*Aedes*) group such as *Stegomyia albopicta, St. desmotes, St. annandalei, St. imitator, Downsiomyia harinasutai* (*Ae. niveus group*), *St. malikuli* and another mosquito group such as *Armigeres* subgenus *Armigeres subalbatus* (*Ar.* (Arm.) *subalbatus*) *Ar*. subgenus *Leicesteria* and *Mansonia dives* were filarial worm positive. For the PCR technique for identified to the species of filarial worm is still on the experiment. In this period, there is no Plasmodium infection in the wild caught Anopheline mosquitoes by PCR procedure experiment.

KEY WORDS : filarial disease, malaria, mosquitoes, filarial vector, malaria vector

Introduction

The prevalence of disease caused by *Plasmodium* spp. 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 both 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 co-endemic area between malaria and filariasis, the population dynamics and biting peak of various mosquito vectors of the malarial and filarial parasites of both disease in the area will be investigated. All of the malarial and filarial parasites from the collected mosquitoes will be identified by the PCR technique.

Materials and Methods

Collection site

The study sites are located in an endemic area with both *Plasmodium spp.* (malaria A1 area) and *Wuchereria bancrofti* infections occurring. The village selected as having low pesticide agricultural use was Ban Mae Num Noy village (MNN), 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 village is surrounded by rubber plantations and hills. There are two large clean pools in the village formed by spring water damming up near the village. There is a small permanent stream nearby.

Adult sampling

Wild adult females various mosquitoes were collected 3 months/ season between December and February in dry season and in June to August in wet season by human bait catches in rubber plantation 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 fourth 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.

For the parasite identification,

- filarial worm that collected from the positive mosquitoes will be identify by the pictorial key for their morphological identification and then they will be preserved at -80^{o}C until use in the PCR technique for identification into the species.

Pictorial key for infective stage of filarial worm identification

Identifications

- To focusing on distance between anal opening and posterior end of the worm bodies.

Nested PCR technique

for *Plasmodium* detection (Snounou and Singh, 2002)

1. **DNA extraction**

Tissue & cells genomic Prep mini spin kit

Plasmodium genomic DNA from mosquito specimens was extracted using Tissue & cells genomic Prep mini spin kit followed the manufacturer's instruction.

1 Each mosquito from the field (after separating the abdomen) was crushed the tissue added with 500 µl of PBS buffer (pH 7.4) and centrifuged for 5 min at 2,000 *g*.

2 All the supernatant was discarded and the pellet was added with 50 µl of lysis buffer type I and 10 µl of Proteinase k.

3 The mixture was vortexed for 15 sec and incubated for 1 hr at 56 ºC.

4 Then, the mixture was added with 500 µl of lysis buffer type IV, vortexed for 15 sec and incubated for 10 min at room temperature.

5 The sample was applied to minicolumn in collection tube, and centrifuged for 1 min at 2,000 *g*.

6 All of the supernatant was discarded, the pellet was added with 500 µl of lysis buffer type IV and centrifuged at 2,000 *g* for 1 min.

7 The supernatant was discarded and the pellet was added with 500 µl of wash buffer type VI and centrifuged at 2,000 *g* for 1 min.

8 The supernatant was discarded and the column was dried by centrifugation for 3 min. The column was transferred to a 1.5 ml microcentrifuge tube.

9 50 µl of pre-warmed Elution buffer type V was added directly into the center of the column.

10 The column was incubated at room temperature for 1 min and then centrifuged at 2,000 *g* for 2 min. to elute DNA.

11 The extracted DNA of each sample was frozen at -20 ºC until used.

2. PCR amplification

The nested PCR was carried out using *Plasmodium* genomic DNA. The specific primer pairs were used to detect the presence of human malaria (PCR product approx 235 bp). Further identification of *Plasmodium falciparum* and *Plasmodium vivax* were performed using species specific primer pairs (PCR product of *P.falciparum* approx 206 bp and PCR product of *P.vivax* approx 121 bp).

Firstly, a DNA fragment about 1.6-1.7 kb of the SSU rDNA gene was amplified using the nested PCR with the first primers **rPLU1**(5'TCA AAG ATT AAG CCA TGC AAG TGA3') and **rPLU5** (5'CCT GTT GTT GCC TTA AAC TTC3') in the primary PCR with the condition previously described by Snounou and Singh (2002) as follow:

1. Initial denaturing at 95 °C for 5 min; then 25 cycles, each comprising denature at 94 °C for 1 min, annealing at 58 °C for 2 min, extension at 72°C for 2 min, final annealing at 58°C for 2 min followed by a final extension at 72°C for 5 min .
2. A 235 bp fragment of the *rPLU* gene was amplified in the secondary PCR using primers **rPLU3** (5'TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT') and **rPLU4** (5'TAC CCG TCA TAG CCA TGT TAG GCC AAT ACC3'). The PCR initiated with 1 cycle of 95 °C for 5 min, 64 °C for 2 min, and 72°C for 2 min, followed by 29 cycles of repeat step,64°C for 2 min and final extension at 72°C for 5 min.

3. For species-specific amplification. The second primers, **rFAL1** (5'TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT3') and **rFAL2** (5'ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC3') were used to amplify 206 bp of *P. falciparum* gene and **rVIV1**(5'CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC3')and rVIV2(5'ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA3') were used to amplify 121 bp of *P.vivax* gene with the condition previously described by Georges Snounou and Balbir Singh as follow: initial denaturing at 95 °C for 5 min; then 30cycles, each comprising denature at 94 °C for 1 min, annealing at 58 °C for 2 min, extension at 72°C for 2 min,final annealing at 58°C for 2 min followed by a final extension at 72°C for 5 min .

3. Agarose gel electrophoresis

PCR product was electrophoresed in 2% agarose gel in 1x Tris/borate/EDTA (TBE)buffer.

A 5 µl aliquot from each PCR product was diluted with 1:5 volume of loading dye and before loading into each well. The size of DNA fragments was estimated by using 100 bp DNA ladder (New England BioLab, USA). The gel was run at a constant voltage of 100 volts at room temperature for at least 30 min, stained with cybergold solution for 30 min. Then, the amplified-PCR product was visualized under UV light.

Results and Discussion

Mosquito captures made from December 2009 to February 2012 (2 years) to study on the distribution of them and the data is showing in Table 1. During this period representing 45 species of various mosquitoes were found, among the total number of wild caught mosquitoes of 14,210 specimens. The greatest proportion of mosquitoes has been recorded in wet season of June and the lowest population was on December in dry season (figure 1). There aer 42 mosquitoes of 9 species compost with *Stegomyia* (*Aedes*) group such as *Stegomyia albopicta, St. desmotes, St. annandalei, St. imitator, St. mediopunctatus* and another mosquito group such as *Armigeres* subgenus Armigeres *subalbatus* (*Ar.* (Arm.) *subalbatus*) *Ar*. subgenus Leicesteria *dolichocephalus* (*Ar.* (Lei.) *dolichocephalus*), *Hulecoeteomyia reinerti* and *Coquilletidia crassipes* were filarial worm positive (table 1). There are 3 mosquito species such as *St. annandalei, St. mediopunctatus* and *Coquilletidia crassipes* positive with *Wuchereria bancrofti*, Likewise, almost of the filarial worm that collected from the wild caught mosquitoes are *Dirofilaria immitis*. For plasmodium detection in Anopheline mosquito group parallel *Anopheles* species complex identifications by PCR were shown in table 3. However, there is no mosquito infected with plasmodium for this period.

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Table1: Total number of wild caught mosquitoes that collected during December 2009 and February 2012.

No.	Month(year)	Total of Mosquitoes
1	December(09)	197
2	January (10)	149
3	February (10)	464
4	June (10)	1,085
5	July (10)	1,100
6	August (10)	1,462
7	December (10)	264
8	January (11)	402
9	February (11)	635
10	June (11)	2,571
11	July (11)	2,682
12	August (11)	1,857
13	December (11)	324
14	January (12)	454
15	February (12)	564
	Total	**14,210**

Table2: Positive mosquito species and filarial worm identifications by PCR technique.

Month	ยุง				ชนิด และจำนวนของพยาธิ filarial ที่พบ				
	Genus - species	No. of dissected	No. of positive	% positive	W. bancrofti	B. malayi	D. immitis	Other	PCR results
December	*Aedes desmotes*	6	2	33.33	-	-	3	-	*D. immitis*
	Armigeres (*Lei*) *dolichocephalus*	11	1	9.09	-	-	-	3	X
	Hulecoeteomyia reinerti	3	2	66.67	-	-	13	-	*D. immitis*
January	-	-	-	-	-	-	-	-	-
February	*Aedes imitator*	12	1	8.33	-	-	8	-	*D. immitis*
June	*Aedes desmotes*	755	16	2.12	-	-	98	6	*D. immitis* /X
	Hulecoeteomyia reinerti	54	1	1.85	-	-	3	-	*D. immitis*
	Armigeres subalbatus	260	2	0.77	-	-	3	-	*D. immitis*
July	*Aedes albopictus*	1,083	3	0.27	-	-	3	-	*D. immitis*
	Aedes annandalei	85	2	2.35	-	-	23	-	*D. immitis*
	Aedes mediopunctatus	15	2	13.33	1	-	7	-	*D. immitis*
	Hulecoeteomyia reinerti	26	2	7.69	-	-	11	-	*D. immitis*
August	*Aedes albopictus*	368	2	0.54	-	-	3	-	*D. immitis*
	Aedes desmotes	59	1	1.69	-	-	-	2	X

	Aedes annandalei	67	1	1.49	5	-	-	-	*W. bancrofti*
	Hulecoeteomyia reinerti	25	2	8.00	-	-	20	-	*D. immitis*
	Armigeres subalbatus	113	1	0.88	-	-	-	6	X
	Coquilletidia crassipes	1	1	100	1	-	-	-	*W. bancrofti*
Total	**9 Positive mosquito species**	**2,943**	**42**	**1.42**	**7**	**-**	**195**	**17**	

X = unknown species of the worms.

Table3: Mosquito identification and Plasmodium detection data.

No.	Old code	Mosquito genus species	New code	PCR product		Remarks
				PF	PV	
1	6/54 39	*An.minimus*	min85	negative	negative	pool
2	6/54 23	*An.minimus*	min86	negative	negative	pool
3	6/54 22	*An.minimus*	min87	negative	negative	pool
4	6/54 24'	*An.minimus*	min88	negative	negative	pool
5	6/54 24	*An.minimus*	min89	negative	negative	pool
6	6/54 24	*An.minimus*	min90	negative	negative	pool
7	6/54 24'	*An.minimus*	min91	negative	negative	pool
8	6/54 24'	*An.minimus*	min92	negative	negative	pool
9	6/54 24'	*An.minimus*	min93	negative	negative	pool
10	6/54 45	*An.minimus*	min94	negative	negative	pool
11	6/54 46	*An.minimus*	min95	negative	negative	pool
12	6/54 46	*An.minimus*	min96	negative	negative	pool
13	6/54 24'	*An.minimus*	min97	negative	negative	pool
14	6/54 21	*An.minimus*	min98	negative	negative	pool
15	6/54 48	*An.minimus*	min99	negative	negative	pool
16	6/54 20	*An.minimus*	min100	negative	negative	pool
17	6/54 23	*An.minimus*	min101	negative	negative	pool
18	6/54 21	*An.minimus*	min102	negative	negative	pool
19	6/54 20	*An.minimus*	min103	negative	negative	pool
20	6/54 46	*An.minimus*	min104	negative	negative	pool
21	6/54 46	*An.minimus*	min105	negative	negative	pool
22	6/54 43	*An.minimus*	min106	negative	negative	pool
23	6/54 22	*An.minimus*	min107	negative	negative	pool
24	6/54 43	*An.minimus*	min108	negative	negative	pool
25	6/54 45	*An.minimus*	min109	negative	negative	pool
26	6/54 23	*An.minimus*	min110	negative	negative	pool
27	6/54 23'	*An.minimus*	min111	negative	negative	pool

28	6/54 24'	*An.minimus*	min112	negative	negative	pool
29	6/54 24'	*An.minimus*	min113	negative	negative	pool
30	6/54 45	*An.minimus*	min114	negative	negative	pool
31	6/54 24	*An.minimus*	min115	negative	negative	pool
32	6/54 24'	*An.minimus*	min116	negative	negative	pool
33	6/54 48	*An.minimus*	min117	negative	negative	pool
34	6/54 46	*An.minimus*	min118	negative	negative	pool
35	6/54 24	*An.minimus*	min119	negative	negative	pool
36	6/54 24'	*An.minimus*	min120	negative	negative	pool
37	6/54 24	*An.minimus*	min121	negative	negative	pool
38	6/54 25	*An.minimus*	min122	negative	negative	pool
39	6/54 24'	*An.minimus*	min123	negative	negative	pool
40	6/54 24'	*An.minimus*	min124	negative	negative	pool
41	6/54 48	*An.minimus*	min125	negative	negative	pool
42	6/54 47'	*An.minimus*	min126	negative	negative	pool
43	6/54 48	*An.minimus*	min127	negative	negative	pool
44	6/54 44'	*An.minimus*	min128	negative	negative	pool
45	6/54 48'	*An.minimus*	min129	negative	negative	pool
46	6/54 47	*An.minimus*	min130	negative	negative	pool
47	6/54 48	*An.minimus*	min131	negative	negative	pool
48	6/54 45'	*An.minimus*	min132	negative	negative	pool
49	6/54 16	*An.minimus*	min133	negative	negative	pool
50	6/54 47'	*An.minimus*	min134	negative	negative	pool
51	6/54 48	*An.minimus*	min135	negative	negative	pool
52	6/54 47'	*An.minimus*	min136	negative	negative	pool
53	6/54 48'	*An.minimus*	min137	negative	negative	pool
54	6/54 48'	*An.minimus*	min138	negative	negative	pool
55	6/54 48	*An.minimus*	min139	negative	negative	pool
56	6/54 45'	*An.minimus*	min140	negative	negative	pool
57	6/54 48	*An.minimus*	min141	negative	negative	pool
58	6/54 47	*An.minimus*	min142	negative	negative	pool

59	6/54 45'	*An.minimus*	min143	negative	negative	pool
60	6/54 48'	*An.minimus*	min144	negative	negative	pool
61	6/54 16	*An.minimus*	min145	negative	negative	pool
62	6/54 48	*An.minimus*	min146	negative	negative	pool
63	6/54 47'	*An.minimus*	min147	negative	negative	pool
64	6/54 47'	*An.minimus*	min148	negative	negative	pool
65	6/54 47	*An.minimus*	min149	negative	negative	pool
66	6/54 48	*An.minimus*	min150	negative	negative	pool
67	6/54 47	*An.minimus*	min151	negative	negative	pool
68	6/54 47'	*An.minimus*	min152	negative	negative	pool
69	6/54 48	*An.minimus*	min153	negative	negative	pool
70	6/54 45'	*An.minimus*	min154	negative	negative	pool
71	6/54 48'	*An.minimus*	min155	negative	negative	pool
72	6/54 48	*An.minimus*	min156	negative	negative	pool
73	6/54 24	*An.minimus*	min157	negative	negative	pool
74	6/54 24	*An.minimus*	min158	negative	negative	pool
75	6/54 48	*An.minimus*	min159	negative	negative	pool
76	6/54 48'	*An.minimus*	min160	negative	negative	pool
77	6/54 48'	*An.minimus*	min161	negative	negative	pool
78	6/54 48	*An.minimus*	min162	negative	negative	pool
79	6/54 48'	*An.minimus*	min163	negative	negative	pool
80	6/54 48	*An.minimus*	min164	negative	negative	pool
81	6/54 45'	*An.minimus*	min165	negative	negative	pool
82	6/54 48'	*An.minimus*	min166	negative	negative	pool
83	6/54 48	*An.minimus*	min167	negative	negative	pool
84	6/54 48	*An.minimus*	min168	negative	negative	pool
85	6/54 47'	*An.minimus*	min169	negative	negative	pool
86	6/54 24	*An.minimus*	min170	negative	negative	pool
87	6/54 48'	*An.minimus*	min171	negative	negative	pool
88	6/54 48	*An.minimus*	min172	negative	negative	pool
89	6/54 48	*An.minimus*	min173	negative	negative	pool

90	6/54 47	*An.minimus*	min174	negative	negative	pool
91	6/54 48'	*An.minimus*	min175	negative	negative	pool
92	6/54 48	*An.minimus*	min176	negative	negative	pool
93	6/54 24	*An.minimus*	min177	negative	negative	pool
94	6/54 48	*An.minimus*	min178	negative	negative	pool
95	6/54 48'	*An.minimus*	min179	negative	negative	pool
96	6/54 16	*An.minimus*	min180	negative	negative	pool
97	6/54 46'	*An.minimus*	min181	negative	negative	pool
98	6/54 43	*An.minimus*	min182	negative	negative	pool
99	6/54 47	*An.minimus*	min183	negative	negative	pool
100	6/54 48'	*An.minimus*	min184	negative	negative	pool
101	6/54 46'	*An.minimus*	min185	negative	negative	pool
102	6/54 48'	*An.minimus*	min186	negative	negative	pool
103	6/54 44	*An.minimus*	min187	negative	negative	pool
104	6/54 48	*An.minimus*	min188	negative	negative	pool
105	6/54 47	*An.minimus*	min189	negative	negative	pool
106	6/54 47	*An.minimus*	min190	negative	negative	pool
107	6/54 48'	*An.minimus*	min191	negative	negative	pool
108	6/54 48	*An.minimus*	min192	negative	negative	pool
109	6/54 48	*An.minimus*	min193	negative	negative	pool
110	6/54 44'	*An.minimus*	min194	negative	negative	pool
111	6/54 46'	*An.minimus*	min195	negative	negative	pool
112	6/54 47	*An.minimus*	min196	negative	negative	pool
113	6/54 48	*An.minimus*	min197	negative	negative	pool
114	6/54 47	*An.minimus*	min198	negative	negative	pool
115	6/54 48	*An.minimus*	min199	negative	negative	pool
116	6/54 42	*An.minimus*	min200	negative	negative	pool
117	6/54 18'	*An.minimus*	min201	negative	negative	pool
118	6/54 42	*An.minimus*	min202	negative	negative	pool
119	6/54 47'	*An.minimus*	min203	negative	negative	pool
120	6/54 16'	*An.minimus*	min204	negative	negative	pool

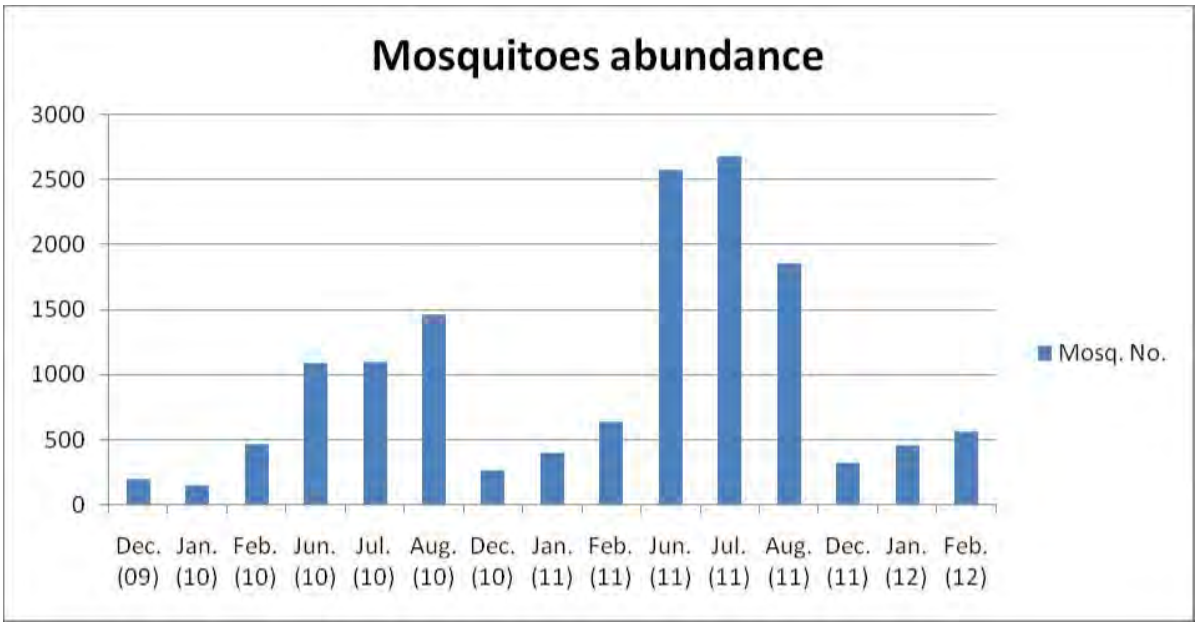
121	6/54 40'	*An.minimus*	min205	negative	negative	pool
122	6/54 40	*An.minimus*	min206	negative	negative	pool
123	6/54 38	*An.minimus*	min207	negative	negative	pool
124	6/54 39	*An.minimus*	min208	negative	negative	pool
125	6/54 18'	*An.minimus*	min209	negative	negative	pool
126	6/54 41	*An.minimus*	min210	negative	negative	pool
127	6/54 18	*An.minimus*	min211	negative	negative	pool
128	6/54 41	*An.minimus*	min212	negative	negative	pool
129	6/54 42'	*An.minimus*	min213	negative	negative	pool
130	6/54 17'	*An.minimus*	min214	negative	negative	pool
131	6/54 15'	*An.minimus*	min215	negative	negative	pool
132	6/54 17'	*An.minimus*	min216	negative	negative	pool
133	6/54 18'	*An.minimus*	min217	negative	negative	pool
134	6/54 16'	*An.minimus*	min218	negative	negative	pool
135	6/54 39	*An.minimus*	min219	negative	negative	pool
136	6/54 16'	*An.minimus*	min220	negative	negative	pool
137	6/54 40	*An.minimus*	min221	negative	negative	pool
138	6/54 42	*An.minimus*	min222	negative	negative	pool
139	6/54 38'	*An.minimus*	min223	negative	negative	pool
140	6/54 39	*An.minimus*	min224	negative	negative	pool
141	6/54 15'	*An.minimus*	min225	negative	negative	pool
142	6/54 14'	*An.minimus*	min226	negative	negative	pool
143	6/54 17	*An.minimus*	min227	negative	negative	pool
144	6/54 40	*An.minimus*	min228	negative	negative	pool
145	6/54 14'	*An.minimus*	min229	negative	negative	pool
146	6/54 18	*An.minimus*	min230	negative	negative	pool
147	6/54 16'	*An.minimus*	min231	negative	negative	pool
148	6/54 18	*An.minimus*	min232	negative	negative	pool
149	6/54 17	*An.minimus*	min233	negative	negative	pool
150	6/54 42	*An.minimus*	min234	negative	negative	pool
151	6/54 18'	*An.minimus*	min235	negative	negative	pool

152	6/54 15	*An.minimus*	min236	negative	negative	pool
153	6/54 42	*An.minimus*	min237	negative	negative	pool
154	6/54 18'	*An.minimus*	min238	negative	negative	pool
163	6/54 20'	*An.dirus*	di1	negative	negative	pool
164	6/54 20	*An.dirus*	di2	negative	negative	pool
165	6/54 39'	*An.dirus*	di3	negative	negative	pool
166	6/54 35	*An.dirus*	di4	negative	negative	pool
167	6/54 21'	*An.dirus*	di5	negative	negative	pool
168	6/54 43	*An.dirus*	di6	negative	negative	pool
169	6/54 45	*An.dirus*	di7	negative	negative	pool
170	6/54 21	*An.dirus*	di8	negative	negative	pool
171	6/54 45	*An.dirus*	di9	negative	negative	pool
172	6/54 43	*An.dirus*	di10	negative	negative	pool
173	6/54 45	*An.dirus*	di11	negative	negative	pool
174	6/54 21	*An.dirus*	di12	negative	negative	pool
175	6/54 22'	*An.dirus*	di13	negative	negative	pool
176	6/54 23'	*An.dirus*	di14	negative	negative	pool
177	6/54 24'	*An.dirus*	di15	negative	negative	pool
178	6/54 24'	*An.dirus*	di16	negative	negative	pool
179	6/54 46	*An.dirus*	di17	negative	negative	pool
180	6/54 22	*An.dirus*	di18	negative	negative	pool
181	6/54 21'	*An.dirus*	di19	negative	negative	pool
182	6/54 46	*An.dirus*	di20	negative	negative	pool
183	6/54 20'	*An.dirus*	di21	negative	negative	pool
184	6/54 44'	*An.dirus*	di22	negative	negative	pool
185	6/54 47	*An.dirus*	di23	negative	negative	pool
186	6/54 44'	*An.dirus*	di24	negative	negative	pool
187	6/54 44	*An.dirus*	di25	negative	negative	pool
188	6/54 46	*An.dirus*	di26	negative	negative	pool
189	6/54 48'	*An.dirus*	di27	negative	negative	pool
190	6/54 44'	*An.dirus*	di28	negative	negative	pool

191	6/54 48'	*An.dirus*	di29	negative	negative	pool
192	6/54 16'	*An.dirus*	di30	negative	negative	pool
193	6/54 14'	*An.dirus*	di31	negative	negative	pool
194	6/54 18'	*An.dirus*	di32	negative	negative	pool
195	6/54 40	*An.dirus*	di33	negative	negative	pool
196	6/54 14'	*An.dirus*	di34	negative	negative	pool
197	6/54 15	*An.dirus*	di35	negative	negative	pool
198	6/54 15'	*An.dirus*	di36	negative	negative	pool
199	6/54 41	*An.dirus*	di37	negative	negative	pool
200	6/54 14	*An.dirus*	di38	negative	negative	pool
201	6/54 15	*An.dirus*	di39	negative	negative	pool
202	6/54 38	*An.dirus*	di40	negative	negative	pool
203	6/54 14'	*An.dirus*	di41	negative	negative	pool
204	6/54 14'	*An.dirus*	di42	negative	negative	pool
205	6/54 40	*An.dirus*	di43	negative	negative	pool
206	6/54 45'	*An.dirus*	di44	negative	negative	pool
207	6/54 44	*An.dirus*	di45	negative	negative	pool
208	6/54 38	*An.dirus*	di46	negative	negative	pool
209	6/54 15'	*An.dirus*	di47	negative	negative	pool
210	6/54 40	*An.dirus*	di48	negative	negative	pool
211	6/54 14'	*An.dirus*	di49	negative	negative	pool
212	6/54 38'	*An.dirus*	di50	negative	negative	pool
213	6/54 48'	*An.dirus*	di51	negative	negative	pool
214	6/54 39	*An.dirus*	di52	negative	negative	pool
215	6/54 39	*An.dirus*	di53	negative	negative	pool
216	6/54 46'	*An.dirus*	di54	negative	negative	pool
217	6/54 45'	*An.dirus*	di55	negative	negative	pool
218	6/54 44'	*An.dirus*	di56	negative	negative	pool
219	6/54 24	*An.dirus*	di57	negative	negative	pool
220	6/54 48'	*An.dirus*	di58	negative	negative	pool
221	6/54 47	*An.dirus*	di59	negative	negative	pool

222	6/54 46'	*An.dirus*	di60	negative	negative	pool
223	6/54 45'	*An.babirostris*	bar3	negative	negative	pool
224	6/54 46'	*An.babirostris*	bar4	negative	negative	pool
225	6/54 24'	*An.babirostris*	bar5	negative	negative	pool
226	6/54 23'	*An.babirostris*	bar6	negative	negative	pool
227	6/54 42	*An.babirostris*	bar7	negative	negative	pool
228	6/54 20	*An.maculatus*	mac1	negative	negative	pool
229	6/54 45'	*An.maculatus*	mac2	negative	negative	pool
230	6/54 43	*An.harisoni*	har1	negative	negative	pool
231	6/54 20'	*An.annularis*	ann1	negative	negative	pool

Graph 1 Population dynamic of total mosquito collected throughout 2 years.



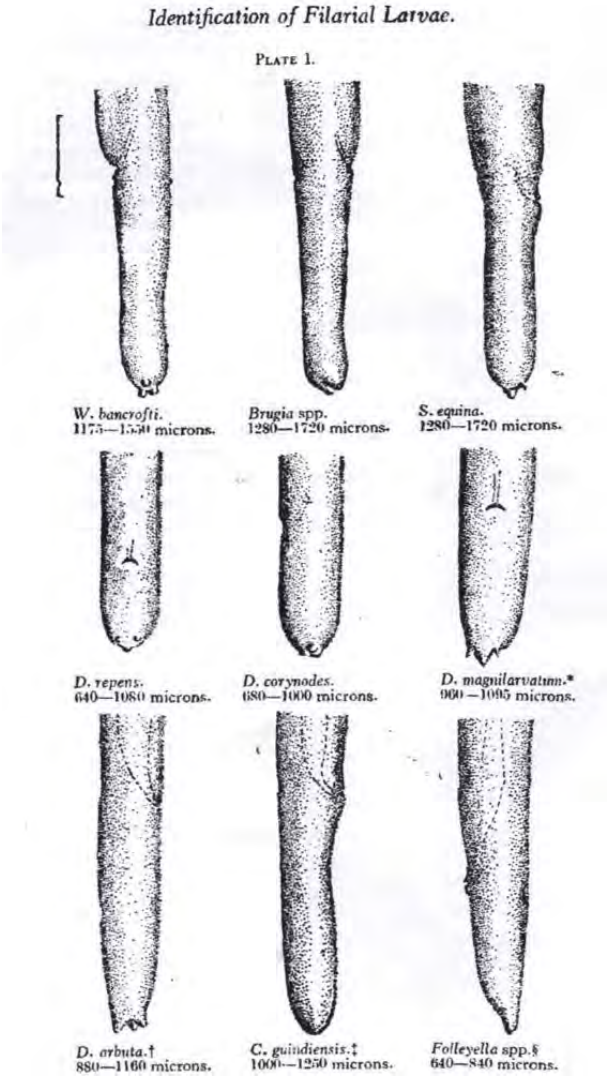


Figure1. Showing anal opening and posterior end of infective stage of filarial worm that collected from infective mosquitoes (G.S Nelson, 1960)

Filarial worms morphology comparison with drawing picture of the pictorial key.

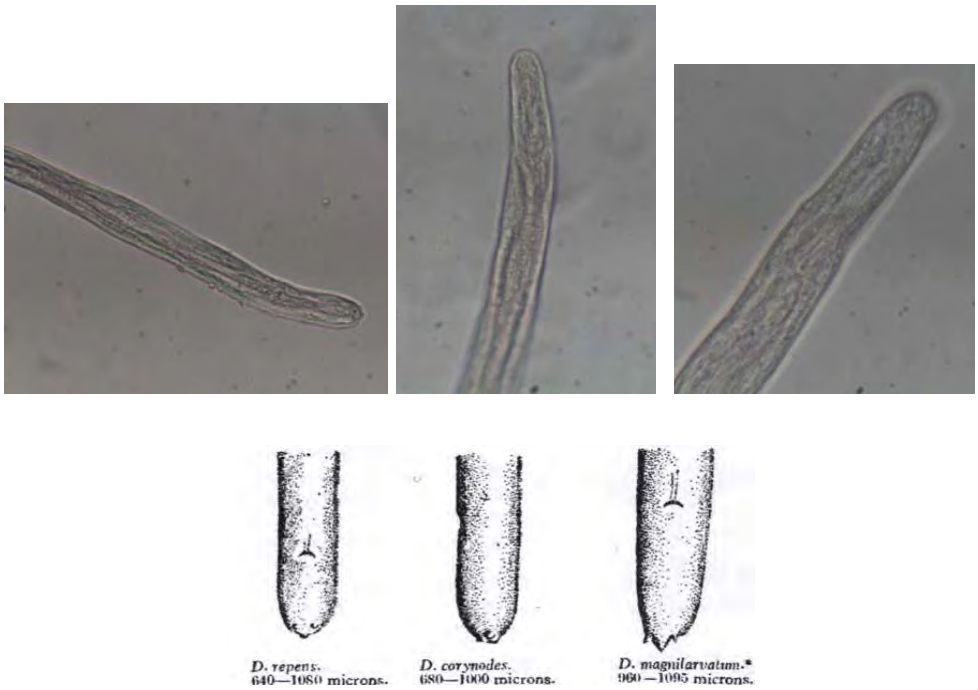


Figure 2 Grouping of *Dirofilaria* spp.

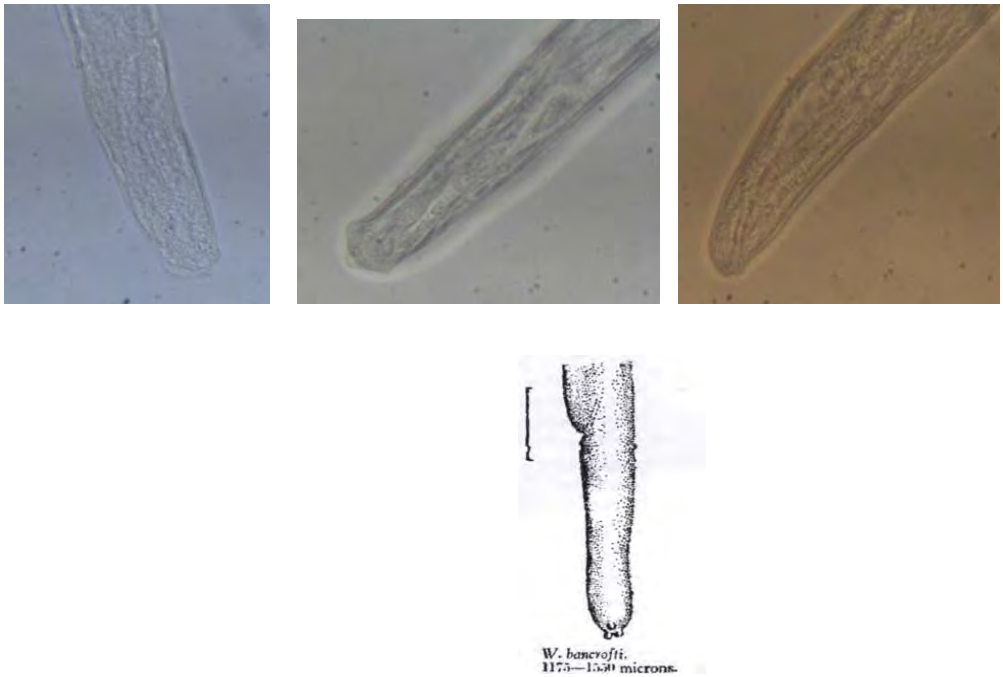
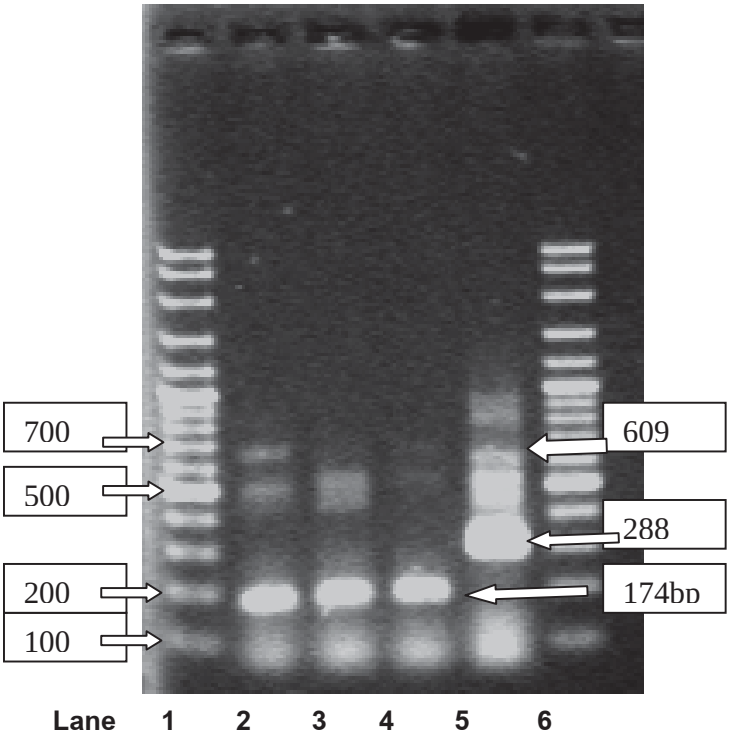


Figure 3 Grouping of *Wuchereria bancrofti*

PCR technique

for *Wuchereria bancrofti* identification

Agarose gel of PCR product from beta-tubulin gene, amplified by BT9/12 and BT 121/122 primers using extracted DNA from Wb positive blood samples as template by TDTU-nested PCR (Pechgit et al, 2011). The PCR products presented both first and second rounds at 609 bp and 174 bp, respectively.



Lane 1, 6: 100 bp DNA Ladder marker

Lane 2: DNA band of Wb with intensity of 1080 mf/ml

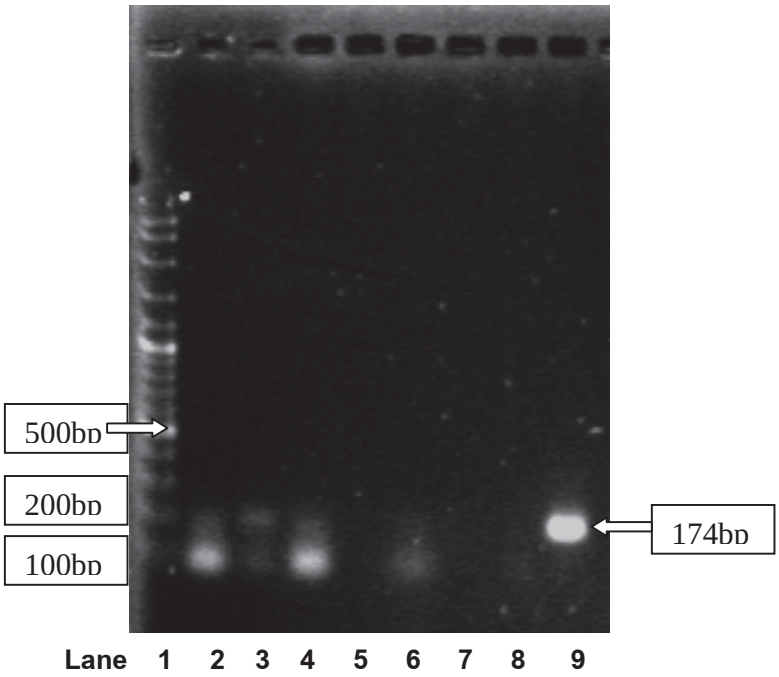
Lane 3: DNA band of Wb with intensity of 252 mf/ml

Lane 4: DNA band of Wb with intensity of 13 mf/ml

Lane 2: DNA band human beta-tubulin gene (for internal control)

-: mf = microfilaria from the patient blood

Agarose gel of PCR product from beta-tubulin gene, amplified by BT9/12 and BT 121/122 primers using extracted DNA from mf in mosquito as template by TDTU-nested PCR (Pechgit et al, 2011). The PCR products presented both first and second rounds at 609 bp and 174 bp, respectively.



Lane 1: 100 bp DNA Ladder marker

Lane 2,**3**: DNA bands of 1 and 2 round PCR **from 1 L3 in mosquito**

Lane 4,5: DNA bands of 1 and 2 round PCR from 5 L3 in mosquito

Lane 6,7: DNA bands of 1 and 2 round PCR from 10 L3 in mosquito

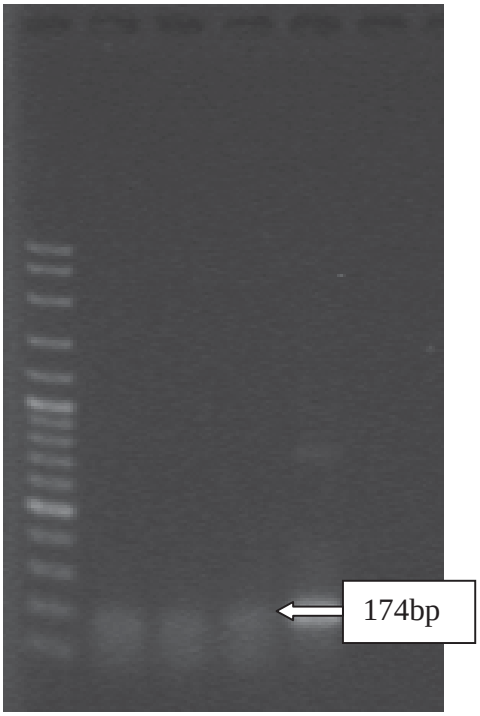
Lane 8,9: DNA band of 1 and 2 round PCR from Wb with intensity of 252 mf/ml (for internal control)

-: L3 = Larva stage 3 (infective stage) from mosquito vector

PCR technique

for *Dirofilaria immitis* identification

Agarose gel of PCR product from beta-tubulin gene, amplified by **BT9/12 and BT 26/27 primers** using extracted DNA from L3 in mosquito as template by nested PCR. The PCR products presented second round 174 bp. ซึ่งเหมือนกับ *Wuchereria bancrofti* bp เพราะใช้ product จาก beta-tubulin gene เหมือนกัน แต่ใช้ primers คนละตัว



Lane 1 2 3 4 5

Lane 1: 100 bp DNA Ladder marker

Lane 2: DNA bands of 2 round PCR from 1 L3 in mosquito

Lane 3: DNA bands of 2 round PCR from 5 L3 in mosquito

Lane 4: DNA bands of 2 round PCR from 10 L3 in mosquito

Lane 5: DNA band of 2 round PCR from *D. immitis* with intensity of 570 mf/ml (for internal control)

Subproject-4 (Dr. Uraiwan Arunyawat)

Molecular population genetics of *Anopheles minimus* complex in Thailand

Summary

This subproject aims firstly to estimate genetic diversity and population genetic structure of *Anopheles minimus* complex, consisting *An. minimus* and *An. harrisoni* in Thailand, and secondly to test population genetic models using the genetic diversity information for understanding evolutionary processes of this malaria vectors. Two important aspects were investigated in this subproject: 1) using single nucleotide polymorphisms data to estimate population genetic parameters in two closly related Minimus mosquito species, 2) to reconstruct phylogenetic trees of malaria vectors presented in Thailand based on multilocus approach of mitocondrial and nuclear DNA sequences. The results from this subprojects have contributed into three parts, presenting seperately in the following section.

Part I

Molecular population genetic studies of *Anopheles minimus* (Diptera: Culicidae), vector of malaria in Thailand

Abstract Understanding the evolutionary factors that shape patterns of genetic variation of the vector species is important to devise new methods for malaria control. In this study, we aim to use population genetic model to find any precise answers to evolutionary questions of *Anopheles minimus*, one of the most widespread malaria vectors in Thailand. We employed a multilocus approach to estimate genetic diversity and to infer population genetic structure of *An. minimus* populations across Thailand by utilizing DNA sequence data for 4 nuclear loci. Our data showed that Kanchanaburi and Chiang Mai populations exhibit low levels of nucleotide diversity, Chantaburi and Surat Thani populations show substantial levels of nucleotide variation, whereas *An. minimus* from Tak province appears to be the most polymorphic populations. Tajima's *D* test was used to measure deviation from neutral equilibrium expectation. Two populations exhibited significant positive Tajima's *D* values, suggesting an excess of intermediate-frequency polymorphism. The estimation of intragenic linkage disequilibrium is high for the populations showing small number of recombination events.

Keywords: *Anopheles minimus*, malaria vector, population genetic analysis, sequence data

Introduction

Mosquito-borne diseases are still a major health problem in many countries. Thailand is one of the countries in tropical region where Mosquito-borne disease is prevalent. *Anopheles minimus* complex is one of the primary malaria vectors that are most widespread in Thailand. Even there are several studies on *An. minimus* complex group but it is still difficult to distinguish from both its species complex and its closely related species. Moreover, there is very limited information on the evolutionary extent of genetic diversity in the malaria vectors endemic in Thailand. Understanding the causes and maintenance of genetic variation is important to control a disease with drugs or vaccine. It is therefore necessary to study population genetics and understanding the nature of the vector species in order to achieve malaria vector control.

In this study, therefore, we apply a multilocus approach to estimate genetic diversity and to infer population genetic structure of *An. minimus* populations across Thailand by utilizing DNA sequence data for 4 nuclear loci. We focus specifically on three objectives, (1) to estimate the patterns and levels of nucleotide diversity in Thai *An. minimus* populations (2) to examine levels of population differentiation among *An. minimus* populations (3) to evaluate pattern of linkage disequilibrium in these studied *An. minimus* populations.

Materials and Methods

Population sampling

Ten adult mosquitoes (*An. minimus*) each population was collected from five different locations, where malaria is still prevalent, across Thailand as shown in table 1. For this study, to resolve the problem of morphological identification, we applied molecular technique to confirm the identification between the two sibling species (*An. minimus* and *An. harrisoni)*, using primers in the ITS2 region developed by Phuc et al. (2003) and Garros et al. (2004)

Choice of neutral DNA markers

Our broad criteria for choosing putative neutral DNA markers are: (i) length of the fragments should be approximately 500-700 bp in order to avoid a difficulty of obtaining the sequences (ii) intron or non-coding region is preferable because intron has been shown to yield substantial

polymorphisms for population genetic analysis. Therefore, in this study we choose the putative nuclear fragments on the X chromosome, using the available sequences on chromosome X of *An. gambiae* (www.ensembl.org) and also the prior studies on this species (Stump et al 2005, Srivastava et al 2009).

We selected some putative genes (as shown in Table 2), same markers as in the study of our collaborator (Dr. Aparup Das at the National Malaria research Institute, New Delhi India), who works also on population genetics study of *An. minimus* and other malaria vectors species endemic in India. For the purpose of analysis more on evolutionary history and species distribution of this malaria vector in a broad view.

Primer design

Our criteria for the primer design followed the approach of Bierne et al. (2000), using EPIC (Exon Primed Intron Crossing) technique. 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, and for the same reason within species amplification PCR artifacts such as null alleles are expected to be less frequent.

Table 3 shows list of primer pairs that we will test for target markers amplification. O8 and O10 primers were designed from the available data of *An. gambiae* on the www.ensembl.org website, and P-primers (P9, P12 and P22) were selected from the study on the X chromosome of *An. gambiae* (Stump et. al, 2005). Both primer groups were designed based on EPIC method.

Genomic DNA isolation and species identification

We extracted genomic DNA of *An. minimus* populations and checked quality on agarose gel electrophoresis. We obtained genomic DNA for all five populations (10 individuals each population). For species identification by molecular technique, we found that both methods (Phuc *et al.* 2003 and Garros *et al.* 2004) were able to identify *An. minimus* (former *An. minimus* species A) between *An. harrisoni* (former *An. minimus* species C) of the Minimus species complex.

DNA amplification and Sequencing

Genomic DNA was isolated from single individual of *An. minimus* from each population using Genomic DNA Mini kit (Tissue) (GeneAid, Biotech Ltd. Taiwan). We used the extraction protocol as provided by the supplier. Then Genomic DNA was quality checked on 1% agarose gel electrophoresis. Polymerase Chain Reaction (PCR) primers and conditions were tested to amplify the putative markers. 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 Seqman and 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 pairwise differences between sequences in a sample (Nei, 1987) as implemented in DNAsp version 5.0 program (Rozas et al. 2003). 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. Moreover, we used the four gamete test (Hudson and Kaplan, 1985) to estimate the minimum number of recombination events (R_m). We also calculated the level of linkage disequilibrium using the average of squared allele-frequency correlations (r^2) in term of Z_{ns} statistic (Kelly 1997).

Results and Discussion

Five primer pairs were able to amplified putative neutral fragments, however, only four primer pairs (P22, P9, O8 and O10) were selected for the study, whereas the PCR product amplified by primer P12 was excluded from further study because the PCR size is much shorter (about 300 bp) than our selected fragment's size (between 500-700 bp). Fragment amplification using four selected primer pairs (P22, P9, O8 and O10) were approximately 400-450 bp, 600-700 bp, 450-500 bp, 600-700 bp, respectively. We sequenced 4 unlinked nuclear loci (P22, P9, O8 and O10) in 5 populations of *An. minimus*, with a total concatenated length of approximately 2 kb per allele.

Levels of Nucleotide diversity

The estimation of nucleotide diversity for each locus and each population was shown in table 4. The number of polymorphism varies from 0 to 16 segregating sites across four loci. Kanchanaburi (KAN) and Chiang Mai (CMI) populations exhibit low levels of nucleotide diversity (θ_w and π ~ 0-0.3 %). Populations from Chantaburi (CTB) and Surat Thani (STR) show substantial levels of nucleotide variation (θ_w and π ~ 0.5-0.9 %), whereas *An. minimus* from Tak (TAK) population seems to be the most polymorphic populations (θ_w and π ~ 0.7-1.1 %).

Test of Neutrality

We used Tajima's *D* statistic (1981) to test for deviation from neutrality. This test measures skews in the frequency spectrum based on two nucleotide variation estimators (θ_w and π). 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 Tajima's *D* values for each locus are given in table 2.

The CMI shows significant positive *D* values at 2 loci (O10 and P9), and the KAN population shows a significant positive *D* value at locus P9, whereas SRT and TAK populations exhibit negative *D* values at 4 loci, except a positive *D* value at locus P22 for the SRT population (figure 1). The statistically significant positive Tajima's *D* values of the *An. minimus* populations from Chiang Mai (CMI) and Kanchanaburi (KAN) suggest an excess of intermediate-frequency variants. This result might be contributed by evolutionary factors such as population bottleneck.

Recombination and Linkage disequilibrium (LD)

The minimum number of recombination events (R_m) was estimated in 5 studied population, using the four-gamete test. Overall, the R_m parameter varies from 0 to 6 events for individual population samples, where *Rm* is lowest for the KAN population and highest for the CTB population (table 5). Moreover, we estimated *Rm* for the "ALL" samples, with pooled of the 5 population samples. The *Rm* estimates of the pooled samples were generally higher than for

individual populations. The haplotype diversity and the LD parameter (using Z_{ns} which is an average of squared allele-frequency correlations (r^2) over all pairwise comparisons) are reported in table 5. As expected, the high levels of LD were found for the populations with low number of recombination events such as the KAN and CMI populations.

na : not available,

All : combined samples based on 5 populations

[#] R_m : minimum number of recombination events (Hudson and Kaplan, 1985)

[&] Z_{ns}: average of squared allele-frequency correlations (r^2) over all pairwise comparisons (Kelly 1997)

Conclusion

Our results present levels of genetic variation of 5 studied *An. minimus* populations for 4 unlinked nuclear loci. We found significantly positive Tajima's *D* values in two populations at two loci, implying population bottleneck might be one of the evolutionary forces that shaped patterns of nucleotide variation within these two populations. Moreover, test of population structure and the analysis of intragenic LD decay with physical pairwise distance might be informative for further association study, as well as for the purpose of understanding evolutionary processes of this malaria vector.

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Table 1 Information of *An. minimus* studied populations

Number	Population location	Population code	Sample size
1	Mae Rim distinct, Chiang Mai province	CMI	10
2	Sai Yok distinct, Kanchanaburi province	KAN	10
3	Mae Sod distinct, Tak province	TAK	10
4	Don Sak distinct, Surat Thani province	SRT	10
5	Gaeng Hang Meaw distinct, Chanthaburi province	CTB	10

Table 2 Genetic information of putative neutral DNA marker (www.ensembl.org)

Gene ID	**Gene Description**	**Gene Size (Kb)**	**Location at Chromosome X**	**No. of Exon**	**Between exons: Intron Length (primer name)**
AGAP000369	Putative GPCR class a orphan receptor 10	2.12	6,791,093-6,793,207	3	2-3: 486 bp (O3)
AGAP000500	NADPH cytochrome P450 reductase	4.75	8,794,244-8,798,989	12	5-6: 415 bp (O4)
AGAP000824	Gbb-60A.	2.88	15,336,624-15,339,506	8	2-3: 680 bp (O8)
AGAP001043	Armadillo segment polarity protein	14.12	20,143,880-20,158,004	5	2-3: 435 bp (O9)
AGAP001076	Cytochrome P450 CYP4G16	9.19	22,937,938-22,947,129	6	3-4: 579 bp (O10)

Table 3 List of primer pairs for amplification (F – Forward primer; R- Reverse primer)

Primer name		Primer sequence (5'-3')	Tm (C)	Gene ID
O8	-F	CGGCCATTGGGCGGACATTT	65	AGAP000824
	-R	ACGCATCTCCGATCCGTGATGATC		
O10	-F	CTGGCTGGGCAACGGACTA	60	AGAP001076
	-R	GGAAAGTAGGGGCAATCAGTTTG		
P9	-F	TGGAAAGTAGGGGCAATCAG	50.9	14481
	-R	GCTGGGCAACGGACTACTTA		
P12	-F	TTTACGTCGTGCAAGAGTGC	45.2	23030
	R	GCCCACGTTCTTCATCGTAT		
P22	-F	CTCTACGGAACGGAGCTGTC	50	04170
	R	CAGGGTCGACCAGTCACATA		

Table 4 Summary of Nucleotide variation and Neutrality test

Locus	Population	Number of Sequence	Analyzed Sites	Segregating sites	θ_w (%)	π (%)	Tajima's *D* values
O8	CMI	20	412	3	0.205	0.255	0.6539
	KAN	20	412	0	0	0	0.0000
	TAK	20	412	13	0.889	0.669	-0.8973
	SRT	20	412	8	0.547	0.441	-0.6564
	CTB	20	412	8	0.514	0.511	-0.2237
O10	CMI	20	609	6	0.278	0.48	2.3174*
	KAN	20	608	4	0.185	0.315	2.0241*
	TAK	20	609	16	0.741	0.459	-1.4135
	SRT	20	609	13	0.602	0.411	-1.1526
	CTB	20	609	16	0.741	0.79	0.2477
P9	CMI	20	594	3	0.142	0.266	2.3163*
	KAN	20	594	5	0.237	0.386	1.9223
	TAK	20	594	15	0.712	0.454	-1.3383
	SRT	20	594	13	0.617	0.363	-1.4912
	CTB	20	594	11	0.522	0.64	0.8001
P22	CMI	20	384	8	0.587	0.685	0.5629
	KAN	20	385	2	0.146	0.052	-1.5128
	TAK	20	382	15	1.107	0.828	-0.9294
	SRT	20	384	13	0.954	1.044	0.3427
	CTB	20	385	9	0.659	0.727	0.3568

Table 5 Estimates of haplotype diversity, LD and recombination parameters

Locus	Population	Haplotype diversity	R_m #	Z_{ns} &
O8	CMI	0.726	1	0.093
	KAN	0	na	na
	TAK	0.932	4	0.083
	SRT	0.911	1	0.042
	CTB	0.895	3	0.048
	All	0.84	6	0.024
O10	CMI	0.626	0	0.759
	KAN	0.647	0	0.625
	TAK	0.968	2	0.102
	SRT	0.879	2	0.071
	CTB	0.937	5	0.288
	All	0.925	8	0.063
P9	CMI	0.526	0	1.000
	KAN	0.611	0	0.644
	TAK	0.986	1	0.074
	SRT	0.921	2	0.049
	CTB	0.937	6	0.170
	All	0.939	7	0.056
P22	CMI	0.895	2	0.153
	KAN	0.1	0	na
	TAK	0.958	1	0.101
	SRT	0.937	3	0.241
	CTB	0.926	3	0.155
	All	0.920	8	0.035

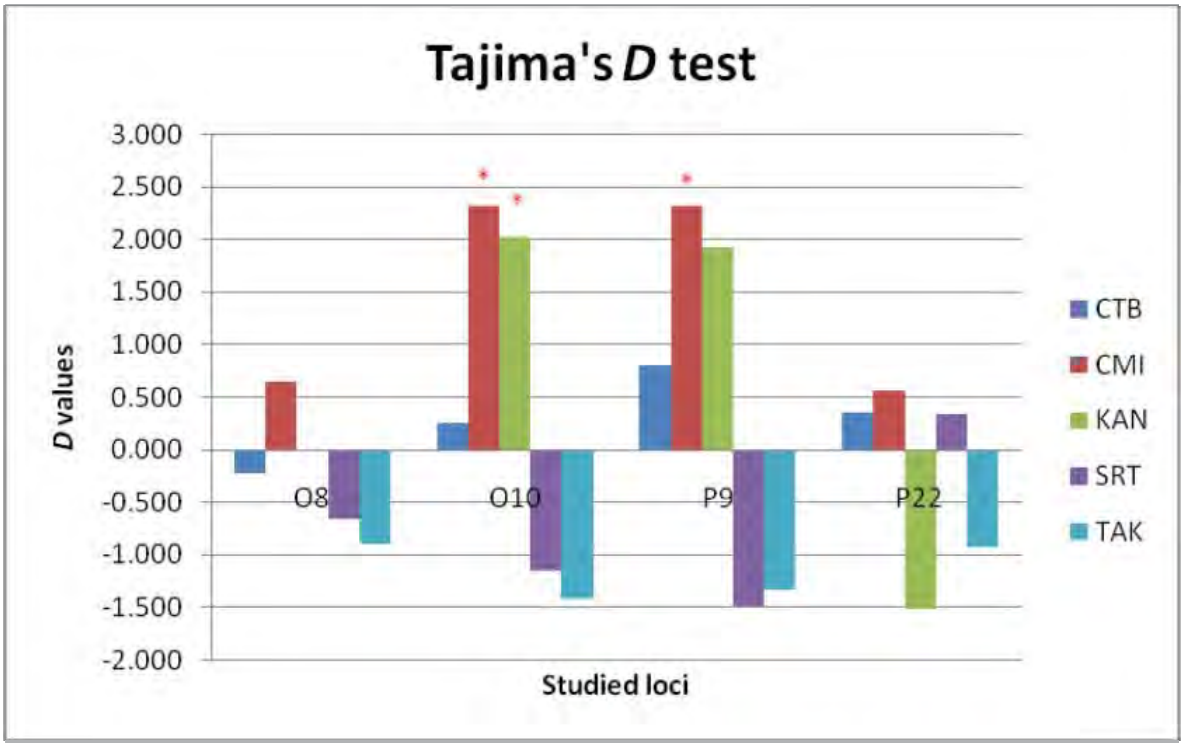

Figure 1 Tajima's *D* values of 5 populations (CTB, CMI, KAN, SRT and TAK) at four studied loci (O8, O10, P9, and P22). "*" indicates a statistically significant departure from neutral expectation (* $P < 0.05$ based on 10,000 coalescent simulations)

Part II

Population genetic study of malaria vector *Anopheles harrisoni* in Thailand

Abstract *Anopleles harrisoni* is one of main malaria vectors in Southeast Asia. In Thailand, *An. harrisoni* is widespread in Sai Yok district, Kanchanaburi province. Investigating pattern and level of genetic diversity is useful for understanding of evolutionary history of this malaria in order to achieve the efficient vector control program. In this study, five putative neutral fragments locating on X chromosome and one fragment on autosome were analyzed using single nucleotide polymorphism data in two populations from Kanchanaburi Province and one population from north-western Vietnam of *An. harrisoni*. This study exhibited low level of nucleotide diversity for all three *An. harrisoni* populations. There is no population differentiation between two mosquito populations from Kanchanaburi Province. Moreover, the statistical tests of neutrality indicated no departure from neutral equilibrium, suggesting that mutation-random genetic drift equilibrium is the cause of observed polymorphisms in the studied populations.

Keywords: *Anopheles harrisoni*, Genetic diversity, Single nucleotide polymorphisms

Introduction

Anopheles harrisoni (former *An. minimus* C) is one of the Minimus Complex species, which is recognized as main malaria vectors in Southeast Asia (Garros et al. 2006, Manguin et al. 2008). In Thailand, Thai-Myanmar and Thai-Cambodia border are still highly malaria transmission areas (WHO 2010). *An. harrisoni* is prevalent in Kanchanaburi province (Poolprasert et al. 2008), and have been found in a large sympatric area with the sibling species, *An. minimus* (former *An. minimus* A) (Manguin et al. 2008). In addition to *An. yaeyamaensis* is formally included in the Minimus Complex (Harbach, 2004). *An. minimus* and *An. harrisoni* are recognized as main malaria vectors in the mainland of the Oriental regions, while *An. yaeyamaensis* can be found only in Ishigaki Island in the Ryukyu Archipelago of Japan, which is a non-malarial area (Manguin et al.

2008). *An. harrisoni* can be found in Vietnam, China, Myanmar, Laos and Thailand, while *An. minimus* can be found in India, Bangladesh, Myanmar, Thailand, Laos, China, and Vietnam. In Thailand, *An. harrisoni* is widespread in 3 provinces; including Kanchnaburi province (Sharpe *et al.*, 1999; Sharpe *et al.*, 2000; Rwegoshora *et al.*, 2002; Kengluecha *et al.*, 2005; Sungvornyothin *et al.*, 2006a; Poolprasert *et al.*, 2008), Tak province (Green *et al.*, 1990; Rattanarithikul *et al.*, 2006), and Chiang Mai province (Sharpe *et al.*, 1999; Sharpe *et al.*, 2000; Rattanarithikul *et al.*, 2006), while *An. minimus* can be found throughout Thailand (Green *et al.*, 1990; Rwegoshora *et al.*, 2002; Rattanarithikul *et al.*, 2006).

Single Nucleotide Polymorphism (SNP) is base substitution or changes in single nucleotide (point mutation) in DNA sequence (Vignal *et al.*, 2002; Halliburton, 2004), occurring by chance (Smith, 2002). SNP is the most common variation at the DNA level (Halliburton, 2004). SNPs are widespread in many regions including non-coding and coding regions (Smith, 2002; Morin *et al.*, 2004). Most SNPs are located in non-coding regions including regulatory regions (Kohn *et al.*, 2006; Jarukamjorn and Jatupornprasert, 2007). Most of SNPs are simple bi-allelic, because the mutation rates of base substitutions show the low frequency (about 10^{-8}-10^{-9}). The probability of point mutation taking place at former position is very low (Vignal *et al.*, 2002; Brumfield *et al.*, 2003). In addition to the tri-allelic SNPs can be found in low frequency (less than 0.1%) (Lai, 2001; Petkovski *et al.*, 2003). Sometimes, one base pair indels (insertions or deletions) are also assumed as the SNPs (Vignal *et al.*, 2002). For average SNP frequencies of diverse taxa, SNPs identifying from a non-coding regions (ncSNPs) can be found every 200-500 base pairs and SNPs identifying from coding regions (cSNPs) can be found every 500-1000 base pairs (Brumfield *et al.*, 2003). Moreover, SNPs can be found in diverse organisms, such as vertebrates, invertebrates and plants (Rafalski, 2002; Andersen *et al.*, 2006; Cohuet *et al.*, 2008). The advance of DNA sequencing technology (fast and cheap) generating abundant sequence information especially SNPs databases is one of many choice for using SNPs (DNA sequencing) (Halliburton, 2004; Morin *et al.*, 2004; Black IV and Vontas, 2007; Lister *et al.*, 2008) which direct sequencing is the simplest process for SNP discovery (Rafalski, 2002). SNPs are considered as an ideal genetic marker (Vignal *et al.*, 2002; Morin *et al.*, 2004), which is extensively used in many research fields, such as human (Guo and Jamison, 2005), wild populations of the endangered Italian wolf (*Canis lupus*) (Andersen *et al.*, 2006; Seddon *et al.*, 2005), immune related genes and randomly chosen genes in *An. gambiae* (Cohuet *et al.*, 2008), crops (corn and soybean) (Rafalski, 2002; Zhu *et al.*, 2003), fungus (Xu *et al.*,

2007), cultivated species (citrus) (Novelli *et al.*, 2004), vertebrates (bird, whale and cattle) (Heaton *et al.*, 2002; Cramer *et al.*, 2008) and insects (Black IV and Vontas, 2007).

Using insecticide is one efficient method for malaria vector controls, however, the issue of insecticide resistance is considerably increasing (WHO, 2009). Thus, the study of genetic variation and vector species distribution is necessary for understanding of evolutionary history that may involve insecticide resistance of the malaria vectors, in order to achieve the efficient vector control program. Presently, most genetic information of the Minimus Complex has been reported on *An. minimus*, (Green *et al.*, 1990, Van Bortel *et al.*, 1999, Van Bortel *et al.*, 2003, Boonsuepsakul *et al.*, 2005, Poolprasert et al., 2008, Dixit *et al.*, 2011). Despite *An. harrisoni* is also defined as main malaria vector, the genetic information of this vector species is still very limited, particularly in Thailand. Therefore, the objective of this study is to evaluate level of genetic diversity of *An. harrisoni* population in Kanchanaburi province where *An. harrisoni* is mostly widespread using single nucleotide polymorphism as a marker to understand the evolutionary background of this malaria vector species.

Materials and Methods

Collection of mosquitoes and species identification

Adult *Anopheles* mosquitoes were collected by human-landing and cattle-bait collection methods from Pu Teuy village (14º17'N, 99º11'E) (KPT, 10 samples) and Bong Ti Noi village (14º15'53.2"N, 98º55'52.8"E) (KBT, 2 samples) in Sai Yok district, Kanchanaburi province, and samples from Khoi village, Hoa Binh province, Vietnam (20º38'11.1"N, 105º09'58.4"E) (VHB, 2 samples) providing by Dr. Sylvie Manguin from University Montpellier I, Faculté de Pharmacie, Montpellier, France were also utilized for comparison. *An. harrisoni* specimens were identified by using morphological keys (Rattanarithikul, 2006). Molecular identification was used to confirm the species identification as following Garros *et al.* (2004).

DNA isolation, PCR amplification and sequencing

Genomic DNA was individually extracted from adult female mosquitoes using the Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan). The primers were developed from intron flanking-region of gene using EPIC (Exon-Primed Intron-Crossing) method (Bierne *et al.*, 2000). The information of gene fragments, primers, the sequence of primers and their annealing temperature are shown in Table 1. PCR amplifications were performed in a final volume of 30 µl, containing approximately

20-50 ηg of genomic DNA, 0.02 units of Taq polymerase (Vivantis), 0.83X PCR buffer (MgCl2 free) (Vivantis), 0.83 mM MgCl2 (Vivantis), 0.4 mM of dNTPs (Eppendorf) and 0.4 pmol of each primer. PCR reactions were carried out using the following cycling profile: 95 ºC for 5 min; 30 cycles of 95 ºC for 30 s, 50-60 ºC for 40 s (annealing temperatures are provided in Table 1), 72 ºC for 1 min; followed by a final extension at 72 ºC for 8 min. PCR products were purified using Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas). The purified PCR products were directly sequenced in both directions using forward- and reverse-specific primers by Macrogen Inc. (Korea). Forward and reverse sequences of each gene fragment were compared and validated with *Anopheles* species using nucleotide BLAST program from the NCBI database.

nucleotide sequences were visually checked between base-calling and chromatograms. Homozygotes and heterozygotes were visually analyzed from the chromatograms. One fluorescent peak pattern was defined as homozygous sequences, by the way, two peaks at a single position in the chromatograms was determined as heterozygous sequences. Sequences were aligned and edited using BioEdit version 7.0.5.3 (Hall, 1999).

Data analysis

Data analyses and molecular population genetic tests were performed using DnaSP version 5.00.07 (Librado and Rozas, 2009). Nucleotide diversity was estimated based on θ_w (Watterson, 1975) and π (Nei, 1987). For test of neutrality, Tajima's *D* (Tajima, 1989) was calculated for each locus, that is a statistical test for discrimination between patterns of diversity evolving random (mutation and random genetic drift; neutrality) and non-random process (e.g. selection, demography), Tajima's *D* statistic measures skews in the frequency spectrum based on π and θ_w parameters. Under neutral evolution, Tajima's *D* value should be close to zero; a negative *D* value indicates an excess of low frequency polymorphisms, and a positive *D* value indicate an excess of intermediate-frequency polymorphisms. The other tests of neutrality were also utilized, e.g. Fu and Li's *D* and *D** statistics (D_{FL} and D^*_{FL}, respectively) (Fu and Li, 1993) as well as Fay and Wu's *H* statistic (Fay and Wu, 2000). The test of Fu and Li is based on comparing the distributions of the mutations between the external and internal branches in the genealogy of a random sample of genes from the population. The excess of mutations in the external branches indicates the presence of purifying or negative selection, while the excess of mutations in the internal branches indicates the presence of balancing (overdominant) selection. Fay and Wu's *H* statistic measures

the differences between θ_H and π. The estimator θ_H is calculated from the frequency of derived segregating sites. Under neutrality the difference between θ_H and π is zero, but negative H value indicates the excess of high frequency variants, assuming the presence of hitchhiking effects (positive selection). The coalescent simulations were used to ascertain the statistical significance of this test, using the segregating sites, no recombination, and 10,000 replications. An outgroup was utilized to estimate the derived state of polymorphism data, using the homologous sequences of *An. gambiae* (accession no. AAAB01008963.1 and EU104640.1). Differentiation among subpopulations was calculated by estimation of the average degrees of gene flow from DNA sequence data which is based on *F* coefficient; F_{ST} (Hudson *et al.*, 1992). The nearest-neighbor statistic (S_{nn}) statistic (Hudson, 2000) was also utilized for estimating differentiation among subpopulations. The permutation test with 10,000 random was utilized to estimate the *p*-value of S_{nn} statistic.

Results and Discussion

Molecular identification of *Anopheles* mosquitoes from each locality was performed. Samples of *An. harrisoni* were precisely distinguished from *An. minimus* based on AS-PCR technique. The results showed that most of morphological identifications of *An. harrisoni* were misidentification. Thus, molecular identification is required for precise classification of mosquito species complex.

Sequence Polymorphism

The intraspecific nucleotide diversity of *An. harrisoni* from KPT, KBT and VHB populations was determined from non-coding regions. A total of 72,016 nucleotides across 6 non-coding fragments were analyzed. The total length of the studied loci ranges from 245 bp to 609 bp. A total of 34 polymorphic sites along 6 fragment regions were identified. The polymorphic sites including 31 single nucleotide substitutions (SNPs) and three insertion/deletion polymorphisms (indels) were observed. All studied fragments showed probable low level of nucleotide polymorphism (Table 2). The highest nucleotide variation was found in locus P12, while no polymorphism was detected in locus ITS2. These results were consistent with the nucleotide diversity studying in the both forms of *An. gambiae* and *An. arabiensis* (Stump et al., 2005) as well as *An. minimus* (Dixit et al., 2011). The fragments P9 and P22 locate near the centromere on the X chromosome, which could imply low recombination in this region. Nucleotide diversity at locus P12 was small, in spite of this locus

locates distal the centromere on the X chromosome. Moreover, low genetic diversity was also detected at fragment O8 and O10 which locate distal and near the centromere on the X chromosome, respectively. This result suggested the low genetic diversity in studied *An. harrisoni*, due to low recombination event and perhaps small population size since they have been found in restricted area.

Test of neutrality

None loci showed statistically significant Tajima's D value, suggesting these studied fragments are under a standard neutral equilibrium. For other tests of neutrality, Fu and Li' D and D* statistic tests of all populations and loci do not show significant departures from neutral equilibrium expectations. And the H test of Fay and Wu do not show significant departures from neutral evolutions, suggesting no signal of selective sweep in all populations (Table 2).

Population differentiation

Estimations of the standard F_{ST} statistic based on Hudson (1992) and Hudson's S_{nn} statistic (2000) across 6 loci in 3 populations were performed. Results of the standard F_{ST} statistic and S_{nn} statistic are shown in Table 3.

The result of population differentiation across 5 loci in 3 populations of *An. harrisoni* showed significant genetic differentiations at all loci (excluding locus ITS2). The average FST estimate was 0.37651, indicating high genetic variation among these populations when compared with others *Anopheles* species (Rongnoparut et al., 1999; Van Bortel et al., 2003; Stump et al., 2005; Poolprasert et al., 2008). In addition, average pairwise estimated population differentiation across 5 loci in 3 populations was also calculated and results are shown in Table 4.

The result of average pairwise estimated population differentiation across 5 loci in 3 populations of *An. harrisoni* showed that no genetic differentiation was observed among 2 populations from Kanchanaburi Province, Thailand (KPT and KBT). Genetic differentiations between VHB and KPT, as well as VHB and KBT were 0.382 and 0.349, respectively. Genetic differentiation between Vietnam and Thailand populations is relatively high, which is most likely due to geographic distance between Vietnam and Thailand.

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Table 1. The information of primers, the sequence of primers and their annealing temperature.

Gene (Primer name)	Gene details			Details of analyzed fragments		Reference
	Location	Putative function	Region	Sequences (5' to 3')	Annealing temp.	
CYP (P9)	X chromosome	Metabolism of xenobiotics	Non-coding	F: TGGAAAGTAGGGGCAATCAG R: GCTGGGCAACGGACTACTTA	51 ºC	Stump et al. (2005)
INPP4 (P12)	X chromosome	Inositol phosphate metabolism	Non-coding	F: TTTACGTCGTGCAAGAGTGC R: GCCCACGTTCTTCATCGTAT	51 ºC	Stump et al. (2005)
CYP (P22)	X chromosome	Metabolism of xenobiotics	Non-coding	F: CTCTACGGAACGGAGCTGTC R: CAGGGTCGACCAGTCACATA	51 ºC	Stump et al. (2005)
Gbb-60A (O8)	X chromosome	Signaling proteins, differentiation	Non-coding	F: CGGCCATTGGGCGGACATTT R: ACGCATCTCCGATCCGTGATGATC	60 ºC	Dixit et al. (unpublished data)
CYP (O10)	X chromosome	Metabolism of xenobiotics	Non-coding	F: CTGGCTGGGCAACGGACTA R: GGAAAGTAGGGGCAATCAGTTTG	58 ºC	Dixit et al. (2011)
ITS2 (ITS2)	Nuclear genome	No function	Non-coding	F: TGTGAACTGCAGGACACAT R: TATGCTTAAATTCAGGGGGT	50 ºC	Garros et al. (2004)

CYP, Cytochrome P450; *INPP4*, Inositol polyphosphate 4-phosphatase; *Gbb-60A*, Glass bottom boat 60A; *ITS2*, Ribosomal internal transcribed spacer 2.

Table 2. Information of data analyses of *An. harrisoni* from each population using multilocus DNA variation.

Loci	KPT population						KBT population						VHB population					
	θ_w	π	*D*	D_{FL}	D^*_{FL}	*H*	θ_w	π	*D*	D_{FL}	D^*_{FL}	*H*	θ_w	π	*D*	D_{FL}	D^*_{FL}	*H*
P9	0.14	0.17	0.55	1.01	-0.12	-2.58	0.18	0.22	1.89	1.44	1.89	0.00	0.27	0.28	-0.06	-0.91	0.16	0.33
P12	0.34	0.12	-1.72	-2.17	-2.38	0.18	0.67	0.62	-0.75	-1.20	-0.75	0.66	0.44	0.40	-0.70	-1.20	-0.70	0.66
P22	0.43	0.36	-0.54	0.12	0.54	-0.35	-	-	-	-	-	-	-	-	-	-	-	-
O8	0.12	0.15	0.61	0.85	0.86	0.47	0	0	-	-	-	-	0.11	0.10	-0.61	1.09	-0.61	1.00
O10	0.04	0.08	1.56	0.62	0.64	0.00	0.18	0.19	0.59	1.44	0.59	-1.00	0.26	0.27	0.16	-0.36	0.16	0.66
ITS2	0	0	-	-	-	-	0	0	-	-	-	-	-	-	-	-	-	-
Mean	0.18	0.15					0.20	0.20					0.27	0.26				

KPT: Pu Teuy village, Sai Yok district, Kanchanaburi province; KBT: Bong Ti Noi village, Sai Yok district, Kanchanaburi province; VHB: Khoi village, Hoa Binh province, Vietnam; Nucleotide diversity (percentage scale), θ_w (Watterson, 1975) and π (Nei, 1987); *D*: Tajima's *D* statistics (Tajima, 1989); D_{FL}, D^*_{FL}: Fu and Li's D and D* statistics, respectively; *H*: Fay and Wu's H statistic (Fay and Wu, 2000).

Table 3. Population differentiation across 5 loci (3 populations) of *An. harrisoni*.

Locus	F_{ST} [a]	S_{nn} [b]	*p*-value of S_{nn}
P9	0.2484	0.6298	0.0162*
P12	0.5836	0.7218	0.0004***
O8	0.6613	0.6285	0.0275*
O10	0.3891	0.6975	0.0012*
ITS2	0.0000	0.7048	1.0000[ns]
Average	0.37651	0.53555	

[a] F_{ST} statistic based on Hudson (1992); [b] S_{nn} statistic based on Hudson et al. (2000); ns, not significant; *, 0.01< P <0.05; ***, P <0.001; No data for locus P22.

Table 4. Average pairwise estimated population differentiation across 5 loci in 3 populations of *An. harrisoni*.

Population	KPT	KBT	VHB
KPT		0	0.382
KBT			0.349
VHB			

Negative F_{ST} were set to zero; KPT: Pu Teuy village, Sai Yok district, Kanchanaburi province; KBT: Bong Ti Noi village, Sai Yok district, Kanchanaburi province; VHB: Khoi village, Hoa Binh province, Vietnam.

Part III

Phylogenetic reconstruction of malaria vectors in Thailand using multilocus DNA sequencing

Abstract Malaria is spread through mosquitoes, belonging to the *Anopheles* genus. Understanding the evolutionary and taxonomic status of closely related malaria vector species is the initial step in a malaria vector control program. In this study, four different approaches (neighbor joining, minimum evolution, maximum likelihood and maximum parsimony) were performed to reconstruct phylogenetic trees for the main malaria vectors which have presented in Thailand based on the sequence information of six DNA fragments from both the nuclear and mitochondrial regions. The results revealed clear evidence that *Anopheles* species separate into three distinct clades: Dirus group, Minimus group and Maculatus group. Interestingly, phylogenetic trees based on different reconstructed algorithms and different gene regions provided congruent phylogenetic status of the mosquito species studied. The phylogenetic relationships of these malaria vector species follow a pattern based on morphological identification. Moreover, estimation of the divergence time among the studied species inferred that *Anopheles* species probably existed around the Eocene and Miocene periods (later than 41 million years ago).

KEY WORDS Phylogenetic inference, Thai *Anopheles* species, multilocus DNA sequence

Introduction

Understanding the evolutionary and taxonomic status of closely related malaria vector species is the initial stage in malaria vector control program. This information may help for further studies and better understanding in the capacity to transmit malaria, genetically determined ecological and evolutionary of genes in insecticide resistance for management of malaria vector control. Mosquito-borne diseases are still a major health problem in many countries (Mehlotra and Zimmerman 2006, Dixit et al. 2010). Thailand is one of the countries in tropical region where malaria is prevalent (WHO, 2008). Malaria is spread through mosquitoes, belonging to *Anopheles* genus (Cann, 1996; Rich and Ayala, 2006). The ability of transmit malaria varies within members of this genus, including innate susceptibility to *Plasmodium*, host choice and longevity (CDC, 2010). Moreover, the vectorial capacity of *Anopheles* mosquitoes involved in malaria transmission remains poorly understood in Thailand.

Anopheles species mostly exist in temperate, subtropical and tropical areas of the world. They can be found in different geographies from hilly to coastal areas (Krzywinski and Besansky, 2003; Harbach, 2011). Most *Anopheles* mosquitoes are species complex which is difficult to distinguish from morphological characteristics (Manguin et al. 2008). The Dirus Complex occurs in tropical evergreen rainforest, cultivated forests fringes along Southeast Asia, and five Dirus mosquito species are found in Thailand including *An. baimaii*, *An. cracens*, *An. dirus*, *An. nemophilous and An. scanloni* (Manguin et al. 2008). Within the Minimus complex, *An. minimus* and *An. harrisoni* can be found in large sympatric areas in the Oriental Region (Manguin et al. 2008). Moreover, *An. aconitus*, *An. varuna*, and *An. pampanai* are considered as the closely related species of the Minimus complex due to ambiguity of overlapping morphological characters (Manguin et al. 2008). The Sundaicus Complex consists of at least four distinct species, including *An. sundaicus* s.s., *An. epiroticus*, *An. sundaicus* D, and *An. sundaicus* E (Dusfour et al. 2007, Manguin et al. 2008). In Thailand, *An. epiroticus* is predominant species in the Sundaicus Complex (Dusfour et al. 2007, Manguin et al. 2008, Sumruayphol et al. 2010) and could transmit malaria in coastal areas (Sumruayphol et al. 2010). Five species of the Maculatus Group have distributed in Thailand, *i.e. An. pseudowillmori*, *An. willmori*, *An. maculatus*, *An. sawadwongporni* and *An. notanandai* (Manguin et al. 2008). Six *Anopheles* mosquitoes are considered as malaria vectors in Thailand. Three species of them have been examined as primary vectors, *i.e. An. dirus*, *An. minimus*, and *An. maculates*, and the remains have been considered as secondary vectors, *i.e. An. aconitus*, *An. sundaicus*, and *An. pseudowillmori* (Ministry of Public Health, 2008).

Most of the taxonomic relationships of mosquito species in the previous studies have been identified based on the morphological characters. However, these taxonomies showed inconsistent and ambiguous relationships among the species. Nowadays, molecular phylogenetic relationships based on DNA and protein sequences comparative can be generated more simple and reliable than only morphological data. For example, the results of morphological and molecular studies (based on ribosomal D3 and mitochondrial COII fragments) of the Afrotropical Funestus and Afro-Oriental Minimus groups were accordance to separate *Anopheles* species into five clades (the Aconitus, Culicifacies, Funestus, Minimus, and Rivulorum subgroups) (Garros et al. 2005a). Phylogenetic inferences of Indian malaria vectors were investigated based on multilocus DNA sequences. The results showed that the tree topologies of COII and ITS2 were congruent, and the estimated divergence time supports the hypothesis of the species radiation of the *Anopheles* genus during the late Cretaceous period (Dixit et al. 2010). Moreover, accurate species identification is particularly important in vector-

borne disease studies. Thus, ambiguous morphological identification between the Funestus group in the Myzomyia Series and the Annularis group in the Neocellia Series were addressed by developing a multiplex-PCR assay. The molecular phylogenetic relationships was consistent with the morphological taxonomy inferring that these species were separated into two distinct monophyletic clades; Myzomyia and Neocellia Series (Swain et al. 2010). The phylogenetic study of the Minimus group confirmed that *An. minimus* in Thailand consists of at least two cryptic species besides species A and C. The divergence time between *An. minimus* A and species C based on the mitochondrial COII and ITS2 regions was estimated to be approximately 0.57 to 1.5 million years. The estimated effective population size suggested that the distribution of *An. minimus* species C was probably larger than had been recognized to date (Sharpe et al. 2000).

Investigations of the phylogenetic relationships among *Anopheles* mosquitoes based on morphological and molecular characteristics of malaria mosquitoes in Thailand is still limited and not covered the whole major malaria vector species. Moreover, most of the phylogenetic studies on *Anopheles* species rely on single locus for the phylogenetic construction. In order to obtain precise phylogenetic tree of *Anopheles* species, applying a multilocus approach with fragments of different functions and chromosomal locations has its advantage. Thus, the purposes of this study are (1) to reconstruct phylogenetic trees among Thai malaria vectors by utilizing multilocus DNA sequences based on both nuclear and mitochondrial regions, and (2) to estimate the divergence time among malaria vectors and related species distributing in Thailand.

Materials and Methods

Mosquito samples

The *Cellia* subgenus of *Anopheles* mosquitoes analyzed for the phylogenetic relationships were focused on malaria vectors presented in Thailand. Taxonomic information of the mosquito species is shown in Table 1.

Retrieval of DNA sequence from database and primer design

Six partial DNA fragments were inclusively chosen from different genome regions, *i.e.* mitochondrial and nuclear genomes, and different DNA regions *i.e.* coding and non-coding regions, and different chromosomal locations, *i.e.* autosome and X-chromosome to investigate the phylogenetic relationships of Thai malaria vectors. The details of DNA fragments used are shown in Table 2.

The published partial sequences of the mitochondrial *COI*, *COII* genes and ITS2 of *rRNA* gene of *Anopheles* mosquitoes widespread in Thailand were retrieved from NCBI database (www.ncbi.nlm.nih.gov). While sequence information of mitochondrial *COI* genes of *An. sawadwongporni* and *COII* gene of *An. epiroticus* is not available, the primers were designed to amplify the partial sequences of these genes in both species. For the *COI* gene, primers were designed based on DNA sequence of the relative species *An. maculates* (accession no: GQ259192.1, EU256336.1, and DQ267690.1). For mitochondrial *COII* gene, primers were designed from full length of this gene. The forward and reverse primers were located on the flanking-regions of the *COII* gene in *Anopheles* mosquitoes which are tRNA-Leu and tRNA-Lys genes, respectively. The complete mitochondrial DNA sequence information of *Anopheles* species were downloaded from NCBI web database. For ITS2 of *rRNA* gene, most of published partial ITS2 sequences of *Anopheles* mosquitoes were retrieved from the NCBI database, excluding *An. scanloni*. The accession numbers of the *Anopheles* species are presented in Table 3

In this study, published DNA sequences of the partial exon and complete coding sequence (cds) of *TOLL6* gene from the whole genome sequence of *An. gambiae* was retrieved and downloaded from NCBI web database for primers design. The accession numbers of *TOLL6* gene of *An. gambiae* are as follows: AM776293.1, AM776294.1, AF444781.1, and XM_320172.2. Moreover, primers were developed from intron flanking-region of Gbb-60A by Stump *et al.* (2005) to amplify Gbb-60A fragment, and primers for CYP fragment amplification were designed by Dixit et al. (personal communication) using

Exon-Primed Intron-Crossing method (Bierne et al. 2000). Information of analyzed DNA fragments and primer sequences are shown in Table 4. The partial sequences of outgroup species were retrieved from database for comparison as shown in Table 5.

Genomic DNA extraction and molecular identification

Genomic DNA of mosquito samples was extracted by using the modification of Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan). Each DNA sample was separated on 1.0% agarose gel (Vivantis, California) in 0.5X TAE buffer (Vivantis, California) for quality and concentration examination, and 1 Kb ladder (Vivantis, California) was use as standard marker. Agarose gel was strained with ethidium bromide and the DNA bands were visualized under ultraviolet light. Subsequently, multiplex allele-specific polymerase chain reaction (AS-PCR) assay was used to identify and confirm species as following Walton et al. (1999), Garros et al. (2004) and Dusfour et al. (2007).

PCR amplification, DNA sequencing and alignment

PCR amplification was performed in 30 µl volume, containing approximately 20-50 ηg of genomic DNA, 0.02 units of Taq polymerase (Vivantis), 0.83X PCR buffer (MgCl2 free) (Vivantis), 0.83 mM MgCl2 (Vivantis), 0.4 mM of dNTPs (Eppendorf) and 0.4 pmol of each primer. The primer sequences and their annealing temperature are given in Table 4. PCR reactions were carried out using the following cycling profile: 95 ºC for 5 min; 30 cycles of 95 ºC for 30 s, annealing temperature for 40 s, 72 ºC for 1 min; followed by a final extension at 72 ºC for 8 min. Three microliters of PCR products were separated on 1% agarose gel in 0.5X TAE buffer (Vivantis, California) and 100 bp ladder (SibEnzyme, Russia) was use as standard marker. Agarose gel electrophoresis was performed for 25 minutes at 100 volts. Gel was strained with ethidium bromide and the PCR product bands were visualized under ultraviolet light. PCR products were purified; the excess primers were digested with Exonuclease I and the dNTPs with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, Ontario). The purified PCR products were directly sequenced in both directions using forward- and reverse-specific primers by Macrogen Inc. (Korea). The nucleotide sequences were manually checked for accuracy between base-calling and chromatograms before editing. Homozygotes and heterozygotes were visually analyzed from the chromatograms. One fluorescent peak pattern was defined as homozygous sequences, by the way, two peaks at a single position in the chromatograms was determined as heterozygous sequences. Nucleotide sequences were aligned and manually edited using BioEdit version 7.0.5.3 computer programs (Hall, 1999).

Phylogenetic analysis and divergence time estimates

Frequency of Nucleotide compositions were obtained by MEGA version 5 computer program (Tamura et al. 2011). Measure for substitution saturation of nucleotides was implemented in DAMBE version 5.2.57computer program (Xia and Xie 2001), tested by Xia's (Xia et al. 2003) and Steel's method (Steel et al. 1993). Transition and transversion substitutions per site were plotted against the evolutionary distance based on Kimura's two parameter substitution model by using DAMBE version 5.2.57computer program (Xia and Xie 2001). When both the transitions and transversions increased with increasing the evolutionary distance indicated that no saturation of substitution is presented.

The mitochondrial coding regions, COII and COII sequences were translated into protein for avoidance of pseudogenes, using MEGA version 5 computer program (Tamura et al. 2011) with the invertebrate mitochondrial genetic code.

For each DNA fragments, four different approaches of phylogenetic tree construction, *i.e.* neighbor joining (NJ), minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) were performed using MEGA version 5 computer program (Tamura et al. 2011). The appropriate nucleotide substitution model for maximum likelihood analysis was performed; the lowest Bayesian Information Criterion (BIC) (Schwarz 1978) value of model tests was considered as the best substitution model. Four independent phylogenetic trees per approaches were constructed supporting with bootstrap methods (10,000 replications). Each method was performed using the heuristic search. Gap positions in aligned sequences were considered as missing data.

Estimation of divergence time, the relative rate test (Tajima 1993) based on the molecular evolutionary clock hypothesis (Morgan 1998) which evolves at a constant rate of gene was firstly implemented in MEGA version 5 computer program (Tamura et al. 2011). The divergence time between mosquito lineages were then estimated using the Bayesian MCMC analyses which is mcmctree program as implemented in PAML version 4.4 computer program (Yang, 2007). The fossil calibration information is required, when the fossil record provides a poor estimate of the divergence and of the actual ages of the taxa in the family Culicidae (Poinar et al. 2000; Harbach, 2007). The estimation of divergence time points of mosquito mtDNA genomes between *Anopheles* and *Aedes* (approximately 145 to 200 million years ago: mya) (Krzywinski *et al.*, 2006) and between *An. minimus* and *An. harrisoni* (approximately 1.1 to 1.3 mya) (Garros *et al.*, 2005b) were utilized to estimate the splits between lineages within subgenus *Cellia*. The most complex nucleotide substitution model of HKY 85, the 10,000

burning periods, the 100,000 numbers of samples, and the 5 sample frequencies were set for computer simulations and performed with four independent analyses.

Results

Nucleotide compositions

Most of the nucleotide sequences of COI, COII, and ITS2 fragments were retrieved from GenBank and some sequences were newly sequenced. Nucleotide compositions of each DNA fragment were presented in Table 6. The results of average base compositions showed that studied mitochondrial DNA fragments (COI and COII) were Adenine Thymine (A-T) rich, while nucleotide compositions of nuclear fragments (O8, P9, TOLL6 and ITS2 fragments) exhibited approximately equal of nucleotide compositions.

Tests of substitution saturation and nucleotide substitution model

Strong substitution saturation (too diverged sequence) is mainly causes the accuracy of phylogenetic reconstruction, reducing phylogentic information contained in sequences (Xia and Lemey 2009). In this study, substitution saturation of sequences was assessed by using Xia's method (Xia et al. 2003) and Steel's method (Steel et al. 1993) as implemented in DAMBE version 5.2.57computer program (Xia and Xie 2001). Concerning the expectation of nucleotide substitution rates among coding and noncoding sequences is different. In coding sequences, nucleotide positions are evolved at different rate; the third codon position is more variable than at the first or second positions (Halliburton 2004). Thus, coding and noncoding sequences were separately considered. The results of substitution saturation test of each fragment indicated little nucleotide substitution saturation meaning that these fragments can be utilized for phylogenetic relationships analysis. Furthermore, the most appropriate substitution model was carried out. The information of selected models from each fragment is provided in Table 7.

Phylogenetic inference with multilocus DNA sequences

Phylogenetic trees were constructed with four different methods based on five partial fragments by heuristic searches using MEGA computer program (Tamura et al. 2011). Phylogenetic relationships based on morphological characters were utilized to compare with molecular relationships in this study. Phylogenetic trees based on four methods from each fragment showed slightly different tree topologies.

Phylogenetic analysis of mitochondrial genes (COI and COII)

Results of the Maximum parsimony topologies based on mitochondrial genome, COI and COII genes are shown in Fig. 1. Phylogenetic analysis of mitochondrial COI fragment (Fig. 1(a)) divided tree into three main clusters. First cluster is monophyly of the Neomyzomyia cluster (*An. dirus*, *An. baimaii* and *An. scanloni*). Second cluster is paraphyletic of Neocellia (*An. maculatus* and *An. sawadwongporni*) cluster relating to the Neomyzomyia cluster, but branch support is poor. Third cluster is the Myzomyia cluster. *An. pampanai* showed improper arrangement from the Myzomyia cluster, and *An. epiroticus* is the basal species of this topology. Moreover, phylogenetic analysis of mitochondrial COII fragment (Fig. 1(b)) separated groups into three clusters. First cluster contains monophyletic group of the Myzomyia Series (*An. minimus*, *An. harrisoni* and *An. pampanai* and *An. aconitus*). Second cluster contains the Pyretophorus (*An. epiroticus*) and Neocellia (*An. maculatus* and *An. sawadwongporni*) Series. Third cluster is basal cluster of the Neomyzomyia cluster (*An. dirus*, *An. baimaii* and *An. scanloni*).

Phylogenetic analysis of nuclear fragments (P9, ITS2, and TOLL6)

Nucleotide sequences of the P9 fragment were newly sequenced from 7 *Anopheles* species. Sequence of *Drosophila melanogaster* was used as outgroup. Result of MP analysis (Fig. 2(a)) separated investigated species into three clusters. First cluster is the Myzomyia cluster (*An. minimus*, *An. harrisoni*). This cluster formed monophyly with second cluster of the Neocellia Series (*An. maculatus* and *An. sawadwongporni*). Third cluster contains the Neomyzomyia Series (*An. dirus* and *An. baimaii*).

Phylogenetic results of NJ, ME, MP and ML analyses generated similar topologies based on the ITS2 fragment. Fig. 2(b) presents phylogenetic tree based on MP approach indicating three main clusters. First cluster is monophyly of the Myzomyia cluster (*An. minimus*, *An. harrisoni*, *An. varuana*, *An. pampanai*, and *An. aconitus*). Second cluster is the Neocellia (*An. maculatus* and *An. sawadwongporni*) cluster, and basal cluster contains the Pyretophorus (*An. epiroticus*) and Neomyzomyia Series (*An. dirus* and *An. baimaii*). Most of branch support showed moderate to strong supporting, except branch between the Pyretophorus and Neomyzomyia Series in MP analysis and most of branches in ML analysis.

Nucleotide sequences of the TOLL6 fragment were newly sequenced from nine *Anopheles* species. Sequence of *Drosophila melanogaster* was used as outgroup. Tree topologies from all analyses are consistent. Fig. 2(c) shows phylogenetic tree based on maximum parsimony reconstruction that investigated species were separated into three main

clusters. First cluster contains monophyly of the Myzomyia cluster (*An. minimus*, *An. harrisoni*, *An. varuana* and *An. pampanai*). Second cluster contains the Pyretophorus (*An. epiroticus*) and Neocellia (*An. maculatus* and *An. sawadwongporni*) Series. The Neomyzomyia Series (*An. dirus* and *An. baimaii*) is paraphyly and basal cluster of these topologies.

In addition, the consistent relationships of the Series taxonomic level were observed in the multilocus phylogenetic analysis. Relationships among main species, the Series of mosquitoes, and analyzed fragments were considered and depicted in Fig. 3. The result showed that arrangements of the Series taxonomic levels between the TOLL6 (nuclear gene) and COII (mitochondrial gene) fragments were similar. The result shows that the Neocellia and Pyretophorus Series were monophyletic in these genes, and this cluster had a close affinity with the Myzomyia Series. Relationships between the P9 and ITS2 fragments were quite consistent (showing a close affinity among the Myzomyia and Neocellia Series), while arrangement of COI fragment was unique (the Neomyzomyia and Neocellia Series were monophyletic).

Estimation of divergence time

Simple statistical methods (the relative rate test) based on the chi-square test were developed by Tajima (1993) to test the molecular evolutionary clock hypothesis. The relative rate test was implemented in the MEGA version 5 computer program (Tamura et al. 2011). A *p*-value less than 0.05 is considered to reject the null hypothesis of the equality of evolutionary rates between lineages. Firstly, no fragment was found that followed the molecular evolutionary clock hypothesis. The results were then reconsidered. Most of the problematic species observed in the COI, P9, TOLL6 and ITS2 fragments were main malaria vectors (data not shown), but for the COII gene fragment, only *An. aconitus* was problematic and *An. varuna* was differently positioned from the species groups of the Myzomyia Series. Thus, these species were excluded from analysis. The relative rate test results between species of *Anopheles* mosquitoes at the COII gene fragment are shown in Table 8. Furthermore, for analysis using the mcmctree computer program (a part of PAML version 4.4 computer program), a sequence data file of analyzed *Anopheles* species based on the COII gene is required to reconstruct the phylogenetic tree. All topologies of each different phylogenetic reconstruction (using the NJ, MP and ML approaches) were considered. The Branch pattern supported by a bootstrap test that satisfied the less-than-50%-majority rule was considered as a questionable branch. The

phylogenetic tree reconstructed using the NJ approach (Fig. 4) with moderate-to-high branch support was chosen for estimating of the divergence time.

The most complex nucleotide substitution model (HKY85; 10,000 burning periods; 100,000 samples; 5 sample frequencies) was used for computer simulations and performed with four independent analyses. Phylogeny and divergence times with their associated 95% confidence intervals for *Anopheles* mosquitoes based on the COII gene fragment are shown in Fig. 5. Lineages of the Myzomyia Series (*An. minimus*, *An. harrisoni*, and *An. pampanai*) were firstly splitted from the Pyretophorus Series (*An. epiroticus*), Neomyzomyia Series (*An. dirus*, *An. scanloni*, and *An. baimaii*), and Neocellia Series (*An. maculatus* and *An. sawadwongporni*), respectively. The divergence time of *An. pampanai* from *An. minimus* and *An. harrisoni* is approximately 6.671 to 38.784 mya. The divergence time between *An. epiroticus* and the Neocellia Series, which approximately presents 0.006 to 2.137 mya of speciation time, is approximately 0.661 to 12.804 mya. The species divergence of the Dirus Complex within the Neomyzomyia Series (*An. dirus*, *An. baimaii* and *An. scanloni*) estimated approximately 0.051 to 7.614 mya. The divergence time of *An. scanloni* from *An. dirus* and *An. baimaii* is approximately 0.271 to 7.614 mya, and the divergence time between *An. baimaii* and *An. dirus* is approximately 0.051 to 2.338 mya.

Discussion

Phylogenetic inference with multilocus DNA sequences

Phylogenetic relationships based on morphological characters were utilized to compare with molecular relationships in this study. Because species classifications based on morphological are not covered species in this study, to examine relationships was performed in taxonomic scale of the series. But these relationships data are incongruent (Foley *et al.*, 1998; Anthony *et al.*, 1999; Sallum *et al.*, 2000). Phylogenetic trees based on four methods from each fragment showed incongruent. When tree topologies of each fragment were considered arrangements between ingroup and outgroup species, appropriate MP analysis was chosen for considering.

The phylogenetic analysis of the mitochondrial COII and nuclear TOLL6 fragment shows that the congruent clusters are supported by morphological relationships of Anthony et al. (1999). Moreover, the phylogenetic relationships based on the mitochondrial DNA data showed that branch supports of species complexes are moderate to strong, while poor branch supports are observed among groups of species complexes and closely related species. This is

consistent with the phylogenetic study of subfamily Anophelinae (Sallum et al. 2002). Sallum et al. (2002) suggested that the mitochondrial DNA data hold little phylogenetic signal, but utilizing for relationships of very recently derived species are exception. Howland and Hewitt (1995) investigated phylogenetic relationships of the family Coleoptera (Ground Beetles) based on the mitochondrial COI sequence data. They suggested that the COI sequence data may be suitable for molecular systematics at lower taxonomic levels of the family. On the other hand, the mitochondrial COI and COII data was not likely suitable to resolve phylogenetic relationships at higher taxonomic level of species complex.

The results of phylogenetic suggest that TOLL6 (Toll-like receptor) fragment appears much more informative and should be useful in phylogenetic reconstruction within these samples of Anopheline mosquitoes because the phylogenetic trees from all approaches were similar tree topology and high bootstrap supports, although bootstrap support for some clade dropped. Additionally, P9 fragment also appears informative signal for phylogenetic reconstruction, but phylogenetic positions of some species were incorrect. These clusters of P9 fragment are supported by morphological relationships of Anthony *et al.* (1999), excepting *An. aconitus*; it showed improper basal position because it belongs to member of the Myzomyia Series. However, the observation of Mardulyn and Whitfield (1999) suggested that informative and useful gene for phylogenetic reconstruction in one taxon properly might not resolve in other taxa, even closely related. Thus, more *Anopheles* species are required for testing phylogenetic information of these regions.

Estimation of divergence time

The molecular evolutionary clock hypothesis was first proposed by Zuckerkandl and Pauling in 1965. The rate of evolution in a protein or DNA molecule is approximately constant over time and among evolutionary lineages (Morgan, 1998). This hypothesis is useful for estimating the evolutionary history and divergence time of organisms having poor fossil records (Hoy, 2003).

The estimated divergence time within this subgenus *Cellia* (late 41 mya) is consistent with divergence between subgenus *Anopheles* and *Cellia*, i.e. approximately 90 to 100 mya (Krzywinski *et al.*, 2006) and after the break-up of Gondwana (approximately 100 mya at the late Cretaceous Period) (Garros *et al.*, 2005b). Speciation between the Aconitus Subgroup (*An. pampanai*) and the Minimus Subgroup (*An. minimus* and *An. harrisoni*) within the Funestus Group of the Myzomyia Series was estimated to have occurred during the late Eocene to late Miocene (Eocene epoch, approximately 34 to 56 mya; Miocene epoch, approximately 5 to 23

mya). The estimated divergence time of the Pyretophorus Series (*An. epiroticus*) and the Neocellia Series occurred during the Miocene (Miocene epoch, approximately 5 to 23 mya). The divergence time estimating between *An. maculatus* and *An. sawadwongporni* (the Neocellia Series) (approximately 2.1 mya; early Pleistocene) is earlier than the previous studies (based on the combined ND5 and COII), occurred during the Pliocene to early Pleistocene (Morgan *et al.*, 2009).

The speciation events of the Dirus Complex are most likely due to Pleistocene forest fragmentation (Pleistocene epoch, approximately 0.0117 to 2.588 mya). During the glacial period of the effects of Pleistocene climatic change, forest-dependent species survived in tropical forest refugia and remained in some areas of mainland Southeast Asia. Then, the allopatric species of the Dirus Complex would have expanded with the extent of forest cover across Southeast Asia in post-gracial recolonization, according to the habitats of these mosquitoes which are only found in tropical forests (O'Loughlin *et al.*, 2008; Morgan *et al.*, 2010; Morgan *et al.*, 2011; Loaiza *et al.*, 2012). This result of divergence times is similar to the allopatric divergence in the speciation of *An. baimaii*, *An. dirus* and *An. scanloni* within the last 1.5 mya: approximately 192-877 thousand years ago (kya) between *An. baimaii* and *An. scanloni*, approximately 204-932 kya between *An. dirus* and *An. scanloni* and approximately 163 kya to 1.53 mya between *An. dirus* and *An. baimaii* (Morgan et al. 2010).

When the estimations of divergence time of mosquito species in this study were considered overall, speciation of these species was estimated to have occurred during the late Miocene to early Plesitocene. This period was considered as an important time for diversification in other forest-dependent taxa across mainland and island Southeast Asia (Morgan *et al.*, 2009). Pliocene climatic change according to increasing forest fragmentation is likely the driving force for this diversification, precipitation levels were high and consequently tropical forest had extensively covered across Southeast Asia (Chandler *et al.*, 1994; Cronin *et al.*, 1994; Ravelo *et al.*, 2004). These data are consistent with habitats of these mosquito species.

Using a single locus may be problematic for estimating divergence events. Thus, the multiple loci following molecular clock hypothesis are required for the estimation of divergence time for further study. However, this estimation of divergence time is the initial step for basic understanding for Anopheline (*Cellia*) mosquitoes. Multiple gene loci, in particular nuclear genes, and more *Cellia* mosquito samples are required for further study in order to understanding the evolution of *Anopheles* (*Cellia*) mosquitoes.

Phylogenetic relationships of *Anopheles* species presented in Thailand were analyzed using multilocus DNA sequence information. The results based on four different reconstructed algorithms as well as different gene regions revealed congruent phylogenetic status of malaria vector species especially at the Series taxonomic level. The nuclear TOLL6 fragment may be an informative gene containing phylogenetic signals to open a new perspective for reconstructing relationships in *Anopheles* mosquitoes. However, more samples of different taxonomic levels are required to confirm the taxonomic positions. Moreover, estimation of the divergence time among the *Anopheles* species studied was performed based on the COII fragment inferring that *Anopheles* species probably existed around the Eocene and Miocene periods (later than 41 million years ago).(Morgan *et al.*, 2009). Pliocene climatic change according to increasing forest fragmentation is likely the driving force for this diversification, precipitation levels were high and consequently tropical forest had extensively covered across Southeast Asia (Chandler *et al.*, 1994; Cronin *et al.*, 1994; Ravelo *et al.*, 2004). These data are consistent with habitats of these mosquito species.

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Acknownledgements

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Table 1. Details of analyzed species; the classifications are based on Harbach (2011).

Species	Taxonomic states	
	Species group	Series
An. dirus	Dirus Complex, Leucosphyrus Subgroup, Leucosphyrus Group	Neomyzomyia
An. scanloni	Dirus Complex, Leucosphyrus Subgroup, Leucosphyrus Group	Neomyzomyia
An. baimaii	Dirus Complex, Leucosphyrus Subgroup, Leucosphyrus Group	Neomyzomyia
An. maculatus	Maculatus Subgroup, Maculatus Group	Neocellia
An. sawadwongporni	Sawadwongporni Subgroup, Maculatus Group	Neocellia
An. epiroticus	Sundaicus Complex	Pyretophorus
An. minimus	Minimus Complex, Minimus Subgroup, Funestus Group	Myzomyia
An. harrisoni	Minimus Complex, Minimus Subgroup, Funestus Group	Myzomyia
An. aconitus	Aconitus Subgroup, Funestus Group	Myzomyia
An. varuna	Aconitus Subgroup, Funestus Group	Myzomyia
An. pampanai	Aconitus Subgroup, Funestus Group	Myzomyia

Table 2. Information of DNA fragments used for phylogenetic reconstruction.

Gene	Chromosome location	Coding/non-coding part
TOLL6	Nuclear genome (Autosome)	Coding
rRNA	Nuclear genome (Autosome)	Non-coding
Gbb-60A	Nuclear genome (X-chromosome)	Non-coding
CYP	Nuclear genome (X-chromosome)	Non-coding
COI	Mitochondrial genome	Coding
COII	Mitochondrial genome	Coding

Note: *rRNA*: ribosomal RNA; *Gbb-60A*: Glass bottom boat 60A (Transforming growth factor beta at 60A); *CYP*: Cytochrome P450; *COI*: Cytochrome c-oxydase I; *COII*: Cytochrome c-oxydase II.

Table 3. Sequence characteristics of mitochondrial *COI*, *COII* and rDNA-ITS2.

Species	*COI*		*COII*		ITS2	
	Length (bp)	Accession no.	Length (bp)	Accession no.	Length (bp)	Accession no.
An. dirus	206	AJ877406.1	555	AJ877470.1	841	U60410.1
An. scanloni	206	AM180859.1	555	AM180873.1	**	**
An. baimaii	206	AJ877510.1	555	HQ403902.1	835	U60411.1
An. maculatus	206	DQ267690.1	555	HQ403945.1	467	FJ526582.1
An. sawadwongporni	206	newly sequenced	555	HQ404164.1	418	FJ526593.1
An. epiroticus	204	AY789200.1	555	newly sequenced	663	AY789168.1
An. minimus	206	HQ877373.1	555	FN433593.1	515	HQ228204.1
An. harrisoni	206	HQ877374.1	555	AF195048.1	461	AF194506.1
An. aconitus	206	AY423055.1	555	HQ403810.1	506	DQ000247.1
An. varuna	206	newly sequenced	555	newly sequenced	495	DQ478879.1
An. pampanai	206	AY423054.1	555	AY486112.1	491	AY737082.1

**Sequence could not be obtained from database.

Table 4. Details of studied DNA fragments and primer sequences for PCR amplification in *Anopheles* species.

Gene (Primer name)	Gene details			Details of DNA fragments		
	Chromosomal location	Putative Function	Region	Primer sequences	Product size (bp)	Annealing temperature
CYP (P9)	X-Chromosome	Metabolism of xenobiotics	Non-coding	F: TGGAAAGTAGGGGCAATCAG R: GCTGGGCAACGGACTACTTA	654	51 ºC
Gbb-60A (O8)	X-Chromosome	Signaling proteins, controls proliferation, differentiation	Non-coding	F: CGGCCATTGGGCGGACATTT R:ACGCATCTCCGATCCGTGATGATC	550	60 ºC
TOLL 6 (TOLL6)	Autosome	Immune signaling pathways	Coding	F: CCAACTCGTTCGTCCATCTG R: CTGGCGAAAGCACACTGAT	755	62 °C
COI (COI)	Mitochondrial genome	Energy-transfer enzymes of respiratory chains	Coding	F: CTGGCTGGGCAACGGACTA R:GGAAAGTAGGGGCAATCAGTTTG	439	50 ºC
COII (COII)	Mitochondrial genome	Component of the respiratory chain and transfer of electrons from cytochrome c to oxygen	Coding	F: TGTGAACTGCAGGACACAT R: TATGCTTAAATTCAGGGGGT	773	50 ºC

Table 5. Information of outgroup species.

Fragments	Outgroup species	Length (bp)	Accession no.
P9	*Drosophila melanogaster*	506	NC_004354
TOLL6	*Drosophila melanogaster*	659	NM_079357.2
COI	*Aedes aegypti*	213	EU352212.1
COII	*Aedes aegypti*	685	EU352212.1
ITS2	*Drosophila melanogaster*	509	EU306667.1

* O8 fragment was excluded from analysis due to very low genetic variation.

Table 6. Average nucleotide compositions of *Anopheles* mosquitoes from each fragment.

Fragment	Average base compositions (%)			
	Thymine	Cytosine	Adenine	Guanine
COI	42.8	16.1	27.0	14.0
COII	38.9	13.4	35.5	12.3
O8	26.5	20.9	27.6	25.0
P9	27.5	21.2	25.2	26.1
TOLL6	22.7	25.9	21.8	29.6
ITS2	20.2	29.0	22.2	28.5

Table 7. The appropriate nucleotide substitution models for likelihood analysis.

Locus	BIC	AICc	*lnL*	Selected model
P9	5459.5	5367.8	-2668.5	T92
TOLL6	5275.1	5142.7	-2551.3	T92+G
COI	1749.9	1593.8	-769.6	TN93+G
COII	4120.9	3917	-1928.4	GTR+G
ITS2	6888.4	6756	-3356.9	K2+G

*O8 fragment was excluded from analysis due to low genetic variation; BIC: Bayesian Information Criterion; AICc: Akaike Information Criterion, corrected; Maximum Likelihood value (*lnL*); T92: Tamura 3-parameter; TN93: Tamura-Nei; K2: Kimura 2-parameter; GTR: General Time Reversible; +G: a discrete Gamma distribution.

Table 8. Relative rate tests between Anopheline mosquitoes at the COII gene fragment.

Species	*Dir*	*Sca*	*Bai*	*Mac*	*Saw*	*Epi*	*Har*	*Min*	*Pam*
Dir		0.14,0.705	1.00,0.317	0.16,0.685	0.16,0.685	0.02,0.896	0.02,0.890	0.02,0.888	0.17,0.680
Sca	*Pam*		0.00,1.000	0.07,0.796	0.27,0.605	0.15,0.696	0.07,0.785	0.72,0.396	1.19,0.276
Bai	*Har*	*Pam*		0.02,0.894	0.07,0.789	0.00,1.000	0.07,0.785	0.08,0.781	0.30,0.586
Mac	*Min*	*Har*	*Pam*		0.00,1.000	0.09,0.763	0.47,0.492	1.85,0.173	0.17,0.680
Saw	*Har*	*Min*	*Har*	*Har*		0.20,0.654	0.47,0.492	1.85,0.173	0.17,0.680
Epi	*Min*	*Pam*	*Min*	*Min*	*Har*		0.83,0.362	1.19,0.276	0.44,0.507
Har	*Epi*	*Epi*	*Epi*	*Dir*	*Dir*	*Dir*		0.06,0.808	0.03,0.872
Min	*Mac*	*Mac*	*Mac*	*Sca*	*Sca*	*Sca*	*Dir*		0.10,0.751
Pam	*Saw*	*Saw*	*Saw*	*Bai*	*Bai*	*Bai*	*Mac*	*Saw*	

Above-diagonal: The chi-square test statistic and *P*-value, respectively; below-diagonal: the species considering as an outgroup between species pair; *Dir*: *An. dirus*, *Sca*: *An. scanloni*, *Bai*: *An. baimaii*, *Mac*: *An. maculatus*, *Saw*: *An. sawadwongporni*, *Epi*: *An. epiroticus*, *Har*: *An. harrisoni*, *Min*: *An. minimus*, *Pam*: *An. pampanai*.

However, branch supports among each cluster are poor and unreliable.

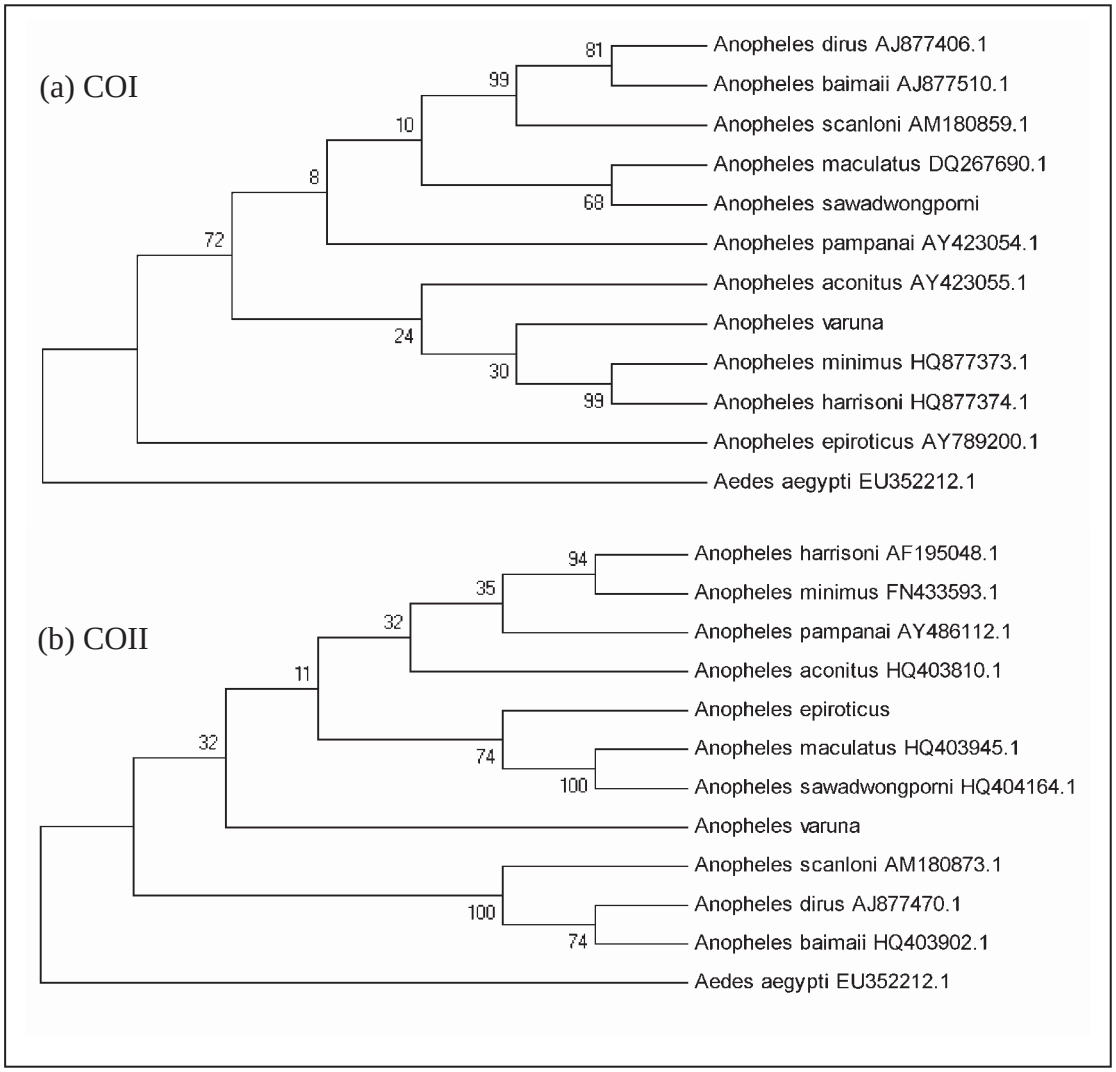

Fig. 1. Phylogenetic trees based on the COI and COII fragment (MP reconstruction);

Fig. 1. Phylogenetic trees based on the COI and COII fragment (MP reconstruction); bootstrap values for maximum likelihood analysis are listed at nodes.

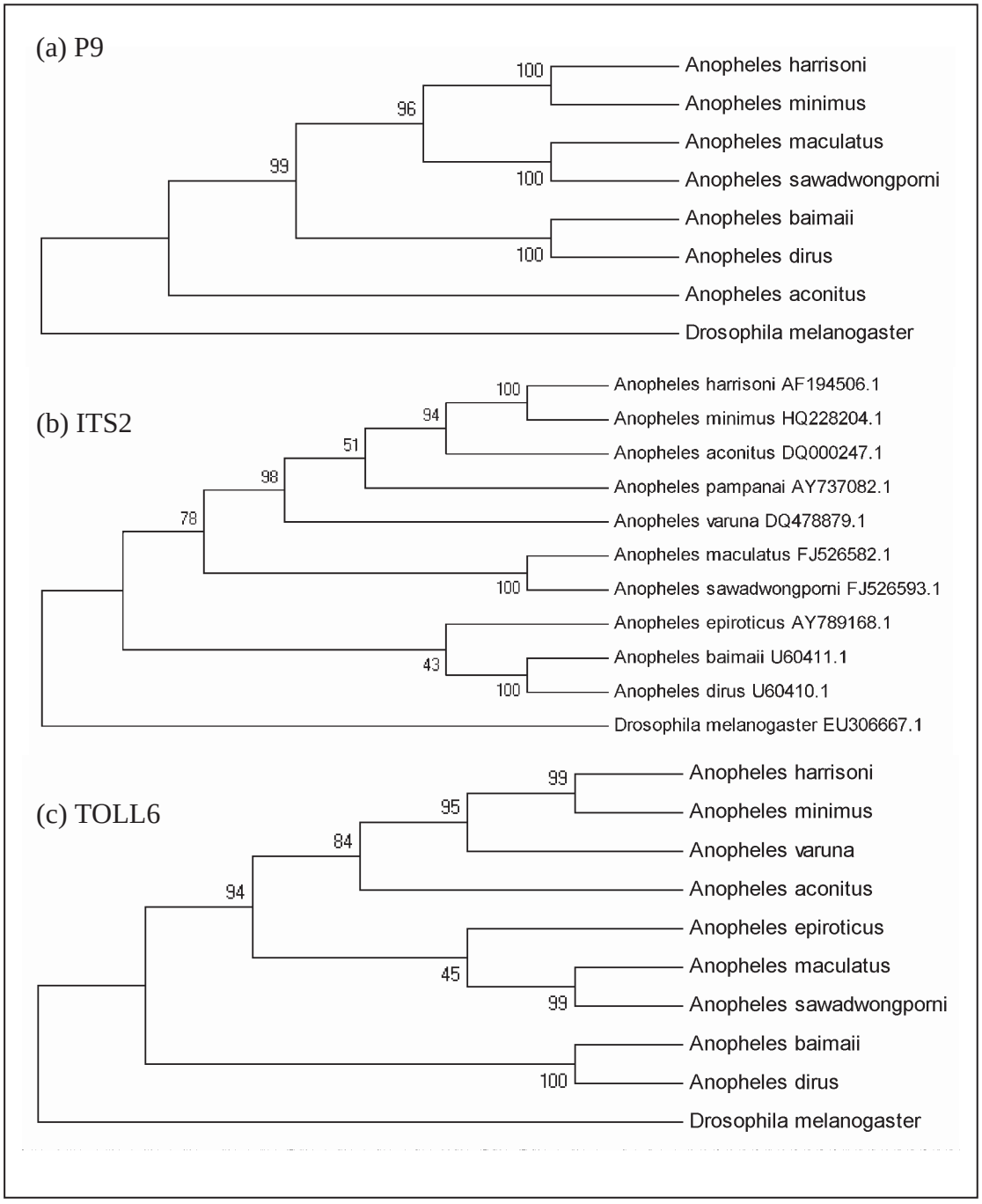

Fig. 2. Phylogenetic trees based on the P9, ITS2 and TOLL6 fragment (MP reconstruction); bootstrap values for maximum likelihood analysis are listed at nodes.

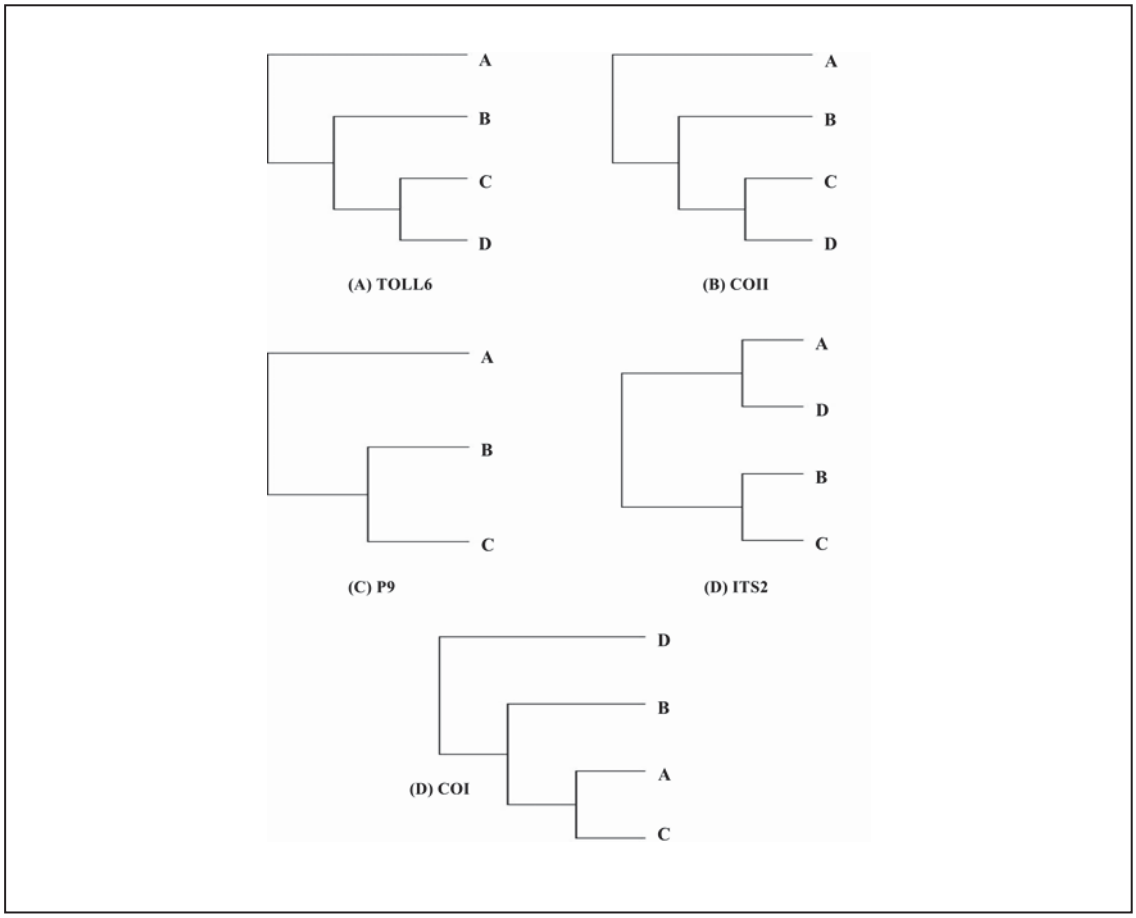

Fig. 3. Illustration of relationships based on MP phylogenetic trees between analyzed fragments and the Series of mosquito taxonomic level; A: Neomyzomyia, B: Myzomyia, C: Neocellia, D: Pyretophorus.

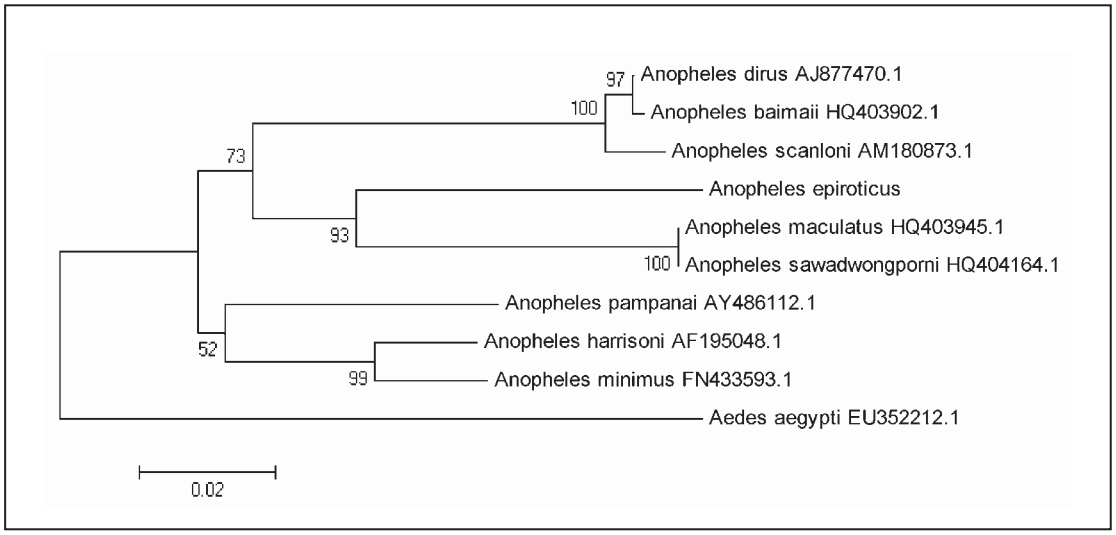

Fig. 4. Neighbor joining phylogenetic tree with the COII fragment in nine *Anopheles* mosquitoes. Bootstrap values for maximum likelihood analysis are listed below nodes.

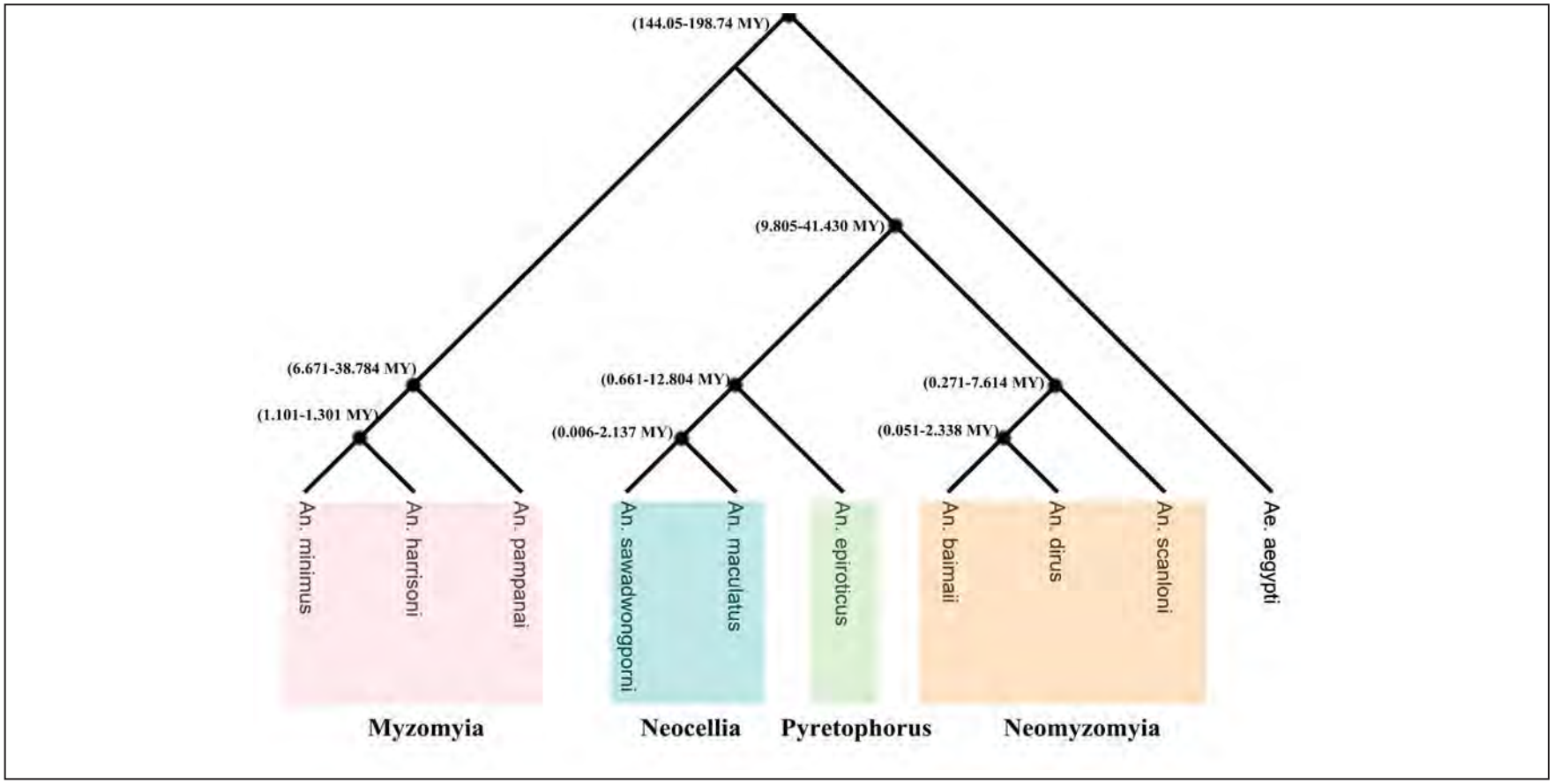

Fig. 5. Phylogeny and estimated divergence times of *Anopheles* mosquitoes from the COII gene fragment based on PAML version 4.4 program.

OUTPUT

Manuscripts for international journals

1. Arunyawat U., P. Phunngam, A. Das, and T. Chareonviriyaphap. Molecular population genetic studies of *Anopheles minimus* (Diptera: Culicidae), vector of malaria in Thailand. *in preparation*
2. Phunngam P, T. Chareonviriyaphap, and U.Arunyawat . Population genetic study of malaria vector *Anopheles harrisoni* in Thailand. *in preparation*
3. Phunngam P, T. Chareonviriyaphap, and U.Arunyawat . Phylogenetic reconstruction of malaria vectors in Thailand using multilocus DNA sequencing. *in preparation*

International conferences/workshop

1. Arunyawat U. P. Phunngam, A. Das, and T. Chareonviriyaphap. Molecular population genetic studies of Anopheles minimus, vector of malaria in Thailand. EMBO Global exchange lecture course on “Molecular and Evolutionary Genetics of malaria”. 21 Nov. – 4 Dec. 2010. New Delhi, India (invited talk)
2. Arunyawat U., P. Phunngam, A. Das, and T. Chareonviriyaphap. Molecular population genetic studies of Thai malaria vector (*Anopheles minimus*). 20-24 March 2011. AMCA 77th Annual Meeting, CA, USA (contributed talk)
3. Phunngam P, T. Chareonviriyaphap, and U. Arunyawat. Phylogenetic reconstruction of malaria vectors in Thailand using multilocus DNA sequencing. 22-26 June 2012 SMBE2012, Dublin Ireland (poster)

Subproject-5 (Dr. Sungsit Sungvornyothin)

Trophic behavior and ecological characteristics of *Anopheles dirus* complex in man-made habitat

Abstract Trophic behavior of *Anopheles dirus* was studied in Kanchanaburi Province, Thailand, as a result from a discovery of unusual type of *An. dirus* breeding site. The characteristic of breeding place was a brick and cement constructed well exposed to the full sunlit with light intensity over 1000 lux. Larval density was 3.7 per dip in average. Adult female mosquitoes were collected from human landing methods during 1800 hr-2400 hr. Female of *An. dirus* complex start to biting at 1900 h with a sharp peak during 1900 – 2000 h. Molecular identification on the dirus complex will be used for species confirmation and validation. The result shown that all specimens which is found in the man-made container are *An. dirus*.

KEY WORDS *Anopheles dirus*, trophic behavior, man-made container, Kanchanaburi, Thailand

Summary

The objective is this proposed study is to characterize the trophic behavior of *Anopheles dirus* and other related malaria vector species in Thailand. This study would help us in understanding the extent of parameters in malaria transmission by *An. dirus* complex and relationship between adult and larva population dynamic and help in further planning of vector control measure in Thailand. Due to the few number of presented *An. dirus* in the study sites, the *An. minimus* and *An. maculatus* are mainly found in Tak Province, were included in this study, to described the blood feeding/biting behavior, host preference and seasonal abundance of *An. minimus* over a two-year period in Tum Sua Village. The result from this subproject was published in the international journals as following:

1. Rungarun Tisgratog, Chatchai Tananchai, Waraporn Juntarajumnong, Siripun Tuntakom, Michael J. Bangs,Vincent Corbel and Theeraphap Chareonviriyaphap. 2012. Host feeding patterns and preference of *Anopheles minimus* (Diptera: Culicidae) in a malaria endemic area of western Thailand: baseline site description. Parasites & Vectors. 5:114.

The work in this subproject was funded by the Thailand Research Fund-Senior Research Scholar Program (RTA5280007) to Theeraphap Chareonviriyaphap. This subproject also

support for Master Degree of Rungarun Tisgratog (1 publication bellow) and Krajana Tainchum, Ph.D. student (project description are added).

Trophic behavior and ecological characteristics of *Anopheles dirus* complex in man-made habitat

Introduction

Anopheles dirus complex is the major malaria vector in South and Southeast Asia. The complex are comprise of at least 7 species. Five of them can be found in mountain areas of Thailand (Sallum *et al*., 2005). Knowing the truly species of complex species is important information to manage the vector and disease control strategies Breeding habitat of *Anopheles dirus* complex is usually temporary, standing or slowly moving water under shade (Obsomer *et al.*, 2007). The human-made container exposed to sun light and maintained the water almost along the year was discovered to be an usual habitat of Anopheles dirus complex in Thailand. To understanding in biological adaption of this medical important species. This study investigates in population dynamic, biting activity and the biological species of *Anopheles dirus* complex.

Materials and Methods

Collection sites

The study area is on the mountain in Wat Promlok, a dhamma retreated temple, in Kanchanaburi Province, Thailand. There is cement stare way to the top of mountain. The breeding place was a brick and cement constructed well exposed to the full sunlit with light intensity over 1000 lux. (Fig. 1).

Mosquito landing collection and larva habitat study

Eight local people volunteers (H1-H8) was sat at the marked site to collect the mosquito landing on their bare legs. The H1 to H3 were sat along the height of mountain. The H4 to H8 was sat on ground (Fig. 5). The mosquitos were collected separately in each hour during 1800 – 2400 hr. The collection was carry out of 2 nights per month. Female of *Anopheles dirus* complex were kept in dry situation for molecular identification. The bricked and cement constructed well was measured light intensity. The larvae collection was done in each month interval. Ten dips of sampling were calculated for larva density.

Molecular identification

Four member species belong to the Dirus complex were clarified the exactly species according to Huong (*et al.,* 2001). The other species of the complex were identified by only morphological characteristic.

Data analysis

Spatial and temporal data (time, date, place, species, number of specimens and environmental conditions) were used in the analysis as appropriate. Chi-square test was used to evaluate the differences in number of Anopheles mosquito among categories at different collection sites. The interaction between number of *Anopheles dirus* captured and the yearly total rainfall at individual site was analyzed using variables with bivariate normal distribution (Kleinbaum et al. 1987). The GLM procedure in SPSS program package (Ver 13, SPSS Inc., Chicago, IL, USA) was used to evaluate the differences between numbers of stable flies collected at each location. All statistical significance was set at $P < 0.05$.

Results

A Molecular identification of the *Anopheles dirus* breeding in human-made container is *An. dirus*. Figure 4 shows the presented major band of polymerase chain reaction with the primer specific to *An. dirus* (species A) and miner band specific to the primer of *An. scaloni* (species C) which interprets as *An. dirus*. During May to September wiith 8 local people volunteers, 175 *An. dirus* were collected. The highest number of *An. dirus* were collected in September while the lowest number were collected in July (Fig 2). Biting activity of *An. dirus* were measured hourly, the peak of activity was during 1900 – 2000 hr. Grouping of human volunteer as 1) On mountain group (H1 – H3) and 2) On ground group (H4 – H8). The on mountain group collected the mosquito as 2.5 time as collected by the on ground group (Fig 3). In details, biting activity of *An. dirus* on each volunteers show direction of the mosquito flying from the top of the mountain to the ground.

High number of *An. dirus* were collectd for all time of collection on human volunteers No.1 (H1) who sat nearest to the bricked well. Number of *An. dirus* declined to the following hour for the H2 and H3. Differ to the on ground group, the number of *An. dirus* increased in the following hour of collection by H5, H6, and H7. Number of the *An. dirus* increased in the last hour of collection on H4 and H8 (Fig 5).

Discussion

Molecular characteristic of *Anopheles dirus* from Wat Promlok Mountain show all bands are presenting to species of *Anopheles dirus*. This is according to some results reports by Houng *et al.*, 2001. Biting activity of *An. dirus* is highest on 1900-2000 hr for the on mountain group volunteer and delayed to the on ground volunteers could interpret the direction to flying to host outside the mountain.

Anopheles dirus has been investigated as the major malaria vector which can adapted very well to the human invading ecology. The unusual breeding site characteristic is an evidence of adaptation of this species. The biting activity and flying direction of *An. dirus* caused us understanding in adapting behavior to survive in human environment.

Acknowledgements

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Figure 1

Figure 2.

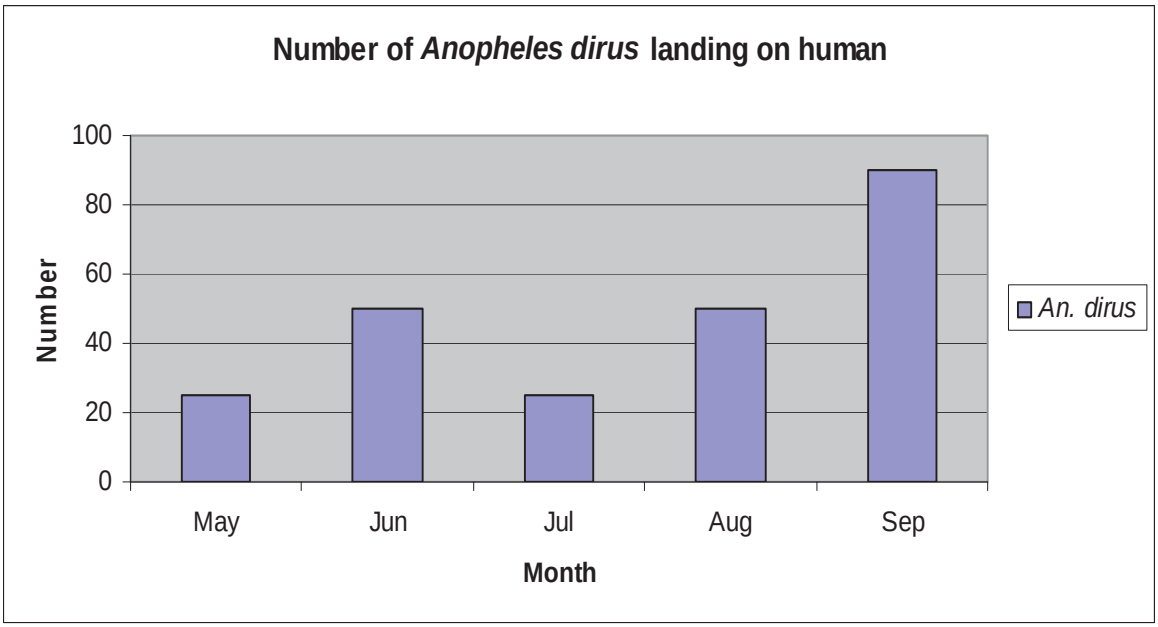

Figure 3.

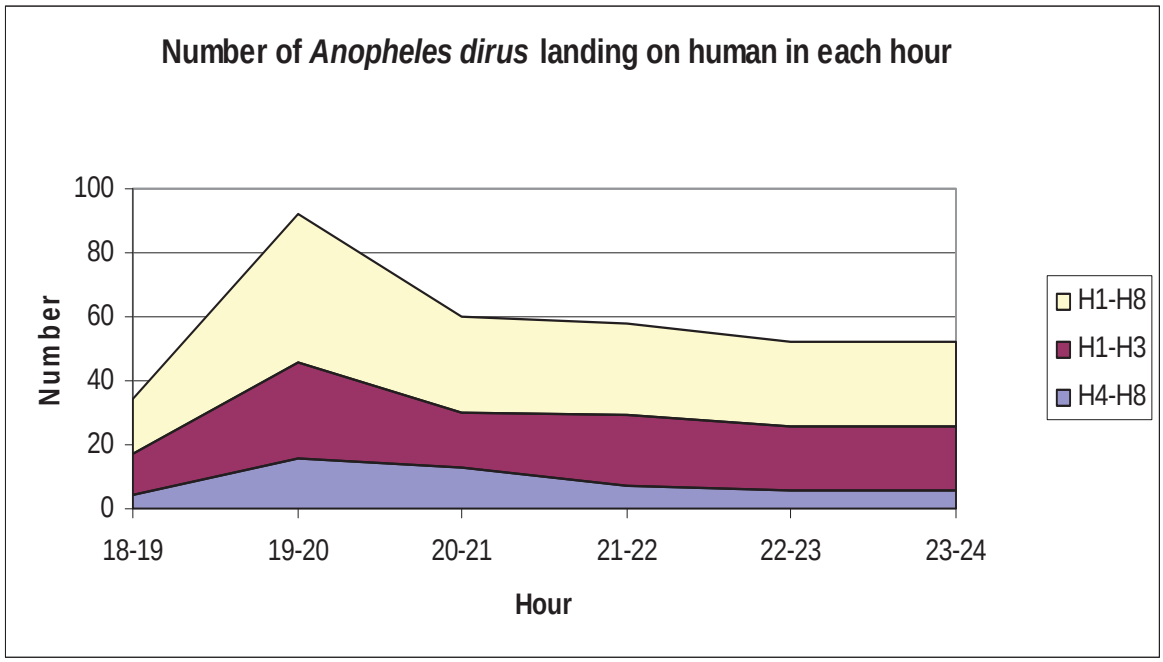

Figure 4.

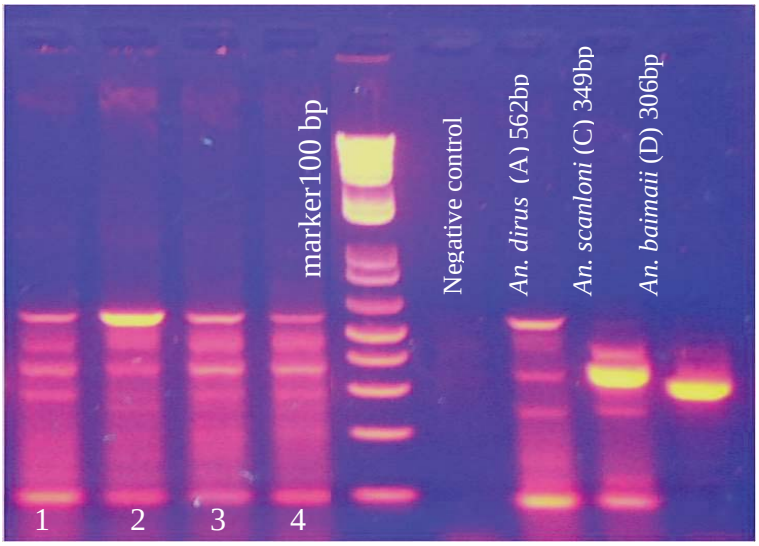

Figure 5.

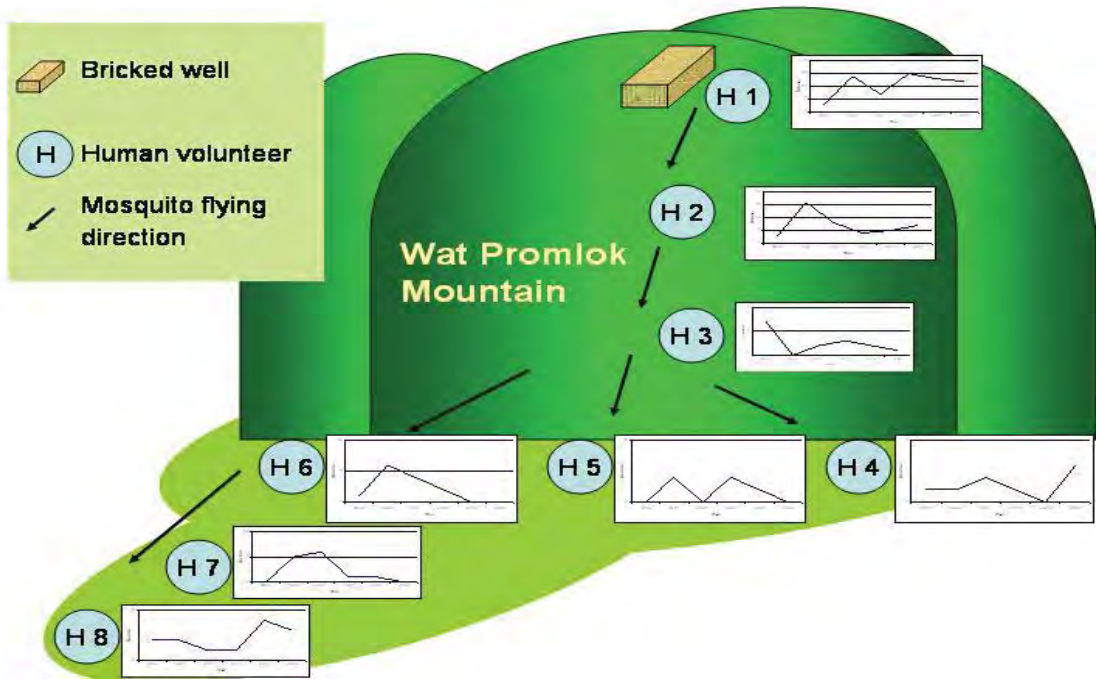

Figure legends

Figure 1. The study site which is located on the mountain and is constructed the cement tank, Kanchanaburi Province.

Figure 2. Number of *Anopheles dirus* from human landing collection in each month interval during May to September 2011.

Figure 3. Hourly biting pattern of *Anopheles dirus* in difference collection place base on three place high level of the mountain(H1-H8).

Figure 4: Multiplex Allele-Specific PCR assay for Dirus Complex. Lanes 1, 2, 4,: *An. dirus;* Lane 5: 100 bp molecular marker; Lane 6: negative control; Lane 7-9: positive control

Figure 5: Biting activity and flying direction model

Objective

To define the vectorial capacities of Anopheles species along the Thai-Myanmar border, to evaluate the co-transmission between *Plasmodium falciparum*, *P. vivax* and *Wuchereria bancrofti* and to study the present of bacteria in mosquito midgut that influence the infection.

Abstract Mosquitoes can be efficient vectors of pathogens and are responsible for the transmission of various parasitic diseases which can have a high human impact, such as agents of malaria and lymphatic filariasis (LF) for the most common vector-borne diseases worldwide. This project propose to study the transmission of *Plasmodium* sp. and *Wuchereria bancrofti*, responsible for malaria and bancrofti filariasis respectively, which occur as co-endemic infections in Thailand. Malaria is a public health priority with a high prevalence of the disease in forested areas, especially along the borders with Myanmar (Tak and Mae Hong Son Provinces) where these two study sites concentrate 55% of all malaria cases nationally encountere. Bancroftian filariasis (LFB) is also endemic along the Thai-Myanmar border, however, only limited data on the prevalence of this disease in Thailand is available. Malaria and LFB are mosquito-borne diseases with *Plasmodium* species, especially *P. falciparum*, *P. vivax* with a prevalence of 50% each nationwide, and rural strains of *W. bancrofti*, sharing the same *Anopheles* vector species.

KEY WORDS *Anopheles minimus*, *Anopheles dirus*, *Plasmodium spp*., *Wuchereria bancrofti*, Bacteria, Thailand

Introduction

In Thailand, malaria is a public health priority with a strong prevalence of this disease in forested regions, in particular along the border with Myanmar where another parasitic disease occurs, Bancrofti filariasis (FLB) for which only limited data are available. Malaria and FLB are mosquito-borne diseases with *Plasmodium* species, especially *P. falciparum*, *P. vivax*, and rural strains of *Wuchereria bancrofti* sharing the same *Anopheles* vector species. In Southeast Asia, *Anopheles* vectors belong to species complexes with different involvement in the transmission of pathogens. Few sibling species of the Dirus complex (7 species), the Minimus complex (3 species) and the Maculatus group (8 species) are involved in malaria and FLB, but specific role of each sibling species has never been studied due to the lack of reliable methods for species identification, now available.

This study will use these techniques for estimating the role of each vector, as well as detecting and identifying the parasites present in mosquitoes. Another aspect, the biodiversity of bacteria in mosquito midgut has been seldom studied, although the first data showed a strong influence of the bacterial flora on the development of oocyst in mosquitoes. This information will evaluate the bacterial biodiversity by amplification of the 16S rNRA sequence. The bacterial biodiversity will be compared among specimens and species in relation to the collections site and the season, and most of all in relation to the positive or negative parasitic infestation in mosquitoes. The project relies on sufficient vector samples in quality and quantity.

Materials and Methodology

Part 1: To investigate the *Anopheles* species and a seasonal abundance of dominant malaria vectors

1.1 Collection sites

Mae Sod district, Tak Province is located in the northern part of Thailand at the border with Myanmar. This is a forested area covered by high trees, lots of vegetation as being an agricultural region and little streams. Recently, 17,164 malaria cases were reported in this area and with a prevalence of 6,041 cases of *Plasmodium falciparum* (35.4%) and 11,013 cases due to *P. vivax* (64.6%) (Ministry of Public Health, 2010).

Sop Moei district is the most southern district of Mae Hong Son Province. This is a mountainous province north of Tak province with the higher transmission period ranging from June to August, during the rainy season (Thimasarn *et al.,* 1995). In 2010, 1,882 cases were

found in this area and malaria parasite has been detected including 848 cases of *Plasmodium falciparum* (46.1%) and 993 cases of *P. vivax* (53.9%) (Ministry of Public Health 2010).

1.2 Collection method and Morphological identification

Anopheline mosquitoes were collected three nights continuously in each site using a standard landing catch technique, including indoor and outdoor human baited landing, and cow baited dwelling, were carried out. Full moon phase was considered in determining time of collections.

Five people will be in charge of live mosquito collections for each period. Collections were performed every hour from sunset to midnight (1800-2400h; first period) and midnight to sunrise (2400-0600h; second period). The first pair of collectors was seat outside a house whereas the second pair will be seating inside a house. One hour landing collection consists of 45 minutes of collection and 15 minutes break. Moreover, one collector was prepared for mosquito captures on cow bed net each 15 minutes at the end of hour. The collectors was 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 was sorted out using morphological criteria for a first identification at the complex or group level using morphological keys by Peyton and Scanlon (1966), Harrison (1980) and Rattanarithikul *et al.* (2006). Mosquitoes were individually stored in labelled-tube (T, M for Tak or Mae Hong Son respectively, with the *Anopheles* group, type of bait and a number corresponding to the collection; an example TAMHI118 refer to Tak, *Anopheles minimus*, indoor human landing, 1st night and time 1800 hr). All specimen were brought back to the laboratory at Kasetsart University and kept frozen (-80°C) at laboratory of Department of Entomology, Kasetsart University until shipped on dry ice to IRD, Montpellier and preserved at -80°C until processed at the Immuno-Physiopathology Lab, UM1, Institute of Research for Development (IRD), Montpellier, France.

1.3 Molecular identification of adult females

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

1.3.1 Mosquito DNA extraction

Head, legs and wings, 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)

1.3.2 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.

2. Data analysis

Analysis of data will include seasons, time periods and different collection methods. Environmental parameters, mean monthly ambient temperature, and accumulative rainfall, will be used to determine association and effect on mosquitoes densities seen in collections. Seasons include wet (June-October), dry (November to February), and hot (March to May). Collection time periods will be 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 will include indoor human-landing, outdoor human-landing, and cow baited collection. Differences in endophily, exophily, and number of species and individuals collected between sites will be analyzed using one -way ANOVA SPSS statistical software. Correlation analysis will be used to investigate the interaction between the number of mosquitoes and environmental data. Comparison statistics will be used for association between adult collection densities.

Part 2: To study the co-transmission of both parasitic diseases, malaria and bancroftian filariasis in dominant and secondary malaria vectors

1. Test population

Legs, head and thorax mosquitoes of the same species and collection will be pooled by 10 and used for DNA extraction using Qiagen kit, then respective qPCR assays, for *Plasmodium* sp. (Parzy et al., unpublished) and conventional PCR for *W.* b*ancrofti* (Farid *et al*. 2001) will be applied for the detection and identification of the parasites in mosquitoes.

2. Detection of *Plasmodium falciparum, P. vivax and Wuchereria bancrofti* in *Anopheles* mosquitoes

Components for 20 µl PCR reactions will be comprised: 2 µl of DNA, 1.8mM $MgCl_2$, 250 µM of each dNTP, 250 pMol of each primer, 2 µl of PCR buffer (10mM Tris–HCl, pH8.3, 50mM KCl), 1.0 unit of Taq DNA polymerase and 11 µl of water. The amplification conditions are: one initial denaturation cycle at 96 °C for 10 min; 30 cycles at 95 °C for 1 min and 60 °C for 5 min; and a final extension at 60 °C for 1 h. Amplification product will be visualize on 2% agarose gel. (Cunha, 2009 or Rao *et al*., 2009).

4. Data analysis

Number of infected mosquito will be separated for four groups: *Plasmodium falciparum* (PF), *Plasmodium vivax* (PV), *Wuchereria bancrofti* (WB) and mix. Mean number of each infected mosquito species will be compared by one-way ANOVA using SPSS software. Also number of infected mosquito in dominant and secondary vector will be evaluated.

Part 3: To estimate the midgut bacterial biodiversity in dominant malaria vector species

1. Test population

Mosquito abdomens of the infected mosquitoes (*Plasmodium sp.* and *Wuchereria bancrofti*) as well as non-infected ones was studied for the detection and identification of the bacteria in mosquito midgut.

2. Abdomen 's mosquito extraction

Each mosquito abdomen was homogenized with 150 µl TE buffer. Each tube was centrifuged at 10,000 rpm/5 min, supernatant was removed. The Ready lyse solution was prepared by Ready lyse and EPPI water in 1:10 and 10 µl of Ready lyse solution was added in each sample tube. A sample was kept at 37 ^{o}C overnight. Proteinase K (PK) and Cell Lysis Solution (CLS) were provided in 1:150, 150 ul was added in each sample. Incubation sample tubes at 65 ^{o}C for 15 min and every 5 min take out all samples for vortex (15 min in 3 times).Sample tube was rest on ice for 5 min. Precipitation was provided by adding 175 ul of the MPC Protein Protection Solution, then vertex for 10 sec. Centrifuge at 10 rct for 10 min, keep supernatant and add 500 ul of Isopropanal was provided. Centrifuge again at 10 rct for 10 min, keep pellet. After that, the pellet was washed with 300 ul of cool 70% Alcohol and centrifuge at 10 rct for 10 min. Remove supernatant and let the pellet dry under flume hoot for 15 min, add 35 ul of TE buffer, keep at room temperature for 3 hrs for DNA loosen. All samples were kept in freezer at -20 ^{o}C.

3. Detection of normal flora in mosquito midgut

In a final volume of 50 ul of PCR reaction will be contained 1 ul of mosquito midgut DNA, 1x of 10x reaction buffer containing 18mM MgCl2 (FastStart High Fidelity Reaction Buffer, Roche®),2mM dNTP, 200 uM each primer, 2.5 unit of FastStart High Fidelity Enzyme Blend (5 U/µl). Negative control (no DNA template) was included in every experiment.

The amplification reaction was performed in a thermocycler: one Initial denaturation at 95°C for 2 min, 30 cycles at 95°C for 1 min, 62°C for 30 sec and 72°C for 1 min, and a final extension at 72°C for 7 min. After amplifying, the DNA products were separated by

electrophoresis in a 1.5% (w/v) agarose gel, colorant by use ethidium bromide (BET) and immersed in TBE buffer.

4. 16S rDNA gene sequencing for bacterial identification

TTGE was performed on a Bio-Rad D-Code apparatus. Two back-to-back 20 cm × 20 cm × 1 mm 8% polyacrylamide (acrylamide:bis ratio, 37.5:1, by weight) gels were prepared in 50X Tris-acetate-EDTA buffer,7 mol/L urea, 0.04g of Ammonium persulfate, 40 ul of Temed. The 6 ul of PCR product was mix with 6 ul of loading dye (0.25% of Bromophenol Blue and 40% D+ saccharose) then, 10 μL of mix was loaded onto the gel. The electrophoresis was carried out at 46 V for 16 h at a constant temperature at 70°C increment of ～0.4 °C/h. The gels were stained in 2 mg/L ethidium bromide for 15 min and imaged with a digital CCD gel documentation system. Confirmation of the nucleotide alteration was performed by direct DNA sequencing of the PCR product from PCR amplified before sequence analysis.

3. Data analysis

Sequence analysis: For preliminary identification, the 16S rRNA genes sequences will be analyzed in BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). The bacterial species will be discussed.

Statistical analysis will be done using SPSS software. The bacterial biodiversity in mosquitoes infected by parasites will be analyzed. Mean number of each bacterial species in each mosquito species, will be compared using independent sample t-test. Correlation test will be performed to observe any relationship between mosquito, parasite and bacterial species. All statistical significant will be set at $P < 0.05$

Results

The Anopheline mosquitoes were collected from two different provincial sites in western part of Thailand, along Thai-Myanmar border. The high prevalence of malaria and filariasis cases in each area was considered for indicating the collection sites. One collecting site in each province was selected, Mae Sod district, Tak Province and Sop Moei district, Mae Hong Son province. The collections will be done every two months interval from February 2011 to January 2013. All Anopheline mosquitoes from two study sites were morphologically sorting.

A total of 4,392 anophelines, 2,532 anophelines was collected in Tak province and 1,860 anophelines was found in Mae Hong Son province during the two months interval in 7 months of study. Tak Province showed higher number of members of three different anopheline vector complexes; *Anopheles minimus* (1,593), *An. maculates* (790) and *An. dirus* (19) more than Mae Hong Son province; *Anopheles minimus* (586), *An. maculates* (895) and *An. dirus* (7) in all seven time collections. On the other hand, more diversity of Anopheles mosquitoes (out of main malaria vectors) were found from Mae Hong son province especially in rainy season, are presented in Table 1. Lower number of collected mosquito was seen in indoor human landing method compared with the others. Cow baited dwelling show higher number of mosquitoes in all species (Table 1). Landing rates by hour and method for *An. minimus* was show in figure 2. The slightly increase number of mosquito in cow baited dwelling was found from 19.00-22.00h (more than 70 mosquitoes).

The higher number of collected mosquito in outdoor human landing method, compared with indoor human landing one, was found in every hour throughout the night. The interaction between environmental factors and mosquito abundance will be determined after 2 years collection.

The mosquito species identification by molecular method was applied to confirm the three main malaria vectors species (Minimus, Dirus and Maculatus complexes).Out of 704 Minimus complex mosquitoes which was done by PCR, 678 mosquitoes from Tak Province are *Anopheles minimus*, 26 mosquitoes from Mae Hong Son, 25 mosquitoes are *An. minimus*, only 1 mosquitoes is *An. aconitus* (Figure 2). The Dirus Complex, 24 mosquito were identified, 19 mosquito from Tak Province comprise 14 *An. dirus* and 5 *An. baimaii*, 6 mosquito from Mae Hong Son including 2 *An. dirus* and 4 *An.baimaii* (Figure 3). The Maculatus complex, 145 mosquitoes were identified by PCR. Tak province, 114 *An. maculates*, 8 *An. swadwongporni*, and 6 *An. dravidicus* which were confirmed by DNA sequencing (Table 3). All 17 specimens from Mae Hong son are *An. swadwongporni* (Figure 4).

The detection for normal flora in mosquito's midgut was studied in 48 mosquitos, the 34 positive mosquitoes were found and 58 positive bands were screened by TTGE. All positive bands were sequenced and the result was shown in Table 2, mostly are *Enteropacter sp*. with 100% identification.

Discussion

Malaria remains an important health threat in rural and forest areas. These areas remain highly conducive to vector-borne diseases due to geography, uncontrolled population

movement and recurring political unrest. One reason why the infection occurs in isolated jungle villages where the inhabitants spend part of the year in rudimentary huts in order to work what remains of the jungle (Somboon et al., 1998). Near the borders, rubber plantations and orchards are also highly conducive to transmission (Singhasivanon et al., 1999) and therefore, adult male population who work near and in these forest plantations are a special group at risk. Malaria transmission in forested areas along the Thai-Myanmar border is intense because of the combination of presence of highly efficient vectors and extensive population movement across the border, especially foreign workers and refugees (Manguin et al., 2010).

The three complexes of malaria vector in Thailand still are Minimus, Dirus and Maculatus complexes. There were prevalent in the Thai-Myanmar border area. Previous studies have showed some biting pattern and trophic preference of members in each complex but only few study which work in all three complexes (Sungvornyothin et al., 2006). In this study will be completed the biting pattern of the three complexes anophelines in near future.

The insecticide residual spray for malaria control program, severally 4 months interval, in the two areas, was introduced to the villager's hut. It may disturb the result of this study. Even though, in this study, the deserted hut was used for indoor human landing collection. Also, the collection was disturbed by many villager stay outside their home during 1900h to 2200h. Higher temperature/ humidity were found in indoor compared with outdoor. However, the result of this study will conduct for implementation of mosquito collection by determining the location etc. In a little while, the molecular study will be started for parasite mosquito detection.

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Table 1. Species diversity and number of Anopheles mosquitoes base on morphological identification, from Mae Sod, Tak Province and Sop Moei, Mae Hong Son Province, Thailand during February 2011to July 2012

Province	Bait	Place	Species*													
			A	B	C	D	E	F	G	H	I	J	K	L	M	N
Tak	Human	Indoor	419	4	86	0	0	0	1	0	1	0	0	0	0	0
		Outdoor	440	14	192	0	0	3	1	0	0	0	0	0	1	1
	Cow		734	1	512	8	12	22	32	5	22	2	1	0	0	18
Mae Hong Son	Human	Indoor	49	1	7	0	0	0	0	0	0	0	0	0	0	0
		Outdoor	141	2	52	0	1	0	0	0	0	0	0	0	0	1
	Cow		396	4	836	6	47	109	49	13	26	1	9	21	1	88
Total			2179	26	1685	14	60	134	83	18	49	3	10	21	2	95

*The letter stand for mosquito species;

A	*An. minimus*	E	*An. jamesii*	I	*An. vagus*	M	*An. kawari*
B	*An. dirus*	F	*An. kochi*	J	*An. stephensi*	N	*An. peditaeniatus*
C	*An. maculatus*	G	*An. barbirostris*	K	*An. subpictus*		
D	*An. nivipes*	H	*An .philippinensis*	L	*An. culicifacies B*		

Table 2. Blast results of bacterial flora detection from Anopheline mosquito ‘s midgut in difference mosquito species.

Code	Species	Descriptions	Number of blasted nucleotides	Blast results	Max ident	Accession n°
3_1 HDA1F	*Anopheles minimus*	mi42, indoor, Tak	170 bp	*Pseudomonas sp.*	98%	HQ290095.1
3_2 HDA1F	*Anopheles minimus*	mi42, indoor, Tak	170 bp	*Pseudomonas sp.*	98%	HQ446870.1
4_1 HDA1F	*Anopheles minimus*	mi43, indoor, Tak	174 bp	*Klebsiella sp.*	97%	Gu003839.1
4_2 HDA1F	*Anopheles minimus*	mi43, indoor, Tak	170 bp	*Pantoea sp.*	97%	AJ001239.1
	Anopheles minimus	mi43, indoor, Tak		*Klebsiella sp.*	97%	Gu003839.1
4_4 HDA1F	*Anopheles minimus*	mi43, indoor, Tak	168 bp	*Enteropacter sp.*	96%	AB695204.1
4_5 HDA1F	*Anopheles minimus*	mi43, indoor, Tak	170 bp	*Enteropacter sp.*	97%	JA194193.1
4_6 HDA1F	*Anopheles minimus*	mi43, indoor, Tak	175 bp	*Enteropacter sp.*	99%	Gu459209.1
8_1 HDA1F	*Anopheles minimus*	mi136,outdoor, Tak	168 bp	*Klebsiella sp.*	96%	Gu003839.1
8_2 HDA1F	*Anopheles minimus*	mi136,outdoor, Tak	172 bp	*Enteropacter sp.*	98%	AB695204.1
8_3 HDA1F	*Anopheles minimus*	mi136,outdoor, Tak	159 bp	*Enteropacter sp.*	100%	HM103364.1
8_4 HDA1F	*Anopheles minimus*	mi136,outdoor, Tak	167 bp	*Enteropacter sp.*	100%	HM103365.1
10_1 HDA1F	*Anopheles minimus*	mi143,outdoor, Tak	162 bp	*Acineiobactor sp.*	92%	JQ039984.1
12_1 HDA1F	*Anopheles minimus*	mi150,outdoor, Tak	174 bp	*Enteropacter sp.*	99%	JN634853.1
12_2 HDA1F	*Anopheles minimus*	mi150,outdoor, Tak	154 bp	*Klebsiella sp.*	100%	JN835542.1
	Anopheles minimus	mi150,outdoor, Tak		*Enteropacter sp.*	100%	JN835523
12_3 HDA1F	*Anopheles minimus*	mi150,outdoor, Tak	168 bp	*Pantoea sp.*	99%	AY659872.1
	Anopheles minimus	mi150,outdoor, Tak		*Enteropacter sp.*	98%	JF832953.1

13_1 HDA1F	*Anopheles minimus*	mi390,cow bait, MHS	165 bp	*Elizabethleingia sp.*	98%	AB680291.1
14_3 HDA1F	*Anopheles minimus*	mi391,cow bait, MHS	171 bp	*Serratia sp.*	99%	JF713819.1
15_1 HDA1F	*Anopheles minimus*	mi393,cow bait, MHS	155 bp	*Serratia sp.*	100%	GU294130.1
16_1 HDA1F	*Anopheles minimus*	mi398,cow bait, MHS	159 bp	*Klebsiella sp.*	97%	JN540016.1
17_1 HDA1F	*Anopheles minimus*	mi403,cow bait, MHS	168 bp	*Enteropacter sp.*	100%	GU459207.1
17_2 HDA1F	*Anopheles minimus*	mi403,cow bait, MHS	169 bp	*Enteropacter sp.*	99%	JN695719.1
17_3 HDA1F	*Anopheles minimus*	mi403,cow bait, MHS	161 bp	*Enteropacter sp.*	99%	JN695719.1
17_4 HDA1F	*Anopheles minimus*	mi403,cow bait, MHS	155 bp	*Enteropacter sp.*	99%	JN695719.1
di3_1 HDA1F	*An.baimaii*	di3, indoor, Tak	170 bp	*Acineiobactor sp.*	98%	FJ386957.1
		di3, indoor, Tak		*Pseudomonas sp.*	98%	JF430802.1
di4_1 HDA1F	*An.dirus*	di4, indoor, Tak	163 bp	*Elizabethleingia sp.*	98%	AB680291.1
di13_2 HDA1F	*An.dirus*	di13, indoor, MHS	142 bp	*Serratia sp.*	91%	FM202483.1
di13_4 HDA1F	*An.dirus*	di13, indoor, MHS	174 bp	*Serratia sp.*	100%	GU294130.1
di24_1 HDA1F	*An.baimaii*	di24,Outdoor, MHS	111 bp	No significant similarity found		
ma1_1 HDA1F	*An.maculatus*	ma1,indoor,Tak	161 bp	*Enteropacter sp.*	94%	GU459207.1
ma1_2 HDA1F	*An.maculatus*	ma1,indoor,Tak	167 bp	*Enteropacter sp.*	94%	GU459207.1
ma1_3 HDA1F	*An.maculatus*	ma1,indoor,Tak	169 bp	*Enteropacter sp.*	95%	GU459207.1
ma1_4 HDA1F	*An.maculatus*	ma1,indoor,Tak	169 bp	*Enteropacter sp.*	94%	GU459207.1
ma1_5 HDA1F	*An.maculatus*	ma1,indoor,Tak	165 bp	*Enteropacter sp.*	94%	GU459207.1
ma26_3 HDA1F	*An.sawadwongporni*	ma26,outdoor,Tak	156 bp	*Enteropacter sp.*	94%	GU459207.1
ma32_4 HDA1F	*An.maculatus*	ma32,outdoor,Tak	170 bp	*Enteropacter sp.*	94%	GU459207.1

ma32_6 HDA1F	*An.maculatus*	ma32,outdoor,Tak	168 bp	*Enteropacter sp.*	94%	GU459207.1
ma32_7 HDA1F	*An.maculatus*	ma32,outdoor,Tak	169 bp	*Enteropacter sp.*	94%	GU459207.1
mi397_1 HDA1F	*An.minimus*	mi397, cow bait, MHS	163 bp	*Enteropacter sp.*	94%	GU459207.1
mi397_2 HDA1F	*An.minimus*	mi397, cow bait, MHS	168 bp	*Enteropacter sp.*	94%	GU459207.1
mi397_3 HDA1F	*An.minimus*	mi397, cow bait, MHS	171 bp	*Enteropacter sp.*	94%	GU459207.1
mi397_5 HDA1F	*An.minimus*	mi397, cow bait, MHS	170 bp	*Enteropacter sp.*	94%	GU459207.1
mi397_6 HDA1F	*An.minimus*	mi397, cow bait, MHS	170 bp	*Enteropacter sp.*	94%	GU459207.1
mi404_1 HDA1F	*An.minimus*	mi404,cow bait, MHS	163 bp	*Enteropacter sp.*	94%	GU459207.1
mi409_1 HDA1F	*An.minimus*	mi409,cow bait, MHS	164 bp	*Enteropacter sp.*	94%	GU459207.1
mi410_1 HDA1F	*An.minimus*	mi410,cow bait, MHS	153 bp	*Enteropacter sp.*	94%	GU459207.1
mi412_1 HDA1F	*An.minimus*	mi412,cow bait, MHS	159 bp	*Enteropacter sp.*	94%	GU459207.1
mi415_1 HDA1F	*An.minimus*	mi415,cow bait, MHS	167 bp	*Enteropacter sp.*	94%	GU459207.1
mi417_3 HDA1F	*An.minimus*	mi417,cow bait, MHS	167 bp	*Enteropacter sp.*	94%	GU459207.1
mi418_1 HDA1F	*An.minimus*	mi418,cow bait, MHS	171 bp	*Enteropacter sp.*	94%	GU459207.1
mi418_2 HDA1F	*An.minimus*	mi418,cow bait, MHS	168 bp	*Enteropacter sp.*	94%	GU459207.1
mi418_5 HDA1F	*An.minimus*	mi418,cow bait, MHS	157 bp	*Enteropacter sp.*	94%	GU459207.1
mi418_7 HDA1F	*An.minimus*	mi418,cow bait, MHS	170 bp	*Enteropacter sp.*	94%	GU459207.1
mi419_1 HDA1F	*An.minimus*	mi419,cow bait, MHS	163 bp	*Enteropacter sp.*	94%	GU459207.1

Table 3. Blast result of 5 *Anopheles dravidicus* samples

Code	Species	Number of blasted nucleotides	Blast results	Max ident	Accession n°
56_5.8 F	*An.dravidicus*	424 bp	*Anopheles dravidicus sp.*	99%	DQ518624.1
107_5.8 F	*An.dravidicus*	430 bp	*Anopheles dravidicus sp.*	99%	DQ518624.2
117_5.8 F	*An.dravidicus*	432 bp	*Anopheles dravidicus sp.*	99%	DQ518624.3
142_5.8 F	*An.dravidicus*	422 bp	*Anopheles dravidicus sp.*	99%	DQ518624.4
168_5.8F	*An.dravidicus*	431 bp	*Anopheles dravidicus sp.*	99%	DQ518624.5

Primers and fragment size for each species

Species	Primer name	Sequence (5′ to 3′)	Size of the product(bp)
Universal forward primer	ITS2A	TGT GAA CTG CAG GAC ACA T	
An. minimus	MIA	CCC GTG CGA CTT GAC GA	310 bp
An. harrisoni	MIC	GTT CAT TCA GCA ACA TCA GT	180 bp
An. aconitus	ACO	ACA GCG TGT ACG TCC AGT	200 bp
An. varuna	VAR	TTG ACC ACT TTC GAC GCA	260 bp
An. pampanai	PAM	TGT ACA TCG GCC GGG GTA	90 bp

Primers and expected fragment size for each species

Species	Primer	Sequence (5´ to 3´)	Size of product(bp)
Universal forward primer	ITS2A	TGT GAA CTG CAG GAC ACA T	
An. dirus	D-U	GCG CGG GGC CGA GGT GG	562 bp
An. scanloni	D-AC	CAC AGC GAC TCC ACA CG	349 bp
An. cracens	D-B	CGG GAT ATG GGT CGG CC	514 bp
An. baimaii	D-D	GCG CGG GAC CGT CCG TT	306 bp
An. nemophilous	D-F	AAC GGC GGT CCC CTT TG	223 bp

Primers and expected fragment size for each species

Species	Primer	Sequence (5´ to 3´)	Size of product(bp)
Universal forward primer	5.8F	ATC ACT CGG CTC GTG GAT CG	
An. maculatus	MAC	GAC GGT CAG TCT GGT AAA GT	180 bp
An. pseudowillmori	PSEU	GCC CCC GGG TGT CAA ACA G	203 bp
An. sawadwongporni	SAW	ACG GTC CCG CAT CAG GTG C	242 bp
An. dravidicus	DRAV	GCC TAC TTT GAG CGA GAC CA	477 bp
Form K	K	TTC ATC GCT CGC CCT TAC AA	301 bp

Primers and expected fragment size for each *Plasmodium* species

Species	Primer	Sequence (5′ to 3′)	Size of product(bp)
P. falciparum	Pf1	CCT GCA TTA ACA TCA TTA TAT GGT ACA TCT	273 bp
	Pf2	GAT TAA CAT TCT TGA TGA AGT AAT GAT AAT ACC TT	
P. vivax	Pv1	AAG TGT TGT ATG GGC TCA TCA TAT G	290 bp
	Pv2	CAA AAT GGA AAT GAG CGA TTA CAT	

Primers and expected fragment size for each Bacterial species

Primer	**Sequence (5´ to 3´)**	**Size of product(bp)**
HDA1f_ GC	ACT CCT ACG GGA GGC AGC AGT	199 bp
HDA2r	GTA TTA CCG CGG CGG CTG CTG GCA	
	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGC GGG G	

Figure 1. Biting pattern and trophic preference of *Anopheles minimus* from Tak province in seven months (February 2011 to January 2012).

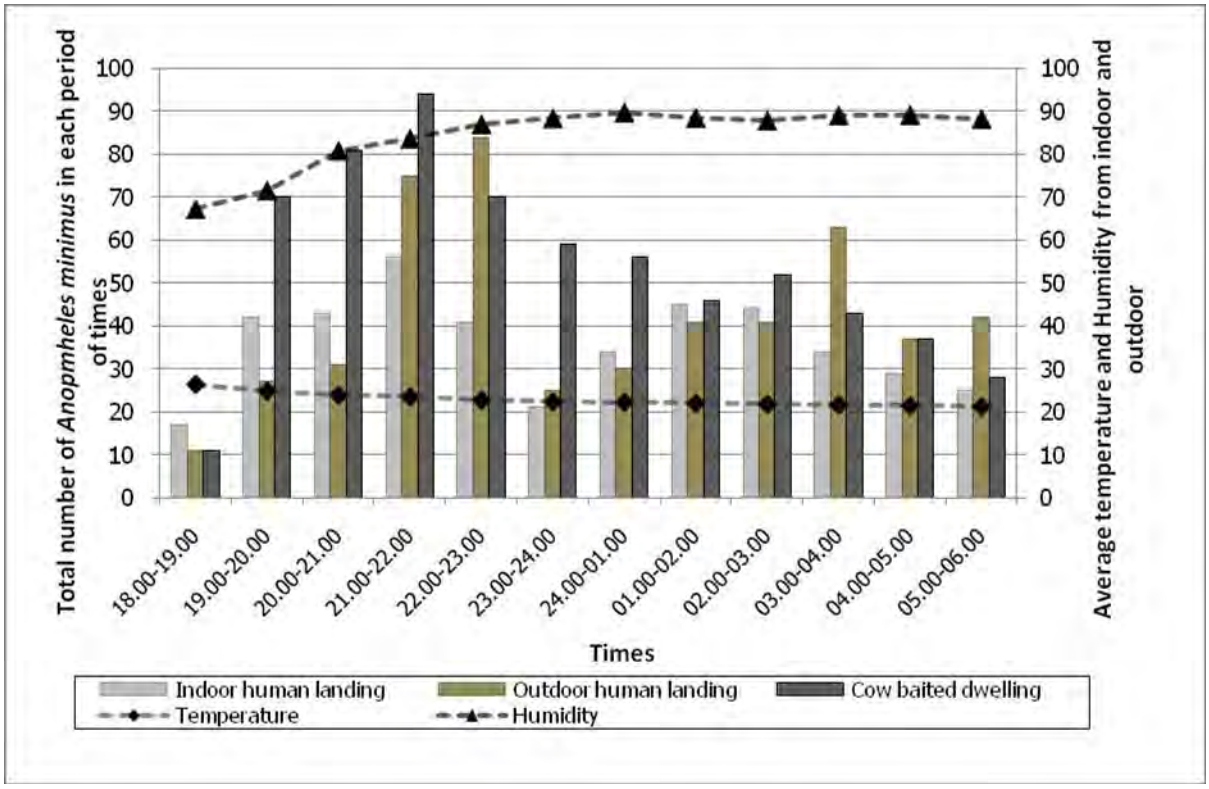


Figure 2. Multiplex Allele-Specific PCR assay for Minimus complex. Lanes 1: *An. aconitus,* Lane 2-7: *An. minimus*; Lane 8: 100 bp molecular ladder; Lane 9: negative control; Lane 10-15: positive control

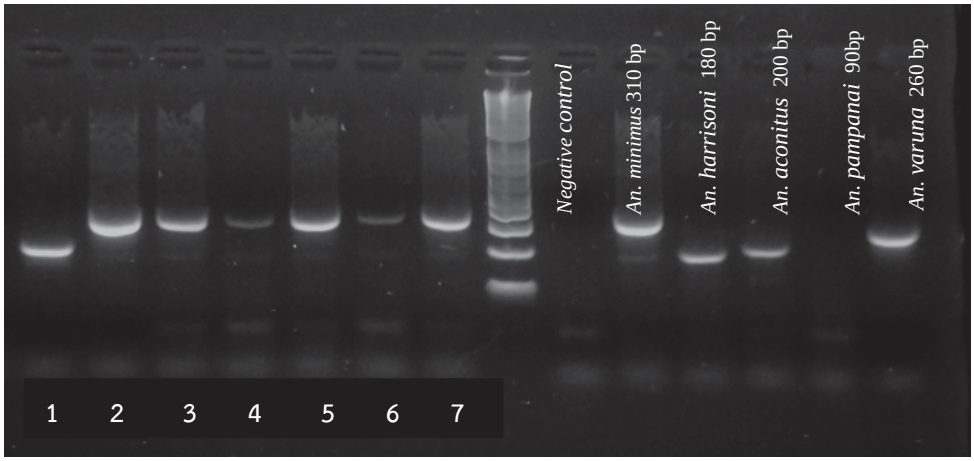


Figure 3. Multiplex Allele-Specific PCR assay for Dirus Complex. Lanes 1, 2, 4, 6-9,11: *An. dirus;* Lane 3, 5, 10, 12 ; *An. baimaii* ; Lane 13: 100 bp molecular ladder; Lane 14: negative control; Lane 15-17: positive control

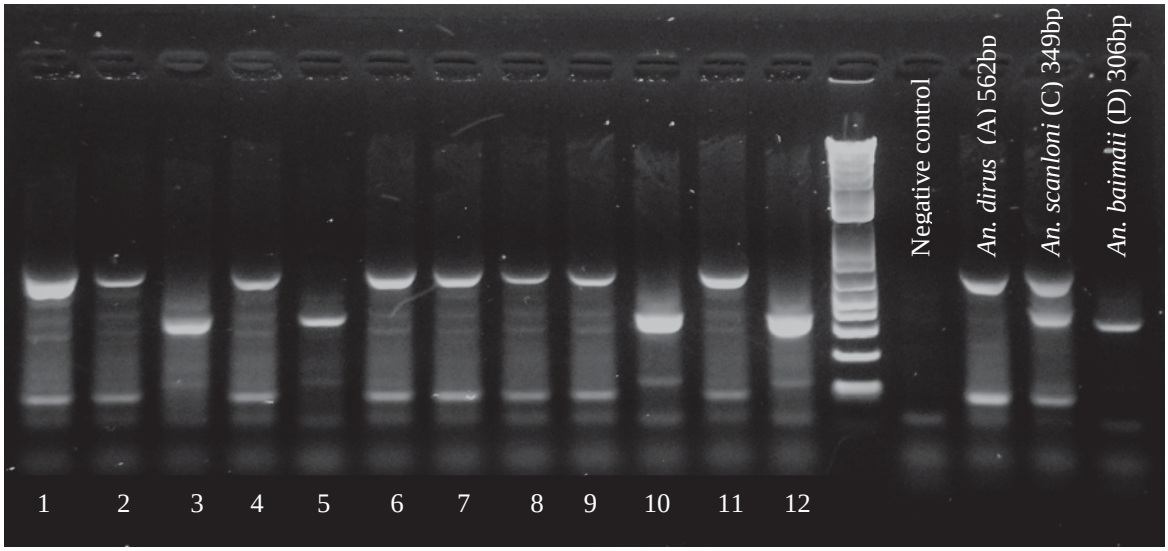
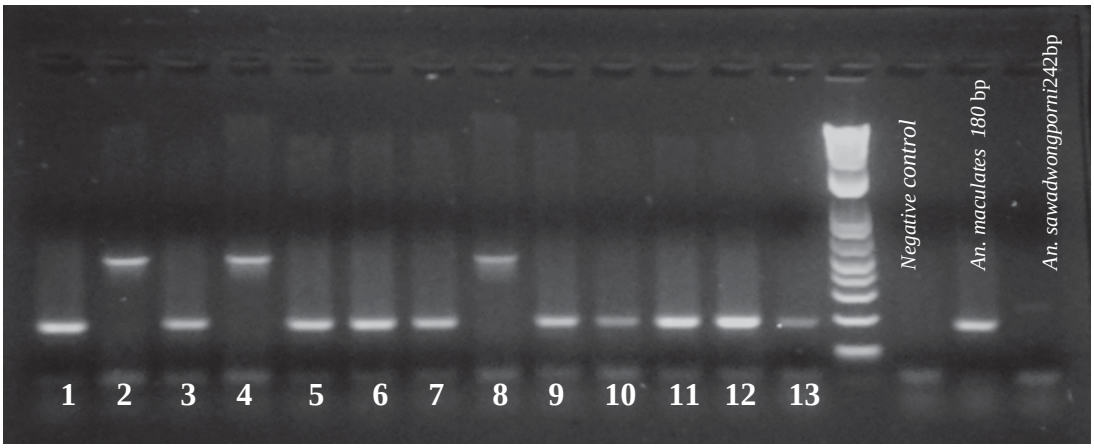


Figure 4. Multiplex Allele-Specific PCR assay for Maculatus Complex. Lanes 1, 3, 5-7, 9-13: *An. maculates;* Lane 2, 4, 8 ; *An. dravidicus*; Lane 14: 100 bp molecular ladder; Lane 15: negative control; Lane 16-17: positive control



Subproject-6 (Dr.Suprada Sukhonthabhirom na Pathalung)

Molecular identification and phylogenetics of *Stomoxys calcitrans* (L.) (Diptera: Muscidae) in Thailand

Introduction

Stable flies belong to the tribe of Stomoxyini (Diptera: Muscidae). The genus *Stomoxys* contains at least eighteen 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. Among these, *Stomoxys calcitrans* (L.), usually called stable fly, is considered as a cosmopolitan species. Both male and female stable flies feed on blood, generally once a day per fly. These insects are aggressive and sometimes bite human in extreme conditions. Although they are quite active at the livestock farms, they are invariably a nuisance insect on beaches and in residential areas used for agricultural purposes with flight range of one mile.

Stable 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 weight gains and production losses. The economic losses of up to a half pound a day in weight gain and 30-40% in milk yield were observed (Hall *et al*., 1982; Mullens *et al*., 1988). In the United States, the estimate loss to beef and dairy industry is of 399$ million annually (Smith *et al*., 1987). High number of flies biting cattle may have an impact on the epidemiology of transmitted diseases. Several stable fly species are considered as vectors of anaplasmosis, trypanosomosis and bovine leucosis virus in dairy cattle (Buxton *et al*., 1985; Mihok *et al*., 1995, 1996; Torr *et al*., 2006).

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 (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). Recently, a more field friendly trap, Vavoua trap, which was originally designed for tsetse fly collection was used for stable fly collection. This trap has proved very efficient to capture *Stomoxys spp* in many regions in African countries (Holloway and Phelps, 1991; Mihok *et al*., 1995) and in La Reunion Island (Gilles *et al*., 2007).

While most studies on stable flies, particularly *S. calcitrans*, have been documented in Africa and the United States, in Thailand, relatively little is known about stable fly distribution. The knowledge of molecular phylogenetics is necessary for the development of models that will provide a better understanding of the geographic distribution of stable flies in Thailand.

Systematic studies have demonstrated that some genes are clearly better than others for reconstructing evolutionary relationships among taxa at particular levels of divergence. The majority of genes used for molecular studies are mitochondrial genes because they are easier to manipulate; are clonally inherited, single copy and non recombining; and are abundant. Ribosomal genes have been studied intensively because of their critical role in protein assembly. As a result of their universal occurrence, sequence and structural conservation and abundance, they have been used for phylogenetic analyses of a wide range of species and divergence levels. These genes have proven useful in molecular phylogenetics (Sharpe *et al*., 2000; Arrivillaga *et al*., 2002; Becerra, 2004; Dsouli, 2009; Morgan *et al*., 2009).

For the phylogenetic analysis of aligned sequences, virtually all methods describe sequence evolution using a model that consists of two components: a phylogenetic tree and a description of the way individual sequences evolve by nucleotide replacement along the branches of that tree. Different models are distinguished by their assumptions or parameterizations regarding the average rates of occurrence of all the possible replacements. There are two approaches to building models of sequence evolution. One approach is to build models empirically using properties calculated through comparisons of large numbers of observed sequences. The alternative approach is for models to be built parametrically on the basis of the chemical or biological properties of DNA. Both methods result in Markov process models, defined by matrices containing the relative rates of occurrence of all possible replacements. From these are calculated the probabilities of change from any nucleotide to any other nucleotide, including the probability of remaining the same, over any period of evolutionary time at any site (Whelan *et al*., 2001).

There are currently two main categories of tree-building methods. The first category is based on discrete characters, which are molecular sequences from individual taxa. The basic assumption is that characters at corresponding positions in a multiple sequence alignment are homologous among the sequences involved. Therefore, the character states of the common ancestor can be traced from this dataset. Another assumption is that each character evolves

independently and is therefore treated as an individual evolutionary unit. The second category of phylogenetic methods is based on distance, which is the amount of dissimilarity between pairs of sequences, computed on the basis of sequence alignment. The distance-based methods assume that all sequences involved are homologous and that tree branches are additive, meaning that the distance between two taxa equals the sum of all branch lengths connecting them.

The algorithms for the distance-based tree-building method can be subdivided into clustering based and optimality based. The clustering-type algorithms compute a tree based on a distance matrix starting from the most similar sequence pairs. These algorithms include an unweighted pair group method using arithmetic average (UPGMA) and neighbor joining. The optimality-based algorithms compare many alternative tree topologies and select one that has the best fit between estimated distances in the tree and the actual evolutionary distances. This category includes the Fitch-Margoliash and minimum evolution algorithms (Xiong, 2006).

Character-based methods are based directly on the sequence characters rather than on pairwise distances. They count mutational events accumulated on the sequences. This preservation of character information means that evolutionary dynamics of each character can be studied. Ancestral sequences can also be inferred. The two most popular character-based approaches are the maximum parsimony (MP) and maximum likelihood (ML) methods.

The parsimony method chooses a tree that has the fewest evolutionary changes or shortest overall branch lengths. By this principle, a tree with the least number of substitutions is probably the best to explain the differences among the taxa under study. This view is justified by the fact that evolutionary changes are relatively rare within a reasonably short time frame. This implies that a tree with minimal changes is likely to be a good estimate of the true tree. By minimizing the changes, the method minimizes the phylogenetic noise owing to homoplasy and independent evolution.

Another character-based approach is ML, which uses probabilistic models to choose a best tree that has the highest probability or likelihood of reproducing the observed data. It finds a tree that most likely reflects the actual evolutionary process. ML is an exhaustive method that searches every possible tree topology and considers every position in an alignment, not just informative sites. By employing a particular substitution model that has probability values

of residue substitutions, ML calculates the total likelihood of ancestral sequences evolving to internal nodes and eventually to existing sequences (Xiong, 2006).

Materials and Methods

Sample collections

Stable fly adults were collected with sweep nets and preserved in 95% ethanol from fourteen localities in Thailand (Fig 1).

- Northern region

Chaiprakan district, Chiang Mai province (N 19° 43', E 99° 8')

Phan district, Chiang Rai province (N 19° 33', E 99° 44')

- Northeastern region

Wang Nam Kheow district, Nakhon Ratchasima province (N 13° 21', E 102° 11')

Mueang district, Khon Kaen province (N 13° 21', E 102° 11')

Waritchaphum district, Sakon Nakhon province (N 17° 16', E 103° 33')

Warin Chamrap district, Ubon Ratchathani province (N 15° 7', E 104° 55')

- Central region

Phatthana Nikhom district, Lop Buri province (N 14° 50', E 100° 59')

Muak Lek district, Saraburi province (N 14° 31', E 100° 52')

- Western region

Kaeng Krachan district, Phetchaburi province (N 12° 35', E 99° 33')

Photharam district, Ratchaburi province (N 13° 44', E 99° 55')

- Eastern region

Wang Sombun district, Sa Kaeo province (N 13° 21', E 102° 11')

Soi Dao district, Chanthaburi province (N 13° 15', E 102° 11')

- Southern region

Rattaphum district, Songkhla province (N 7° 7', E 100° 14')

Mueang district, Phatthalung province (N 7° 38', E 99° 56')

DNA extraction, amplification and sequencing

Genomic DNA was extracted from individual flies using DNeasy tissue kit (QIAGEN) according to the instructions of the manufacturer. The internal transcribed spacer 2 (ITS 2), cytochrome c oxidase subunit I (CO I) and cytochrome b (Cyt b) regions were amplified using the polymerase chain reaction (PCR).

Nucleotide sequences of the primers are as follows.

ITS 2 amplification:

ITS2A (5′-TGTGAACTGCAGGACACAT-3′) and

ITS2B (5′-TATGCTTAAATTCAGGGGGT-3′) (Sharpe *et al*., 2000)

CO I amplification:

C1-J-2183 (5′-CAACATTTATTTTGATTTTTTGG-3′) and

TL2-N-3014 (5′-TCCATTGCACTAATCTGCCATATTA-3′) (Simon *et al*., 1994)

Cyt b amplification:

CB-J-10933 (5′-GTTTTACCTTGAGGACAAATATC-3′) and

CB-N-11526 (5′-TTCAACTGGTCGAGCTCCAATTCA-3′) (Simon *et al*., 1994)

The ITS 2, CO I and Cyt b amplifications were done in 20 μl reaction volume: 12.4 μl dH_2O, 2 μl 10x PCR buffer, 1.3 μl of 25 mM $MgCl_2$, O.2 μl dNTPs (5 mM each), 1 μl of 25 μM forward and reverse primers, 2 μl DNA template, and 0.5 U of Taq DNA polymerase (Red Gold Star®). PCR amplification was done with initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 95°C for 40 s, annealing at 54°C for ITS 2, 47°C for CO I, and 49°C for Cyt b, and extension at 72°C for 1 min, and final extension at 72°C for 7 min. Five microlitres of PCR product were run on a 1% agarose gel. DNA sequencing was conducted on an automated sequencer.

Molecular identification

The species-specific primers were designed based on ITS 2 sequence variation of *S. calcitrans* and *S. sitiens* from GenBank (Fig 5). The ITS2A primer was used as forward primer. The species-specific primers were reverse primers as follows.

S. calcitrans (5′-CCAAGGTATAATTATATATGTATATG-3′)

S. sitiens (5′-AAAGTATATTGTTTTAATATGGCG-3′)

Each species-specific primer was tested the specificity in both species. The PCR amplifications were conducted in the same condition with ITS 2 amplification.

Phylogenetic analyses

Sequences were aligned in CLUSTAL W (Thompson *et al*., 1994) with default parameters, by first aligning all ingroup taxa and then adding the outgroup taxa using the profile alignment option. The Akaike Information Criterion (AIC) implemented in Modeltest (Posada and Crandall, 1998) was used to select the best-fit model for the combined CO I and Cyt b dataset. Phylogenies were inferred using maximum parsimony (MP) and maximum likelihood (ML) approaches. Analyses were conducted for the combined CO I and Cyt b dataset. For all analyses, *Haematobia irritans* and *Musca domestica* were specified as the outgroup. For comparison, phylogenies were estimated using the MP and ML approaches in PAUP* v4.0b10. ML analyses used the models selected by Modeltest for the nucleotide characters. Full heuristic MP and ML searches were performed with random sequence additions and tree bisection-reconnection (TBR) branch swapping. Bootstrap resampling with 1000 replicates was employed to assess nodal support.

Results

The stomoxyine flies were collected from fourteen localities within six geographical regions of Thailand: northern region, Chiang Mai and Chiang Rai provinces; northeastern region, Nakhon Ratchasima, Khon Kaen, Sakon Nakhon and Ubon Ratchathani provinces; central region, Lop Buri and Saraburi provinces; western region, Phetchaburi and Ratchaburi provinces; eastern region, Sa Kaeo and Chanthaburi provinces; southern region, Songkhla and Phatthalung provinces (Fig 1).

The total number of stomoxyine flies collected in each location was summarized in Table 1.

Genomic DNA was extracted from *S. calcitrans*, *S. indicus, S. sitiens, S. bengalensis* and *S. uruma* specimens in each collection site. The DNA extractions were amplified the internal transcribed spacer 2 (ITS 2), cytochrome c oxidase subunit I (CO I), and cytochrome b (Cyt b) regions. The sizes of PCR products were approximately 480 bp, 820 bp, and 600 bp, respectively (Fig 2, 3 and 4). The species-specific primers were designed for *S. calcitrans* and *S. sitiens* based on ITS 2 sequence variation (Fig 5). Each species-specific primer was specific in each species. The size of PCR products was approximately 360 bp (Fig 6 and 7).

Sequences obtained in this study have been deposited in GenBank (Table 2). The combined CO I and Cyt b alignment for 22 individuals contained 1,344 bp with 272 were parsimony informative. When the combined CO I and Cyt b dataset was executed through Modeltest, the General Time Reversible + Invariable site + Gamma distribution (GTR+I+G) model was selected as most appropriate for these data with the following parameter settings. Base frequencies: freq A = 0.29900; freq C = 0.13030; freq G = 0.13880; and freq T = 0.43190. The MP and ML bootstrap consensus tree topologies were almost identical with only minor differences within species clades. Bootstrap support for *S. calcitrans*, *S. uruma*, *S. indicus,* and *S. bengalensis* was 100% under MP and ML analyses (Figure 8 and 9).

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Table 1. Total number of stomoxyine flies collected from fourteen localities in Thailand.

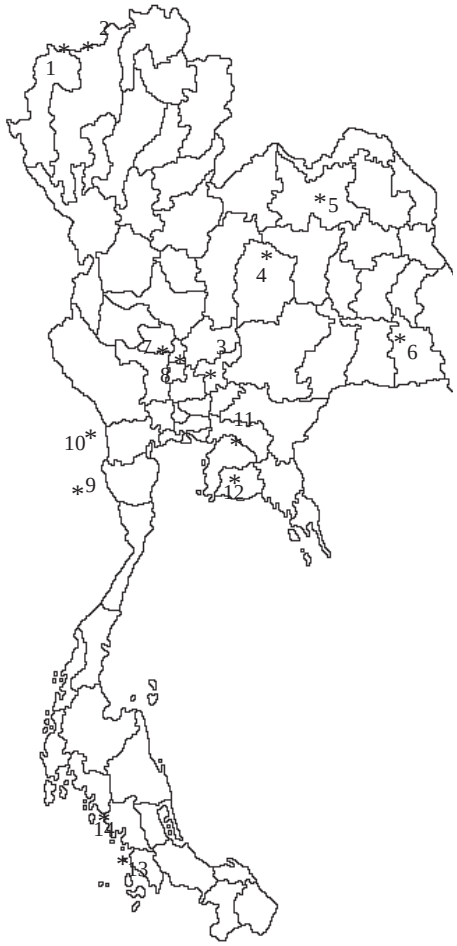
Collection sites (Provinces)	No. of stomoxyine flies				
	S. calcitrans	*S. indicus*	*S. sitiens*	*S. bengalensis*	*S. uruma*
Chiang Mai	52	45	0	0	0
Chiang Rai	66	15	6	1	0
Nakhon Ratchasima	273	125	26	1	0
Khon Kaen	18	7	7	0	0
Sakon Nakhon	29	10	9	0	0
Ubon Ratchathani	10	24	2	4	0
Lop Buri	41	8	26	1	0
Saraburi	53	3	4	0	0
Phetchaburi	96	0	1	0	14
Ratchaburi	49	36	21	1	0
Sa Kaeo	18	23	3	0	0
Chanthaburi	53	1	4	0	0
Songkhla	64	10	2	0	0
Pattalung	48	26	16	0	0

Table 2. Sample locations, gene region sequenced, and GenBank Accession numbers

Species / Locations	GenBank Accession numbers CO I	Cyt b	ITS 2
S. calcitrans			
Chanthaburi	JQ905846	JQ914836-42	JQ914959-61
Chiang Mai	JQ905847	JQ914843-45	JQ914962-63
Chiang Rai	JQ905848-50	JQ914846-49	JQ914964-65
Khon Kaen	JQ905851	JQ914850-53	JQ914966-68
Lop Buri	JQ905852	JQ914854-56	JQ914969-71
Nakhon Ratchasima	JQ905853	JQ914857-59	JQ914972-73
Phetchaburi	JQ905854-56	JQ914860-63	JQ914974-75
Pattalung	JQ905857-64	-	JQ914976-78
Ratchaburi	JQ905865-67	-	JQ914979-81
Saraburi	JQ905868-70	-	JQ914982-84
Sa Kaeo	JQ905871-74	JQ914864-67	JQ914985-87
Songkhla	JQ905875-77	-	JQ914988-90
Sakon Nakhon	JQ905878-80	JQ914868-70	JQ914991-93
Ubon Ratchathani	-	-	JQ914994-96
Greece	JQ905968-75	JQ914939-46	JQ915044-49
Gabon	-	JQ914937-38	JQ915042-43
S. indicus			
Chanthaburi	JQ905881-83	JQ914871-72	-
Chiang Mai	JQ905884-90	JQ914873-77	JQ914997-98
Chiang Rai	JQ905891-97	JQ914878-81	-
Khon Kaen	JQ905898-903	JQ914882-84	JQ914999
Lop Buri	JQ905904-10	JQ914885-86	-
Nakhon Ratchasima	JQ905911-17	JQ914887-92	-
Pattalung	JQ905918-19	JQ914893-97	JQ915000
Ratchaburi	JQ905920-22	JQ914898-99	-
Saraburi	JQ905923-25	-	-
Sa Kaeo	JQ905926-30	-	-

Songkhla	JQ905931-33	JQ914900-03	-
Sakon Nakhon	JQ905934-39	JQ914904-07	JQ915001
Ubon Ratchathani	JQ905940-45	JQ914908-09	JQ915002-03
S. sitiens			
Chanthaburi	JQ905946-47	JQ914910	JQ915004-06
Chiang Rai	JQ905948-50	-	JQ915007-09
Khon Kaen	JQ905951	JQ914911-13	JQ915010-12
Lop Buri	-	JQ914914-20	-
Nakhon Ratchasima	JQ905952-56	JQ914921-25	JQ915013-15
Phetchaburi	JQ905957-58	-	JQ915016-18
Pattalung	JQ905959	JQ914926	JQ915019-22
Ratchaburi	JQ905960-61	JQ914927-29	JQ915023-25
Saraburi	JQ905962	JQ914930-31	JQ915026-29
Sa Kaeo	-	JQ914932	JQ915030-32
Songkhla	JQ905963	JQ914933	JQ915033-36
S. uruma			
Phetchaburi	JQ905964-67	JQ914934-36	JQ915037-41
S. bengalensis			
Chiang Rai	JQ905838-40	JQ914829-30	JQ914947-49
Lop Buri	JQ905841-42	JQ914831	JQ914950-52
Nakhon Ratchasima	JQ905843-45	JQ914832-33	JQ914953
Ratchaburi	-	-	JQ914954-56
Ubon Ratchathani	-	JQ914834-35	JQ914957-58

Figure 1. Collection sites of stomoxyine flies in Thailand.



*** Collection sites**

1: Chaiprakan district, Chiang Mai province

2: Phan district, Chiang Rai province

3: Wang Nam Kheow district, Nakhon Ratchasima province

4: Mueang district, Khon Kaen province

5: Waritchaphum district, Sakon Nakhon province

6: Warin Chamrap district, Ubon Ratchathani province

7: Phatthana Nikhom district, Lop Buri province

8: Muak Lek district, Saraburi province

9: Kaeng Krachan district, Phetchaburi province

10: Photharam district, Ratchaburi province

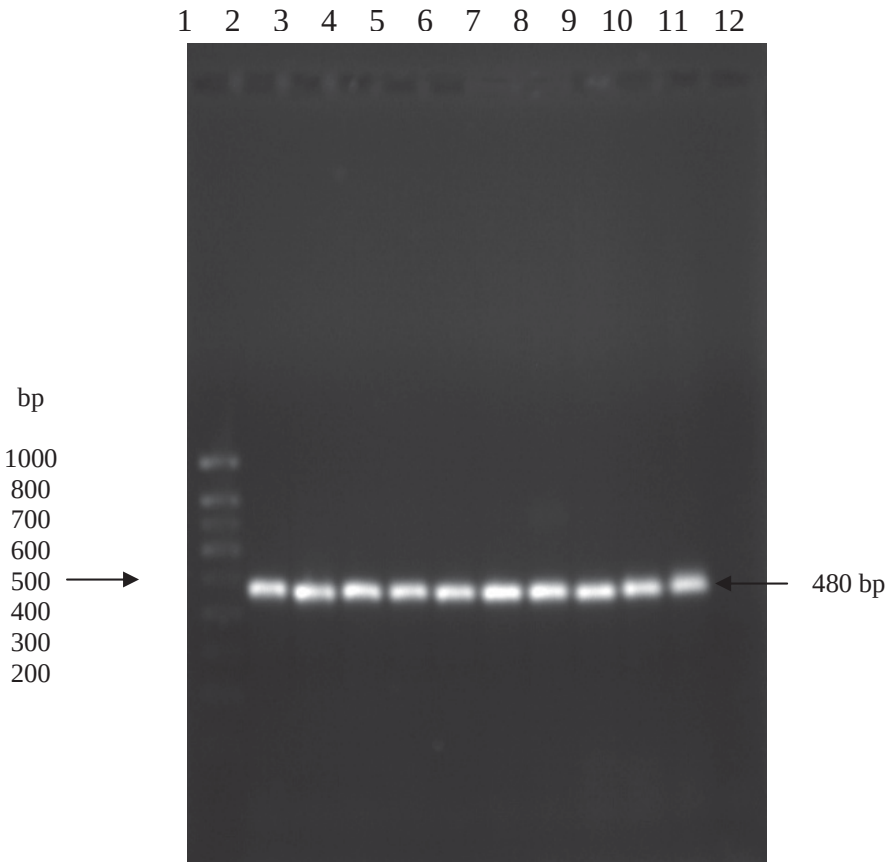
11: Wang Sombun district, Sa Kaeo province

12: Soi Dao district, Chanthaburi province

13: Rattaphum district, Songkhla province

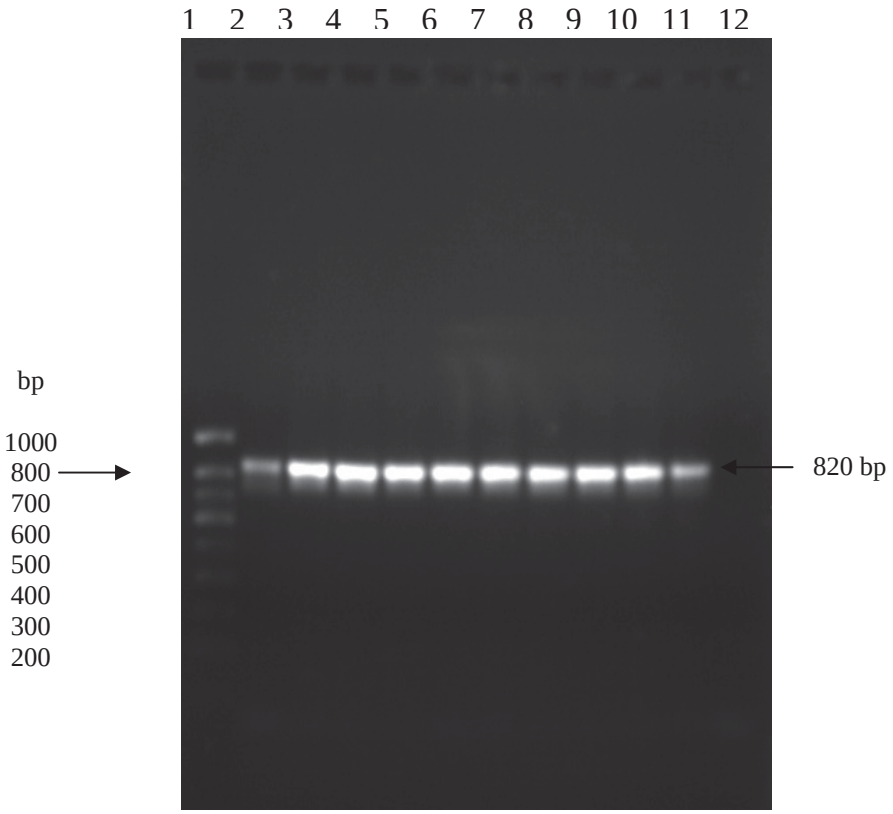
14: Mueang district, Phatthalung province

Figure 2. The ITS 2 amplification of stomoxyine flies



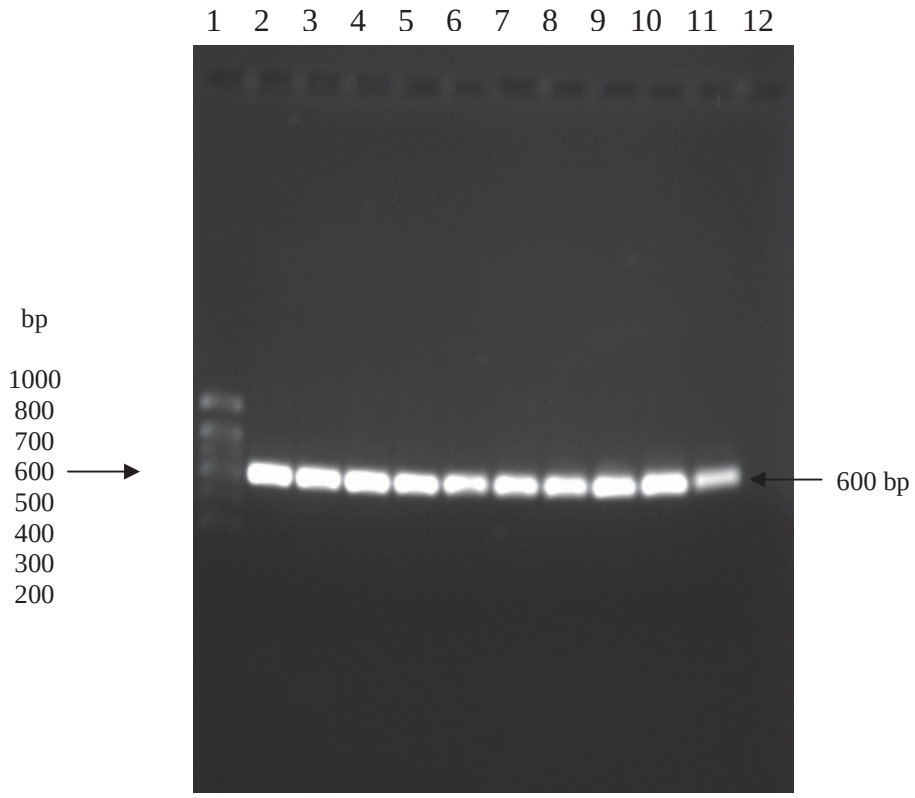
1: PCR marker, 2-3: *S. calcitrans*, 4-5: *S. sitiens*, 6-7: *S. indicus*, 8-9: *S. uruma*, 10-11: *S. bengalensis*, 12: negative control

Figure 3. The CO I amplification of stomoxyine flies



1: PCR marker, 2-3: *S. calcitrans*, 4-5: *S. sitiens*, 6-7: *S. indicus*, 8-9: *S. uruma*, 10-11: *S. bengalensis*, 12: negative control

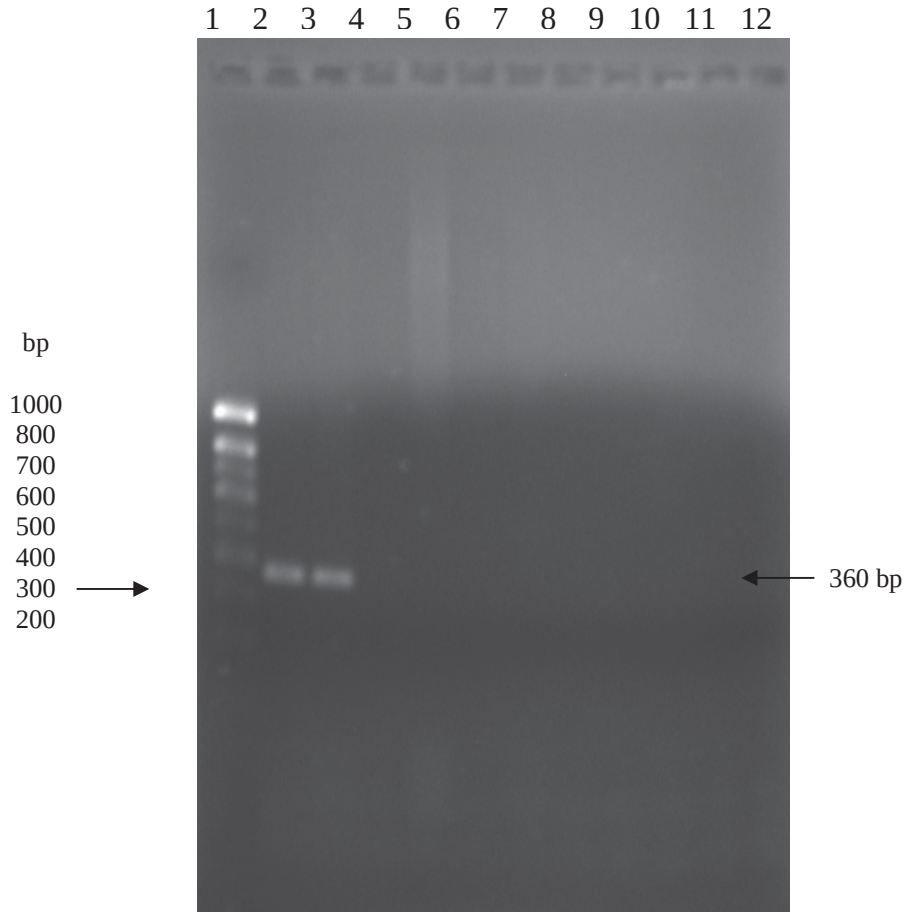
Figure 4. The Cyt b amplification of stomoxyine flies



1: PCR marker, 2-3: *S. calcitrans*, 4-5: *S. sitiens*, 6-7: *S. indicus*, 8-9: *S. uruma*, 10-11: *S. bengalensis*, 12: negative control

Figure 5. The alignment of ITS 2 sequences of *S. calcitrans* and *S. sitiens* from GenBank. Stars (*) indicate the identity of nucleotides within the alignment. Arrows (⟶) indicate the direction of species-specific PCR amplification.

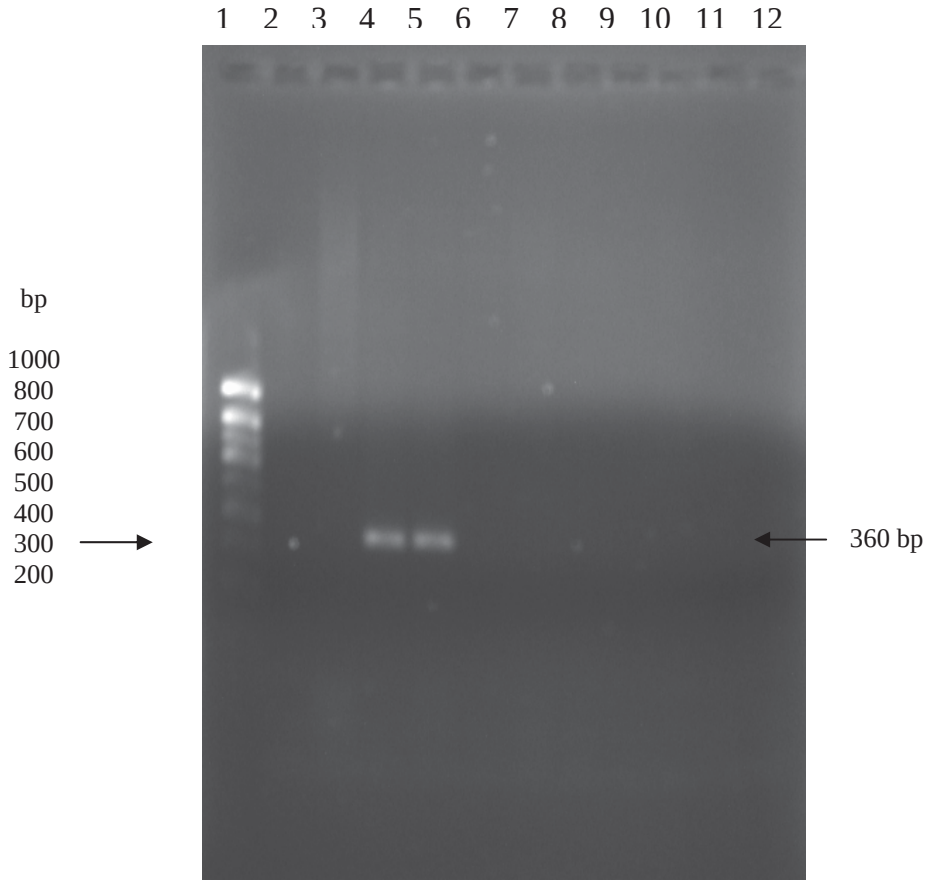
Figure 6. The PCR amplification of stomoxyine flies with *S. calcitrans* specific primer



1: PCR marker, 2-3: *S. calcitrans*, 4-5: *S. sitiens*, 6-7: *S. indicus*, 8-9: *S. uruma*,

10-11: *S. bengalensis*, 12: negative control

Figure 7. The PCR amplification of stomoxyine flies with *S. sitiens* specific primer



1: PCR marker, 2-3: *S. calcitrans*, 4-5: *S. sitiens*, 6-7: *S. indicus*, 8-9: *S. uruma*, 10-11: *S. bengalensis*, 12: negative control

Figure 8. Maximum parsimony consensus tree for *Stomoxys* flies and outgroups based on the combined analysis of CO I and Cyt b genes

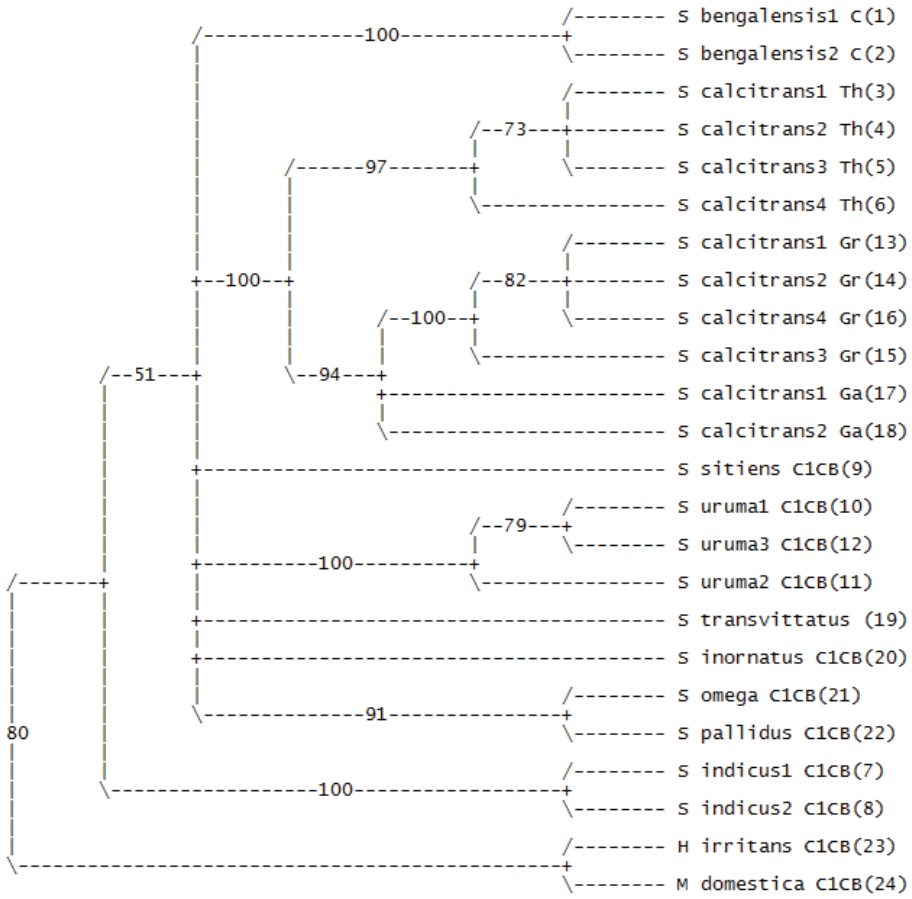
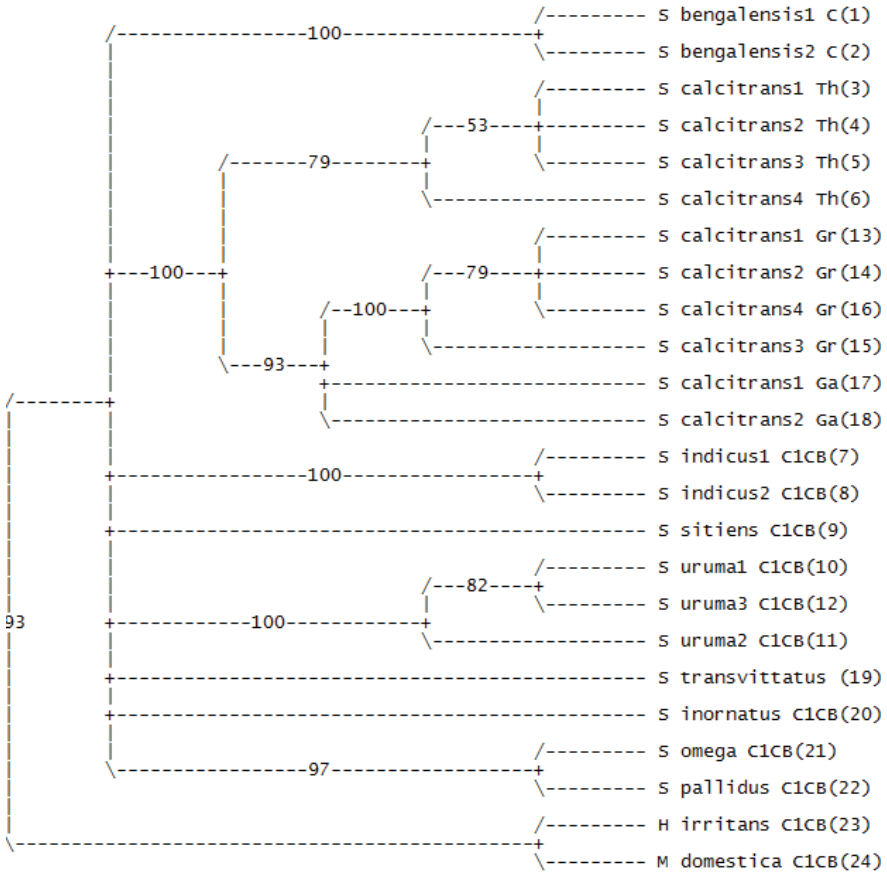


Figure 9. Maximum likelihood consensus tree for *Stomoxys* flies and outgroups based on the combined analysis of CO I and Cyt b genes



Subproject 7 (Dr. Jumnongjit Phasuk)

Circadian activity of *Stomoxys calcitrans* in Thailand

Summary

- A total of 2,520 individuals belonging to four species were collected. The following 4 species were captured: *S. bengalensis* Picard, 1908, *S. calcitrans* (Linnaeus, 1758), *S. indicus* Picard, 1908 and *S. sitiens* Rondani, 1873
- There were significant differences in total numbers of *Stomoxys* species between months on both farms. The highest number of *Stomoxys* species was recorded in September. The most abundant species was *S. calcitrans*.
- The total number of males and females of *S. calcitrans* collected on both farms were not differed significantly between time intervals, but there were more males higher than females. The interaction between sex and time was not significant.
- The weather parameters when related to *S. calcitrans* showed a significant positive correlation with relative humidity.

Abstract Knowledge of seasonal abundance and flight activity pattern are required to design effective management programs for insect pests of human and livestock. In this study, the seasonality and daily flight activity of *Stomoxys* species were observed on two dairy farms. Data was assessed throughout one year using the Vavoua traps starting from September 2010 to August 2011. A total of 2,520 individuals belonging to four species were collected. The total numbers of *Stomoxys* species showed significance on both farms for each month. The *Stomoxys* species peaked in September. *Stomoxys calcitrans* was caught all over the year and the most abundant species for this study. The total number of males and females of *S. calcitrans* collected on both farms were not differed significantly between time intervals, but there were more males higher than females. The interaction between sex and time was not significant. The weather parameters when related to *S. calcitrans* showed a significant positive correlation with relative humidity.

Keywords: daily flight activity, dairy cattle, seasonal abundance, stable flies, Thailand

Introduction

The stable flies belong to subfamily Stomoxyinae in family Muscidae (Diptera). Among 18 *Stomoxys* species described, 6 species are recorded from Thailand, of which one is cosmopolitan, *S. calcitrans* (Linnaeus, 1758) (Zumpt, 1973; Tumrasvin and Shinonaga, 1978). Stable flies resemble the house fly but can be easily distinguished by especially the piercing-sucking mouthparts. Stable fly mouthparts are conspicuous long feature and projecting straight forward from under the head. They are important and widely distributed insect pests of livestock, wildlife and sometimes human. Adult stable flies of both sexes are blood-sucking flies and causing painful bites and significant blood loss to their host. High populations of biting activity can reduce animal productivity and disturbance of feeding 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). Moreover, they may act as both biological and mechanical vectors for several pathogens such as trypanosomes (Sumba et al., 1998). Stable flies may also act as intermediate host of the nematode *Habronema* (Traversa et al., 2008). In Thailand, little is known about the presence of different stomoxyine fly species, their distribution and biology. However, Masmeatathip et al. (2006) described the seasonal abundance of *Stomoxys* species in Thailand. Muenworn et al. (2010a, 2010b) conducted stable fly survey and reported their distribution in Thailand. More understanding the seasonal and daily activity of the flies will facilitate and make fly control programs more effective. This study has as its 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 Amphur Muak Lek, Saraburi Province (Figure 1), located in the central highlands of Thailand, an area of high plains and plateaus about 140 km Northeast of Bangkok. There are three seasons in the year: hot (March to May), rainy (June to October) and dry (November to February). Farm 1 (14° 48' N, 101° 17' E; 460 m a.s.l) has 10 cows and farm 2 (14° 47' N, 101° 15' E; 355 m a.s.l) has 57 cow in milk production.

Specimen collection. Four Vavoua traps (Laveissière and Grébaut, 1990) were installed to represent different types of environment at each dairy farm. Stable flies were observed monthly during a one year study (September 2010 to August 2011). Flies were captured every one hour between 06:00 a.m. and 18:00 p.m. After trapping, flies in the Vavoua traps (Figure 2) were killed using ethanol spray and preserved in 80% ethanol. Air temperature, relative humidity and light intensity were also recorded every hour. The specimens were brought back to the

Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand for the identification according to Zumpt (1973) and Tumrasvin and Shinonaga (1978).

Data analysis. Chi-squared test was used to examine the total numbers of stable flies among months. The total numbers of each sex of stable flies collected were analyzed using a one-way ANOVA at each collection sites. Two-way ANOVA (sex and time as factors) was conducted. The weather parameters as air temperature, relative humidity and light intensity on the abundance of stable fly species were tested using Pearson's correlation analysis. The data were analyzed using SPSS (Version 17, SPSS Inc., Chicago, IL, U.S.A.). All statistical significance was set at $P < 0.05$.

Results

A total of 2,520 individuals of *Stomoxys* species were collected during the 1- year study (Table 1). Out of the total, 1622 were trapped at farm 1 and 898 were caught at farm 2. The following 4 species were captured: *S. bengalensis* Picard, 1908, *S. calcitrans* (Linnaeus, 1758), *S. indicus* Picard, 1908 and *S. sitiens* Rondani, 1873 (Figure 3). The chi-square test showed significance when the total numbers on both farms for each month were compared (Farm 1: χ^2 = 1559, d.f. = 11, P = 0.000; Farm 2: χ^2 = 784.6, d.f. = 11, P = 0.000). The highest number of *Stomoxys* species was recorded in September. The most abundant species was *S. calcitrans* (89-96 %) which was found on both farms. Other species were caught in lower numbers. The diurnal flight activity of males and females of *Stomoxys* species is showed in Table 2. Analysis of total number of males and females of *S. calcitrans* collected on farm1 was not differed significantly between time intervals (F = 1.035, d.f. = 11, 132, P = 0.420, and F = 0.750, d.f. = 11, 132, P = 0.689, respectively) (Table 3). Again, analysis of total number of males and females collected on farm 2 was not differed significantly between time intervals (F = 1.227, d.f. = 11, 132, P = 0.276, and F = 0.890, d.f. = 11, 132, P = 0.552, respectively). Numbers of *S. calcitrans* were significantly affected by sex (Farm 1: F = 34.364, d.f. = 1, 264, P = 0.000; Farm 2: F = 13.303, d.f. = 1, 264, P = 0.000). There was no time effect (Farm 1: F = 1.310, d.f. = 1, 264, P = 0.219; Farm 2: F = 1.659, d.f. = 1, 264, P = 0.083) and no sex and time interaction (Farm 1: F = 0.711, d.f. = 1, 264, P = 0.727; Farm 2: F = 0.658, d.f. = 1, 264, P = 0.778). During the whole collection period, *S. calcitrans* was not related to air temperature and light intensity but showed a positive correlation with relative humidity (r = 0.253, P = 0.000).

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 genera *Stomoxys* collected from both farms. The results showed that the number of *Stomoxys* species on farm 1 was significantly more than farm 2. Stable fly population varies between farms were influenced by environmental conditions, temperature, precipitation and the suitable trap locations (Lysyk, 1993; Gilles et al., 2007; Pitzer et al., 2011). Although *Stomoxys* species commonly was active throughout the year, *S. calcitrans* was the most abundant followed by *S. indicus* (Muenworn et al., 2010b). The peak *Stomoxys* species occurred in September, corresponding to the rain season, this finding was similar with Masmeatathip et al. (2006) and Muenworn et al. (2010b). The number of *Stomoxys* species increase during rainfall due to the widespread increase in suitable breeding site. An immature habitat has to maintain appropriate environmental conditions such as an optimal temperature, humidity, and rainfall for the eggs to hatch and the larvae to survive and successfully develop to pupae and adults (Cruz-Vazquez et al., 2004).

For this study we analyzed the data of *S. calcitrans* only. We assumed that this species was relatively more abundant than the others and can play a significant role. The numbers of *S. calcitrans* males were significantly higher than females, coincided with the reported of Masmeatathip et al. (2006) and Muenworn et al. (2010b). More *S. indicus* females were collected than males at both farms and similarly finding was reported by Masmeatathip et al. (2006).The sex ratio s of *S. sitiens* were variable between farms and only one male of *S. bengalensis* was seen in farm 2. Further studies are needed to evaluate whether these differences are associated with locations or other factors to attract *Stomoxys* species. In previous studies, *S. calcitrans* was reported that has a bimodal pattern of feeding with peaks at 10.00 a.m. and 4.00 p.m. (Masmeatathip et al., 2006; Muenworn et al., 2010b). In our study, hourly collections of *S. calcitrans* indicated that daily activity was not significant. However, the numbers of *S. calcitrans* increased throughout the day until 14.00 p.m. and gradually decreased in number toward at 18.00 p.m. *Stomoxys indicus* and *S. sitiens* showed population peaks in the early morning and in the late afternoon, similarly to the finding of (Masmeatathip et al., 2006). Stable flies require blood for successful mating and ovarian development, but also they require nectar as a supplemental energy as well for successful blood-feeding (Taylor and Berkebile, 2008). Műller et al. (2012) conducted studies in diurnal feeding behaviour of the three *Stomoxys* species in Mali and found that bimodal blood-feeding and unimodal sugar-feeding activity periods. The differences in feeding activity patterns are dependent upon protein

and nectar sources, traps locations and sampling methods. Our results showed that the daily activity patterns of *S. calcitrans* can be influenced by relative humidity. The current study is a step in the direction of planning and developing insects control systems.

Acknowledgements

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Table 1. Monthly distribution and relative abundance (RA) of *Stomoxys* spp. captured on two dairy farms, Amphur Muak Lek, Saraburi Province from September 2010 to August 2011.

		Month													
Farm 1		Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Total	RA (%)
S. calcitrans	Male	404	50	72	102	64	60	174	79	47	65	18	71	1206	74.35
	Female	119	13	16	17	7	19	45	38	31	22	8	20	355	21.89
S. indicus	Male	9	3	1	0	0	0	0	4	2	0	0	0	19	1.17
	Female	15	5	9	0	1	0	0	2	1	1	0	0	34	2.1
S. sitiens	Male	2	2	1	0	0	0	0	0	0	0	0	0	5	0.31
	Female	1	1	1	0	0	0	0	0	0	0	0	0	3	0.18
Total		550	74	100	119	72	79	219	123	81	88	26	91	1622	100
Farm 2															
S. bengalensis	Male	0	0	0	0	0	0	0	0	0	1	0	0	1	0.11
	Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. calcitrans	Male	145	62	11	32	3	1	34	50	38	26	35	98	535	59.58
	Female	70	17	8	6	1	1	4	16	37	33	27	48	268	29.84
S. indicus	Male	4	3	2	1	0	0	0	1	0	0	0	1	12	1.34
	Female	15	1	3	2	0	0	0	1	0	1	2	0	25	2.78
S. sitiens	Male	16	2	0	2	0	0	0	1	0	3	0	0	24	2.67
	Female	20	1	2	1	0	0	0	0	0	6	2	1	33	3.67
Total		270	86	26	44	4	2	38	69	75	70	66	148	898	100

Table 2. Numbers of *Stomoxys* spp. captured per hour on two dairy farms in Amphur Muaklek, Saraburi Province from September 2010 to August 2011.

Farm 1		Time											
		06.00-	07.00-	08.00-	09.00-	10.00-	11.00-	12.00-	13.00-	14.00-	15.00-	16.00-	17.00-
S. calcitrans	Male	19	76	131	132	98	124	130	148	117	111	90	30
	Female	8	21	23	36	39	50	37	30	29	30	25	27
S. indicus	Male	4	0	1	0	0	0	1	0	0	0	1	12
	Female	3	1	0	0	0	0	0	0	3	2	2	23
S. sitiens	Male	0	0	0	0	0	0	1	0	0	0	0	4
	Female	0	0	0	0	0	0	0	0	0	0	0	3
Total		34	98	155	168	137	174	169	178	149	143	118	99
Farm 2													
S. bengalensis	Male	0	0	0	0	0	0	0	0	0	0	0	1
	Female	0	0	0	0	0	0	0	0	0	0	0	0
S. calcitrans	Male	11	31	45	31	45	24	76	70	60	64	56	22
	Female	9	8	15	28	28	31	31	29	26	23	29	11
S. indicus	Male	1	1	0	0	0	0	0	0	0	0	1	9
	Female	9	2	0	0	0	0	0	0	1	0	4	9
S. sitiens	Male	3	3	0	1	1	1	1	0	1	2	1	10
	Female	8	2	5	0	0	0	0	0	1	4	5	8
Total		41	47	65	60	74	56	108	99	89	93	96	70

Table 3. Mean number of *Stomoxys calcitrans* males and females captured per hour on two dairy farms in Amphur Muaklek, Saraburi Province from September 2010 to August 2011.

Time	Numbers of *S. calcitrans* captured (mean ± SE)			
	Farm 1		Farm 2	
	Male	Female	Male	Female
06.00-07.00	1.58 ± .66	0.67 ± .31	0.92 ± .43	0.75 ± .279
07.00-08.00	6.33 ± 2.77	1.75 ± 1.14	2.58 ± .86	0.67 ± .31
08.00-09.00	10.92 ± 5.30	1.92 ± .69	3.75 ± 1.99	1.25 ± .55
09.00-10.00	11.00 ± 4.08	3.00 ± 1.09	2.58 ± .85	2.33 ± .69
10.00-11.00	8.17 ± 3.63	3.25 ± 1.23	3.75 ± 2.55	2.33 ± 1.45
11.00-12.00	10.33 ± 4.66	4.17 ± 1.50	2.00 ± .48	2.58 ± .84
12.00-13.00	10.83 ± 3.45	3.08 ± 1.50	6.33 ± 2.09	2.58 ± .74
13.00-14.00	12.33 ± 4.01	2.50 ± 1.02	5.83 ± 2.45	2.42 ± 1.16
14.00-15.00	9.75 ± 1.97	2.42 ± .56	5.00 ± 1.48	2.17 ± .74
15.00-16.00	9.25 ± 1.84	2.50 ± .62	5.33 ± 1.52	1.92 ± .65
16.00-17.00	7.50 ± 3.23	2.08 ± .56	4.67 ± 1.47	2.42 ± .89
17.00-18.00	2.50 ± .75	2.25 ± 1.07	1.83 ± .63	.917 ± .34

SE, standard error.

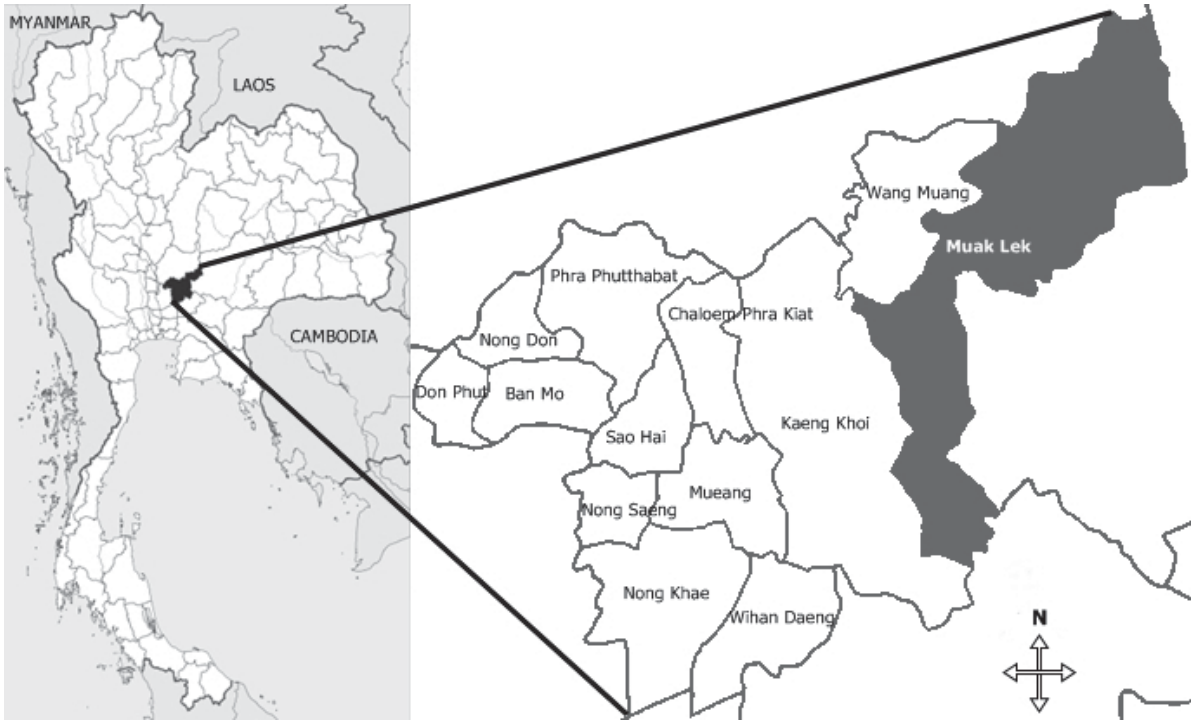

Figure 1. Location of Amphur Muak Lek (study area) in Saraburi Province, Thailand.

Figure 2. Vavoua traps. A; Farm 1 and B; Farm 2.

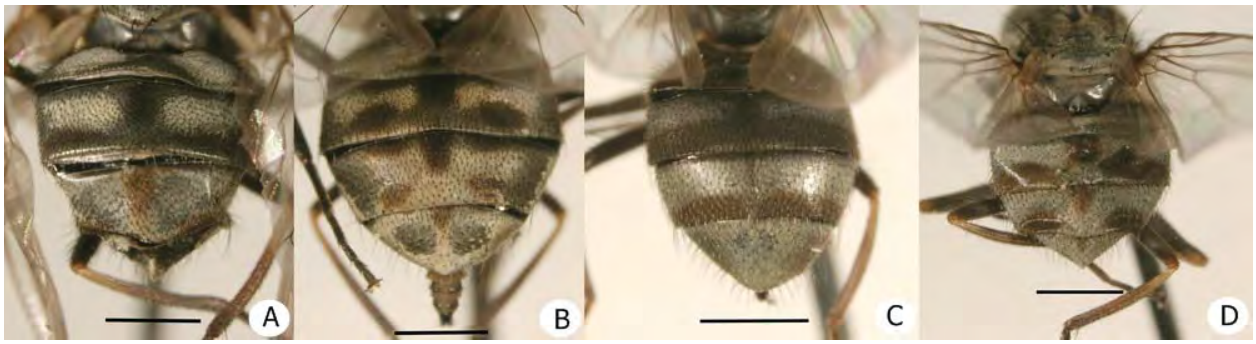

Figure 3. The abdominal patterns of *Stomoxys* spp., A; *S. bengalensis*, B; *S. calcitrans*, C; *S. indicus* and D; *S. sitiens.* Scale bar = 1 mm.

Output

The output from this project is a more detailed knowledge of the seasonality and daily flight activity of *Stomoxys* species and also is likely to give a step in the direction of planning and developing insects control systems.

Output ที่ได้จากโครงการ

1.รายชื่อคณะผู้ร่วมวิจัยในโครงการทุนส่งเสริมกลุ่มวิจัย

รายชื่อกลุ่มวิจัย

ชื่อ-นามสกุล	เริ่มเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
1.ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ	ศาสตราจารย์	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	ที่ปรึกษาโครงการ	ศาสตราจารย์	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	อาจารย์
2. ดร.วรรณภา สุวรรณเกิด	-	กรมควบคุมโรค กระทรวงสาธารณสุข อ.เมือง จ.เชียงใหม่ 50000	ผู้ร่วมวิจัย	-	กรมควบคุมโรค กระทรวงสาธารณสุข อ.เมือง จ.เชียงใหม่ 50000	นักวิชาการสาธารณสุข ชำนาญการพิเศษ
3. ดร.วราภรณ์ จันทรจำนงค์	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	ผู้ร่วมวิจัย	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	อาจารย์
4. ดร.จินรภา โพธิกสิกร	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิ	ผู้ร่วมวิจัย	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิ	นักวิทยาศาสตร์
5. ดร.อุไรวรรณ อรัญวาสน์	-	ภาควิชาพันธุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	ผู้ร่วมวิจัย	-	ภาควิชาพันธุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	อาจารย์
6. ดร.สังสิทธิ์ สังวรโยธิน	-	ภาควิชากีฏวิทยาทางการแพทย์ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล	ผู้ร่วมวิจัย	-	ภาควิชากีฏวิทยาทางการแพทย์ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล	อาจารย์
7. ดร.สุภราดา สุคนธาภิรมย์ ณ พัทลุง	-	กลุ่มกีฏและสัตววิทยา กรมวิชาการเกษตร กระทรวงเกษตรและสหกรณ์	ผู้ร่วมวิจัย	-	กลุ่มกีฏและสัตววิทยา กรมวิชาการเกษตร กระทรวงเกษตรและสหกรณ์	นักวิชาการเกษตร
8. ดร.จำนงจิต ผาสุข	-	ภาควิชาปรสิตวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	ผู้ร่วมวิจัย	-	ภาควิชาปรสิตวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	อาจารย์
9. นางสาวมณฑาทิพย์ คงมี	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาเอก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	คาดว่าจบปีการศึกษา ปลาย 2554
10. นายวิถี เหมือนวอน	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาเอก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	คาดว่าจบปีการศึกษา ต้น 2555
11. นางสาววรรณภา ฤทธิสนธิ์	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาเอก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	คาดว่าจบปีการศึกษา ต้น 2557
12. นางสาวสุนัยนา สะท้านไตรภพ	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาเอก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	คาดว่าจบปีการศึกษา ต้น 2557

ชื่อ-นามสกุล	เริ่มเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
13. นางสาวกราญ์จนา ถาอินชุม	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต	นักศึกษาปริญญาเอก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา	คาดว่าจบปีการศึกษา ต้น 2557

		บางเขน			บางเขน	
14. นางสาวนริศรา มาลัยทอง	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาโท	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา บางเขน	สำเร็จการศึกษาภาคต้น 2554
15. นางสาวสุพัตรา เขียวระยับ	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาโท	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา บางเขน	คาดว่าจบปีการศึกษา ปลาย 2554
16. นางสาวกรวิกา สุวรรณศิลป์	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาโท	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา บางเขน	คาดว่าจบปีการศึกษา ปลาย 2554
17. นางสาวรุ่งอรุณ ทิศกระโทก	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาโท	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา บางเขน	สำเร็จการศึกษาภาคปลาย 2553
18. นายสุนทร พิมพ์นนท์	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสต บางเขน	นักศึกษาปริญญาโท	-	ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศา บางเขน	คาดว่าจบปีการศึกษา ปลาย 2555
19. นางสาวอุมารินทร์ บุญเกื้อ	-	ภาควิชาพันธุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	นักศึกษาปริญญาโท	-	ภาควิชาพันธุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ บางเขน	คาดว่าจบปีการศึกษา ปลาย 2555

2. ผลงาน

2.1 ผลงานตีพิมพ์ในวารสารนานาชาติ

2.1.1 Thanispong K, Achee NL, Bangs, MJ, Grieco JP, Suwondkerd W, Prabaripai A, Chareonviriyaphap T. 2009. Irritancy and repellency behavioral responses of three strains of *Aedes aegypti* expsed to DDT and alphacypermethrin. ***J. Med. Entomol.*** 46: 1407-1414. (IF: 1.925, 2010) (เอกสารแนบ 1)

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2.1.5 Suwannachote N, Grieco JP, Achee NL, Suwonkerd W, Wongtong S, Chareonviriyaphap T. 2009. Effects of environmental conditions on the movement patterns of *Aedes aegypti* (Diptera: Culicidae) into and out of experimental huts in Thailand. ***J. Vec. Ecol*** 34: 267-275. (IF: 1.256, 2010) (เอกสารแนบ 5)

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2.1.8 Malaithong N, Polsomboon S, Poolprasert P, Prabaripai A, Bangs MJ, Suwodkerd W, Pothikasikorn J, Akarathnakul P, Chareonviriyaphap T. 2010. Human landing patterns of Anopheles dirus sensu lato (Diptera: Culicidae) in experimental huts treated with DDT or deltamenthrin. *J. **Med. Entomol.***47: 824-831. (IF: 1.925, 2010) (เอกสารแนบ 8)

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2.1.14 Tisgratog R, Tananchai C, Bangs MJ, Tainchum K, Juntarajumnong W, Prabaripai A, Chauhan KR, Pothikasikorn J. Chareonviriyaphap, T. 2011, Chemically induced behavioral responses in *Anopheles minimus* and *Anopheles harrisoni* in Thailand. ***J. Vec. Ecol.* 36**: 321-331. (IF: 1.256, 2010) (เอกสารแนบ 14)

2.1.15 Chuaycharoensuk T, Juntarajumnong W, Boonyuan W, Bangs MJ, Akratanakul P, Thammapalo S, Jirakanjanakit N, Tanasinchayakul S, Chareonviriyaphap,T. 2011. Frequency of pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*

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2.1.17 Juntarajumnong W, Pimnon S, Bangs, MJ, Thanispong K, Chareonviriyaphap T. 2012. Discriminatiing lethal concentrations and efficacy of six pyrethroids for control of Ae. aegypti in Thailand. ***J. Amer. Mosquito Control Assoc.*** 28: Jan 2012 (IF: 1.066, 2010) (เอกสารแนบ 17)

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District, Nakhon Ratchasrima Province, Thailand***. Parasite*** 19 epub ahead of print (IF: 1.700:2011) (เอกสารแนบ 23)

2.2 ผลงานตีพิมพ์ในวารสารวิชาการระดับชาติ

2.2.1 Muenworn V, Duvallet G, Tuntakom S, Akratanakul P, Chareonviriyaphap T. 2010. Stable fly (Diptera: Musidae) distribution in Thailand. *Kasetsart J. (Natural Science)* 44: 44-52. (เอกสารแนบ 24)

2.2.2 Pimnon S, Juntarajumnong W, Chareonviriyaphap T. 2012. Diagnostic doses of two pyrethroids currently used for the control of *Aedes aegypti* L. (Diptera: Culicidae), a vector of dengue. *Kasetsart J. (Natural Science)* (เอกสารแนบ 25)

2.3 หนังสือ

2.3.1 Chareonviriyaphap T. 2012. INSECTICIDES; PEST ENGINEERING. Chapter Title *Behavioral Responses of Mosquitoes to Insecticides* 201-225: In Tech Publishing Company: 538 pp. ISBN 978 953 307 895 3.

2.4 **ผลงานตีพิมพ์ในวารสารระดับนานาชาติ Manuscripts Accepted**

2.4.1 Tainchum K, Polsomboon S, Achee NL, Grieco JP, Suwonkerd W, Ferdinan S, Prabaripai A, Chareonviriyaphap T. Evaluation of the resting behavior of *Aedes aegypti* (Diptera: Culicidae) based on tactile response to two types of fabrics. *J. Med. Entomol* (Accepted) (เอกสารแนบ 26)

2.5 ผลงานตีพิมพ์ในวารสารระดับนานาชาติ Manuscripts Pending Review

2.5.1 Ferdinand S, Tainchum K, Polsomboon S, Achee NL, Grieco JP, Suwonkerd W, Prabaripai A, Chareonviriyaphap T. Utilization of BG sentinel trap *for Aedea egypti* surveillance. *PLos One* (Submitted). (เอกสารแนบ 27)

2.5.2 Suwansirisil K, Grieco JP, Prabaripai A, Chareonviriyaphap T. Excito repellency of 4 essential oils against *Aedes aegypti* and *Culex quinquefasciatus*. *J. Amer. Mosq. Control Assoc.* (Submitted) (เอกสารแนบ 28)

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2.6 ผลงานตีพิมพ์ในวารสารระดับนานาชาติ Preparation

2.6.1 Chareonviriyaphap T, Suwonkerd W. Review: Insecticide Response of Mosquitoes in Thailand from 2000-2011. *BMC* (open access) (เอกสารแนบ 30)

2.6.2 Pothikasikorn J, Chareonviriyaphap T, Noonpakdee W and Boonplueang R. Technique for preservation of microfilariae of *Wuchereria bancrofti* (เอกสารแนบ 31)

2.6.3 Phasuk J, Prabaripai A and Chareonviriyaphap T. Seasonality and daily flight activity of stable flies (Diptera: Muscidae) on dairy farms in Saraburi Province, Thailand (เอกสารแนบ 32)

2.6.4 Suwonkerd W, Suwannachote N and Charoenviriyaphap T. Mark- Release-Recapture study to measure dispersal of *Aedes albopictus* (Skuse) vector of dengue fever and Chikungunya in Chiang Mai province, northern Thailand. (manuscript in preparation). (เอกสารแนบ 33)

2.6.5 Suwonkerd W, Suwannachoteand N and Charoenviriyaphap T. Assessment of pupal Index in high and low endemic dengue fever areas in northern Thailand, prospective of effective control of dengue vectors (manuscript in preparation). (เอกสารแนบ 34)

2.6.6 Suwonkerd W, Suwannachote N and Charoenviriyaphap T. Seasonal pattern of *Aedes albopictus (Skuse)* vector of Dengue fever and Chikungunya in municipality and rural areas of Chiang Mai Province, northern Thailand. (เอกสารแนบ 35)

2.6.7 Suwonkerd W, Suwannachoteand N and Charoenviriyaphap T. Comparative on a seasonal trend of susceptibility of *Aedes aegypti* against temephos in Chiang Mai province, northern Thailand. (เอกสารแนบ 36)

2.7 ผลงานที่ไปเสนอแบบปากเปล่า

2.7.1 ดร.สังสิทธิ์ สังวรโยธิน ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ มลรัฐแคลิฟอเนียร์ ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2554)

2.7.2 ดร.วราภรณ์ จันทร์จำนงค์ ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ มลรัฐแคลิฟอเนียร์ ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2554)

2.7.3 ดร.อุไรวรรณ อรัญวาสน์ ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ มลรัฐแคลิฟอเนียร์ ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2554)

2.7.4 ดร.วรรภา สุวรรณเกิด ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ มลรัฐแทกซัส ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2555)

2.7.5 ดร.จำนงค์จิต ผาสุข ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control

Association ที่ มลรัฐแทกซัส ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2555)

3.การนำผลงานไปใช้ประโยชน์ในเชิงพาณิชย์ หรือเชิงสาธารณะ หรือเชิงนโยบาย

3.1 เชิงพานิชย์: เครื่องมือคัดกรองและทดสอบประสิทธิภาพสารไล่แมลงและจำหน่ายให้กับหน่วยงานต่าง ๆ ดังนี้

ต่างประเทศ

1. ประเทศสหรัฐอเมริกา	4 ชุด
3. ประเทศฟิลิปปินส์	4 ชุด
2. ประเทศบาลีซ	4 ชุด
4. ประเทศอินโดนีเซีย	4 ชุด
5. ประเทศบินิน อัฟริกา	4 ชุด
6. ประเทศนอร์เวย์	4 ชุด

ในประเทศ

1.สำนักโรคติดต่อนำโดยแมลง กรมควบคุมโรค กระทรวงสาธารณสุข	4 ชุด
2.คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์	4 ชุด
3.คณะเภสัชศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ	4 ชุด

3.2 เชิงสาธารณะ

3.2.1ผลิตสารสมุนไพรไล่ยุง สารไล่ยุงที่ผลิตได้ผ่านการทดสอบประสิทธิภาพด้วยเครื่องมือคัดกรองและทดสอบประสิทธิภาพสารไล่แมลงให้เป็นสารสมุนไพรไล่ยุงสูตรต่างๆ อาทิ ครีม ยาหม่อง โลชั่น เจล โดยดำเนินการภายใต้การสนับสนุนของสำนักงานกองทุนสนับสนุนการวิจัย (สกว) นำไปช่วยผู้ประสพอุทกภัยน้ำท่วมในปี 2554

3.2.2ผลงานวิจัยเรื่อง push-pull concept ในการควบคุมยุงนำโรคไข้เลือดออก ซึ่งกำลังเตรียมต้นฉบับตีพิมพ์ในวารสารนานาชาติ ได้ใช้เป็น model ต้นแบบในการศึกษาสารไล่แมลงให้กับหน่วยควบคุมยุงพาหะนำโรค ขององค์การอนามัยโลก

3.2.3นำไปใช้ในการเรียนสอน นิสิตปริญญา ตรี โท เอก ข้อมูลทางวิชาการจากภาคสนามและห้องปฏิบัติการมีประโยชน์อย่างมากในการนำมาสอนเป็นกรณีศึกษาให้นิสิตทั้งระดับปริญญาตรี โท เอก

3.2.4 ศูนย์ทดสอบและตรวจสอบประสิทธิภาพสารเคมีให้กับหน่วยงานภาครัฐ เอกชน ทั้งในและต่างประเทศ

<u>ห้องปฏิบัติการ:</u> BASF Company ได้ขอให้ทีมวิจัยจัดตั้งห้องปฏิบัติการกลางเพื่อศึกษาประสิทธิภาพสารเคมีของ Long Lasting Net เพื่อทดสอบประสิทธิภาพของ Product ตามมาตรฐานขององค์การอนามัยโลก

ภาคสนาม: IRD ประเทศฝรั่งเศสภายใต้การสนับสนุนขององค์การอนามัยโลก (WHO) ขอให้ร่วมพัฒนาการศึกษาเพื่อประเมินผลกระทบของ long lasting netting (LLN) ที่มีต่อยุงกันปล่องนำโรคมาลาเรีย ภาคสนาม phase ที่ 2

3.3ข้อมูลการเฝ้าระวังโรคที่ติดต่อโดยแมลงแก่หน่วยงานสาธารณสุขและปศุสัตว์ ข้อมูลเหล่านี้สามารถนำไปใช้เป็นข้อมูลเพื่อพัฒนาแผนการเฝ้าระวัง การควบคุมป้องกันโรค ที่มีแมลงเป็นพาหะ ข้อมูลบางส่วนสามารถนำไปพัฒนาเพื่อนำไปใช้ในระดับปฏิบัติต่อไป โดยสามารถช่วยลดงบประมาณของประเทศไปได้โดยเฉพาะการสั่งซื้อ ชุด Diagnostic dose จากองค์การอนามัยโลก

3.4องค์ความรู้ทางวิชาการ ข้อมูลที่ได้รับเป็นองค์ความรู้ใหม่ น่าสนใจที่ได้รับจากงานวิจัยของนักวิจัยแต่ละท่านซึ่งใช้เวลาตั้งแต่การเตรียม การจัดการ การศึกษาวิจัย การวิเคราะห์และการนำเสนอข้อมูลสู่สาธารณะชน

4.วิทยากรรับเชิญในการประชุมที่สำคัญระดับชาติและระดับนานาชาติ

4.1 Technical Advisor Group (TAG) Vector of malaria in Asian and southeast Asain countries. 20-22 August 2008 Technical Advisor Group (TAG) from Asia on Global Malaria Mapping Project, KEMRI University of Oxford-Wellcome trust Collaborative, UK.

4.2 Invited Speaker. Impact of insecticides on mosquito vectors transmitted diseases. 2-6 April 2008. Invited speaker in Symposium. Annual Meeting of AMCA, Nevada, U.S.A.

4.4 Scientific Organizing Committee 2008 The Second International Conference on Dengue and Dengue Haemorrhagic Fever, Phuket Thailand, 15-17 Oct 2008.

4.8 Invited scientist to assist in research training at USU, MD, U.S.A. 26 March 2009

4.9 Invited scientist to participate in research coordination meeting on a novel push-pull strategy to reduce host seeking *Aedes aegypti* inside home 17 November 2009, USDA Washington DC., U.S.A.

4.10 Invited scientist to attend the ASTMH annual meeting from 18-22 November 2009, Washington DC., U.S.A.

4.11 External reviewer: Songkhlanakarin Journal of Science and Technology: Toxicity of oil and crude extract from thiam (*Azadirachta excelsa* Jack.) seed kernel to *Aedes aegypti* larvae, 23 June 2009.

4.12 วิทยากร “โครงการพัฒนาศักยภาพบุคลากรรับผิดชอบโรคติดต่อนำโดยแมลง” สำนักงานป้องกันควบคุมโรคที่ ๑๑ นครศรีธรรมราช ที่ สธ ๐๔๑๗/๑๖๓๕ ลงวันที่ ๓ มิถุนายน ๒๕๕๒

4.13 วิทยากร หลักสูตรผู้ควบคุมการใช้วัตถุอันตรายเพื่อใช้รับจ้าง ภาควิชากีฏวิทยา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์ร่วมกับสำนักงานคณะกรรมการอาหารและยา ที่ ศธ ๐๕๑๓.๑๐๒๐๒/๑๗๑๑ ลงวันที่ ๒๙ เมษายน ๒๕๕๒

4.14 วิทยากร “Thailand Research Expo 2009” เรื่องยุงมหันตภัยใกล้ตัวควบคุมได้ด้วยชีววิธี ที่ วช ๐๐๐๙/๕๒๓๑ ลงวันที่ ๒๐ กรกฎาคม ๒๕๕๒

4.15 Invited Speaker. Insecticide resistance in mosquito vector in Thailand. International Forum for Surveillance and Control of Mosquitoes and Mosquito-Borne Diseases, Beijing, China, May 25-29, 2009.

4.16 Invited Speaker. Impacts of insecticide on adult mosquito behaviors. The Sixth Arbovirus Surveillance and Mosquito Control Workshop. American Mosquito Control District (AMCD), St Augustine, Florida, USA. 31 March- April 2 2009

4.17 Invited Speaker. Household protection from *Aedes aegypti:* Exploiting repellency behavior and prospects for use of botanical repellents. The fifth International Congress of Vector Ecology. Belex-Antalya Turkey 12-16 October 2009, Turkey

4.18 รับเชิญให้เขียนบทความเรื่อง ‘การแบ่งสารเคมีตามแนวคิดใหม่” เนื่องในโอกาส ๖๐ ปี มาลาเรียประเทศไทย วารสารโรคติดต่อนำโดยแมลง ปีที่ ๖ (๑) ตามหนังสือเชิญที่ กระทรวงสาธารณสุข สธ ๐๔๒๒.๑๑/๓๘ ลงวันที่ ๘ มกราคม ๒๕๕๓

4.19 วิทยากร “การจัดการแมลงและสัตว์พาหะในอุตสาหกรรมอาหาร” สมาคมวิทยาศาสตร์และเทคโนโลยีทางอาหารแห่งประเทศไทย วันที่ ๑๘ พฤษภาคม ๒๕๕๓ ตามหนังสือเชิญที่ ส.อ.ท. ๒๕๕๓/ว.๐๕๒ ลงวันที่ ๗ เมษายน ๒๕๕๓

4.20 Scientific Organizing Committee 2010 International Malaria Colloquium (IMC), Bangkok, Thailand, 1-3 Dec 2010.

4.21 Invited speaker. Long lasting insecticidal net meeting in Thailand, Marriott Resort and Spa, Bangkok, Thaiuland 16 February 2011

4.22 Invited to write a book chapter “Behavioral responses of malaria vectors, *Anopheles minimus* complex, to three classes of agrochemicals in Thailand” INSECTICIDE, INTECH Open access Publisher March 02, 2011

4.23 Scientific Organizing Committee: Insecticide resistance in mosquito vector in Thailand. International Forum for Surveillance and Control of Mosquitoes and Mosquito-Borne Diseases, Beijing, China, May 25-29, 2009.

5.รางวัลที่ท่านได้รับระหว่างรับทุนส่งเสริมกลุ่มวิจัย

นักวิจัยในโครงการที่ได้รับรางวัลหรือได้รับทุนวิจัยอื่นในระหว่างที่ท่านรับทุนส่งเสริมกลุ่มวิจัย

5.1 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัล นักวิจัยที่มีผลงานวิจัยตีพิมพ์ในวารสารมาตรฐานสากลสูงสุดปี 2551สาขาเกษตรศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ปีที่ได้รับ 2551

5.2 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัล ผลงานวิจัยตีพิมพ์ระดับนานาชาติปี 2551 ผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ในฐานข้อมูล Science Citation Index Explanded มหาวิทยาลัยเกษตรศาสตร์จำนวน 5 ผลงาน ปีที่ได้รับ 2551

5.3 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัล ผลงานวิจัยตีพิมพ์ในฐานข้อมูลมาตรฐานสากลปี ๒๕๕๒ มหาวิทยาลัยเกษตรศาสตร์ จำนวน 1 ผลงาน ปีที่ได้รับ 2552

5.4 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัลผลงานวิจัยตีพิมพ์ระดับนานาชาติปี 2551 ผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ในฐานข้อมูล Science Citation Index Explanded มหาวิทยาลัยเกษตรศาสตร์จำนวน 5 ผลงาน ปีที่ได้รับ 2551

5.5 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัลผลงานวิจัยตีพิมพ์ระดับนานาชาติปี 2552 ผู้สร้างสรรค์ผลงานวิจัยตีพิมพ์ในฐานข้อมูล Science Citation Index Explanded มหาวิทยาลัยเกษตรศาสตร์จำนวน 5 ผลงาน ปีที่ได้รับ 2552

5.6 ชื่อนักวิจัย ศาสตราจารย์ ดร.ธีรภาพ เจริญวิริยะภาพ

ได้รับรางวัลผลงานวิจัยตีพิมพ์ระดับนานาชาติมหาวิทยาลัยเกษตรศาสตร์ ปี 2553 ผู้สร้างสรรผลงานวิจัยตีพิมพ์ กลุ่ม 1 ในฐานข้อมูล Science Citation Expanded ปีที่ได้รับ 2553

6. นักวิจัยในโครงการที่ได้รับรางวัลหรือได้รับทุนวิจัยอื่นในระหว่างที่ท่านรับทุนส่งเสริมกลุ่มวิจัย

6.1 ชื่อนักวิจัย ดร. วรรณภา สุวรรณเกิด

ได้รับรางวัล/ทุนวิจัย กองทุนโลก (Global Fund) **ปีที่ได้รับ** 2555-2559

หัวข้อเรื่อง Mapping and distribution of a dynamic potential malaria vectors along the Thai-Myanmar border, behavior, bionomics and their insecticide susceptibility, way forwards for effective control

6.2 ชื่อนักวิจัย ดร.อุไรวรรณ อรัญวาสน์

ได้รับรางวัล/ทุนวิจัย ทุนพัฒนาอาจารย์ใหม่ สกว และ สกอ **ปีที่ได้รับ** 16/3/2552-15/3/2554

ได้รับรางวัล/ทุนวิจัย ทุน ScRF จากคณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์**ปีที่ได้รับ** 2553-2554

ได้รับรางวัล/ทุนวิจัย ทุนจากสถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์ **ปีที่ได้รับ** 2553-2555
ได้รับรางวัล/ทุนวิจัย ทุนจากสภาวิจัยแห่งชาติ **ปีที่ได้รับ** 2555

6.3 ชื่อนักวิจัย ดร.สังสิทธิ์ สังวรโยธิน
ได้รับรางวัล/ทุนวิจัย ทุนวิจัย สกว **ปีที่ได้รับ** 2552-2553
ได้รับรางวัล/ทุนวิจัย มหาลัยวิจัย **ปีที่ได้รับ** 2554-2556

6.4 ชื่อนักวิจัย ดร.วราภรณ์ จันทรจำนงค์
ได้รับรางวัล/ทุนวิจัย สวพ.ม.เกษตร **ปีที่ได้รับ** 2553-2554
ได้รับรางวัล/ทุนวิจัย ทุนวิจัย สกว **ปีที่ได้รับ** 2553-2555

6.5 ชื่อนักวิจัย ดร.สุภราดา สุคนธาภิรมย์ ณ พัทลุง
ได้รับรางวัล/ทุนวิจัย ทุนรัฐบาลไทย **ปีที่ได้รับ** 2555

7. กิจกรรมอื่น ๆ ที่เกี่ยวข้อง ได้แก่

นิสิตไปเสนอผลงาน ฝึกอบรม

7.1 นส.วาสนา บุญญวน เดินทางไปทำงานวิจัยในต่างประเทศกับ Prof P Kholer (U of Florida) ณ ประเทศอเมริกา

7.2 นส. กราญ์จนา ถาอินชุม (1 กันยายน 2554-30 มกราคม 2555) และ นส.วรรณภา เดินทางไปทำงานวิจัยที่เมือง Montpalier ประเทศฝรั่งเศสกับ Prof. S Manguin (1 กรกฎาคม-30 ตุลาคม 2554)

7.3 ส่งนิสิตปริญญาเอกไปเสนอผลงานวิจัยแบบปากเปล่าที่เมืองปักกิ่ง ประเทศจีน 3 คน (22-27 พฤษภาคม 2554)

7.4 นส.ทิพวรา ช่วยเจริญสุข นิสิตในทีมปริญญาเอก เดินทางไปทำงานวิจัยที่เมือง Montpalier ประเทศฝรั่งเศสกับ Prof. S Manguin (1 ธันวาคม 2554-30 เมษายน 2555)

7.5 นส.นริศรา มาลัยทอง นิสิตในทีมปริญญาโท เดินทางไปทำงานวิจัยที่เมือง ประเทศฝรั่งเศสกับ Dr.Marc Desquesnce (25 เมษายน 2554-25 มิถุนายน 2554)

7.6 นายฉัตรชัย ทนันไชย นิสิตในทีมปริญญาโท เดินทางไปทำงานวิจัยที่เมือง Montpalier ประเทศฝรั่งเศสกับ Prof. S Manguin (1 มิถุนายน 2552-30 สิงหาคม 2552)

7.7 นส.กรวิกา สุวรรณศิลป์ นิสิตในทีมปริญญาโท เดินทางไปทำงานวิจัยที่เมือง Montpalier ประเทศฝรั่งเศสกับ Prof. S Manguin (1 ตุลาคม-30 ธันวาคม 2552)

7.8 นักวิจัยในทีม (ดร.วราภรณ์ จันทรจำนง ดร.สังสิทธิ์ สังวรโยธิน ดร.อุไรวรรณ อรัญวาสน์) ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ แคลิฟอร์เนีย ประเทศสหรัฐอเมริกา (22-28 กุมภาพันธ์-2554)

7.9 นักวิจัยในทีม (ดร.จำนงค์จิต ผาสุข ดร.วรรภา สุวรรณเกิด) ได้ไปเสนอผลงานแบบปากเปล่าที่ American Mosquito Control Association ที่ มลรัฐแทกซัส ประเทศสหรัฐอเมริกา (22-29 กุมภาพันธ์ 2555)

7.10 นักวิจัยในทีม 2 คน (Dr. Uraiwan and Dr Jinrapa) เดินทางไปทำงานวิจัยในต่างประเทศกับนักวิจัยที่ปรึกษาโครงการ (Prof Sylvie Manguin) ณ ประเทศฝรั่งเศส เป็นเวลา 1 เดือน (Dr. Uraiwan: Sep-Oct 2010) และ เวลา 1 เดือน (Dr Jinrapa: November 2010)

7.11 ส่งนิสิตปริญญาโทไปเสนอผลงานวิจัยแบบ Poster ที่เมือง Atlanta, GA ประเทศสหรัฐอเมริกา ณ AJTMH Conference (Nov 2-6, 2010)

มีการสร้างเครือข่ายงานวิจัยกับนักวิจัยต่างชาติดังนี้

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ภาคผนวก

Irritancy and Repellency Behavioral Responses of Three Strains of *Aedes aegypti* Exposed to DDT and α-Cypermethrin

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Irritancy and Repellency Behavioral Responses of Three Strains of *Aedes aegypti* Exposed to DDT and α-Cypermethrin

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ABSTRACT This study quantified both contact irritancy and noncontact repellency behavioral responses of three strains of *Aedes aegypti* (L.) (one long-term colony and two F1-F2 generation field-caught strains) to field application rates of DDT (2 g/m^2) and α-cypermethrin (ACyp) (0.025 g/m^2) by using an excito-repellency test chamber. The colony The colony strain (USDA) was completely susceptible to DDT and ACyp. One field strain was collected from Chiang Mai (CM) Province, northern Thailand, and was characterized as tolerant (reduced susceptibility) to DDT and completely susceptible to ACyp. The second field strain, collected from Kanchanaburi (KAN) Province, western Thailand, was highly resistant to DDT but fully susceptible to ACyp. All three strains exhibited marked irritancy to contact with ACyp, with more pronounced escape responses occurring in the two field strains. With DDT, the KAN strain demonstrated the lowest escape response during both contact and noncontact trials, whereas a greater response was seen in trials conducted with CM and USDA strains. With exposure to ACyp, repellency was less profound than irritancy but still resulted in a significant escape response compared with paired controls without insecticide ($P < 0.05$). DDT elicited both irritancy and repellency responses but comparably greater spatial repellency than ACyp. Findings indicate ACyp functions primarily as a strong contact irritant, whereas DDT functions as a relatively strong noncontact repellent in the strains tested. The higher the degree of physiological resistance to DDT, the greater the apparent suppression of both behavioral avoidance responses. Most importantly, observations using susceptible, tolerant, and resistant *Ae. aegypti* strains show that behavioral responses that can interrupt human–vector contact still occur regardless of degree of physiological susceptibility to compounds tested.

KEY WORDS *Aedes aegypti*, contact irritancy and noncontact repellency, α-cypermethrin, DDT

Dengue is the most significant human mosquito-borne viral pathogen in the world and presents a major public health problem in many tropical and subtropical countries (Gubler 1998, Guzman and Kouri 2002). The disease is transmitted by *Aedes aegypti* (L.), a notoriously efficient vector that invariably resides in close association with humans (WHO 2008). Typically, *Ae. aegypti* breeds in household water storage containers and preferentially feeds indoors during daylight (Christophers 1960, Gubler 1998). *Ae. aegypti* also prefers to rest indoors in darken and undisturbed places, complicating control of this vector (Reiter and Gubler 1997). Generally, *Ae. aegypti* has a flight range of <400 m, although some studies indicate that *Ae. aegypti* is capable of occasionally dispersing over much longer distances in search of oviposition sites or bloodmeals (Scott et al. 2000, Harrington et al. 2001). Despite progress, an effective and commercially available dengue vaccine is not yet available; therefore, the prevention and control of disease transmission rely almost exclusively on vector control strategies.

Mosquito behavior in response to chemical exposure is a critical component in the epidemiology of vector-borne disease transmission. The use of chemical barriers has historically been used to exploit these behavioral responses for the purpose of inhibiting mosquitoes from preferentially feeding on humans, ingesting infectious bloodmeals, or transmitting pathogens to susceptible hosts (Elliott 1972). The natural reaction of mosquitoes to avoid insecticide-treated surfaces is a general phenomenon; yet, behavioral responses, including contact irritancy and noncontact repellency of adult mosquitoes exposed to insecticides, remain poorly studied. This remains true despite

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that quantifying behavioral responses to insecticides, in addition to toxicity, has long been recognized as an important aspect in understanding how various chemicals function to control vector and the diseases they carry (Muirhead-Thomson 1960, Grieco et al. 2007). Whereas considerable data have been gathered on the impact of test compounds on *Anopheles* species responsible for malaria transmission, far fewer attempts have been made at describing the function and response of chemicals on other mosquito species (Kennedy 1947, Brown 1964, Lal et al. 1965, Moore 1977). This knowledge will allow better decision making on pesticide development, selection, and application methods (Muirhead-Thomson 1960, Roberts et al. 2000, Grieco et al. 2007).

Synthetic compounds, including organophosphates, carbamates, and pyrethroids, have been used with varying degrees of success in national public health vector control programs to control dengue vectors (Reiter and Gubler 1997). Since 1994, the Ministry of Public Health (MOPH 2007) in Thailand has recommended the use of deltamethrin for emergency vector control and adulticiding during dengue outbreaks and remains the only compound used in government-sponsored public health control programs for adult vector control (Chareonviriyaphap et al. 1999). Recent work has reported increased deltamethrin resistance in several field populations of *Ae. aegypti* in Thailand (Jirakanjanakit et al. 2007). The distribution of resistance is raising awareness for the need of alternative insecticides or newer, more innovative methods of controlling mosquito vectors. α-Cypermethrin (ACyp), another pyrethroid, is currently being used in Thailand homes for the protection against indoor biting mosquitoes and other arthropod pests. Therefore, it is important to quantify the chemical actions of ACyp against various *Ae. aegypti* populations from Thailand before any large scale investment and use in public health programs.

An excito-repellency (ER) test chamber system was developed as an experimental tool to evaluate contact irritancy and noncontact repellency behavioral responses of mosquitoes in the laboratory and field (Chareonviriyaphap et al. 1997, Roberts et al. 1997). Since the introduction of this assay system, modifications and improvements have been made that allow greater ease of setup and accuracy in evaluating the innate behavioral response of mosquitoes exposed to varying doses of residual insecticides (Chareonviriyaphap et al. 2002, Tanasinchayakul et al. 2006). The objective of this study was to use a modified version of the ER test chamber to evaluate contact irritancy and noncontact repellency responses of three strains of *Ae. aegypti* populations to ACyp and DDT. Although DDT is not currently being used for vector control in Thailand, this chemical had played a historically pivotal role in malaria control throughout the country and was used in the current study as a chemical standard for high degree of noncontact repellency (Grieco et al. 2007) in comparison with ACyp.

Materials and Methods

Mosquito Strains. Three *Ae. aegypti* strains were used in this study. Two field strains were collected as larvae and/or pupae from containers located in Ban Pu Tuey, Ta-Soa Subdistrict, Sai-Yok District, Kanchanaburi Province (KAN strain) and Ban Pang Mai Deang, Mae Taeng District, Chiang Mai Province, of Thailand (CM strain). The USDA laboratory strain was provided by the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. This strain has been maintained continuously in colony for >40 yr.

All three strains were maintained in isolation from one another in an insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. *Ae. aegypti* were reared in a temperature-controlled room at 25 ± 5°C and 80 ± 10% RH, with an approximate natural photoperiod of 12:12 (L:D) h. Female and male adults were provided with cotton pads soaked with 10% sugar solution beginning on first day of emergence and held in separate 30- by 30- by 30-cm screen cages. The natural mated female mosquitoes were permitted to feed on blood from restrained live guinea pigs on day 4 postemergence. Two days after blood feeding, 10-cm-diameter oviposition dishes containing moist filter paper were placed in the cages for egg deposition. The eggs were dried at room temperature for 1–2 d and then immersed with water in hatching trays. On first 2 d after hatching, ≈250 larvae of each strain were transferred to plastic rearing trays (20 by 30 by 5 cm) containing 1,500 ml of tap water and ≈2.5 g of ground fish pellets. Pupae were transferred daily from larval trays to emergence cups and placed directly into screened 30-cm^3 cages. Adults were provided cotton soaked with 10% sucrose solution from time of eclosion to 24 h before test assays. Only F1 and F2 generations of KAN and CM strains and succeeding F1 and F2 progeny from the newly established long-term USDA strain were used in test trials.

Insecticides. Chemicals from two insecticides groups were used in the behavioral tests: pyrethroids and organochlorines. The pyrethroid was α-cypermethrin [(1*a*(S),3*a*-(±)-cyano-(3-phenoxyphenyl) methyl 3-(2,2-dichloroethynyl)-2,2 dimethyl cyclopropanecarboxylate] (95% purity) obtained from BASF Corp., Chicago, IL. The organochlorine was DDT [1,1′-(2,2,2-trichloroethylidene) bis (4-chlorobenzene); 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane] (98% purity) purchased from Sigma-Aldrich (St. Louis, MO) (CAS 50-29-3, product no. 386340).

Insecticide-Impregnated Netting Material. Polyester netting material, impregnated with field rate applications of ACyp (0.025 g/m^2) and DDT (2 g/m^2) (Najera and Zaim 2002) was prepared using acetone diluents as described previously (Grieco et al. 2007). Netting was soaked with chemical solutions in individual metal pans overlaid with a weighted smaller pan, allowing complete and even absorption of the chemical solution. Additional netting was treated with acetone as diluent only to serve as untreated controls.

Treated material was allowed to air dry for 60 min before use in the assay.

WHO Susceptibility Tests. All three strains were tested to determine background susceptibility patterns against ACyp and DDT by using the WHO diagnostic test kit (WHO 1998a). Impregnated papers (12 by 15 cm) were prepared in the laboratory using technical grade ACyp and DDT in acetone at field recommended concentrations (Najera and Zaim 2002, WHO 2006), specifically 0.05% ACyp (WHO 1998b) and 4% DDT (WHO 1998a). Additional filter-paper was impregnated with 2 ml of acetone mixed with carrier at the ratio of 1.34–0.66 silicon oil (Dow Corning 556 cosmetic grade, 3.6 mg/cm^2) to serve as untreated controls. Using a mechanical aspirator, batches of 25 non–blood-fed, sugar-starved, 3–5-d-old female mosquitoes were placed into respective holding tubes (control and treatment) and exposed to impregnated papers for 1 h to ACyp or 30 min to DDT (WHO 2009). Immediately after exposure, the number of "knockdown" mosquitoes was recorded, and all specimens transferred into corresponding clean holding tubes provided with 10% sucrose cotton pads. Mortality was observed at 24 h postexposure and results expressed as percentage mortality, and if applicable, corrected for control mortality using Abbott's formula (Abbott 1925). Four replicates of paired control and treatment exposures were performed for each strain and chemical.

Behavioral Tests. An ER test system was used to evaluate the behavioral responses of *Ae. aegypti* to ACyp and DDT at recommended field application rates (Roberts et al. 1997, Chareonviriyaphap et al. 2002, Tanasinchayakul et al. 2006). Each test series consisted of two treatment chambers containing insecticide-treated netting and two paired control boxes containing solvent-only-treated material. Each chamber was configured for either contact irritancy evaluation (i.e., mosquitoes allowed to make tarsal contact with insecticide-treated nets) or noncontact repellency (i.e., insecticide-treated material protected behind a mesh screen barrier). For each trial, one test chamber was configured for contact irritancy and the other test was configured for noncontact repellency with matching control chamber.

Fifteen non–blood-fed, sugar-starved, 4–5-d-old female mosquitoes were carefully introduced into each of the four chambers by using a mouth aspirator. Mosquitoes were allowed a 3-min resting period to acclimatize to test chamber conditions, after which the escape funnel was opened to begin the observation period. Mosquitoes escaping from each chamber into the receiving cage, a 6- by 6- by 6-cm paper carton connected to the exit portal, were recorded at 1-min intervals for a period of 30 min. All tests were performed between 0800 and 1630 hours. Each trial configuration (contact irritancy or noncontact repellency, mosquito strain, chemicals) was replicated four times. Upon completion of the exposure period, the number of mosquitoes remaining inside each chamber was recorded. All knockdown and live mosquitoes that escaped and/or remained inside the chambers were removed by mechanical aspirator, placed into respective cups by category, and provided with 10% sugar to assess 24-h mortality.

Table 1. Susceptibility of *Ae. aegypti*[a] strains to diagnostic doses of α-cypermethrin and DDT

Chemical	Dose[b] (%)	Strain[c]	No. mosquitoes[d]	% mortality (mean ± SE)
α-Cypermethrin	0.05	CM	100	100
		KAN	99	98.0 ± 1.15
		USDA	100	100
DDT	4.0	CM	98	37.1 ± 5.54
		KAN	100	2.00 ± 2.00
		USDA	100	100

[a] F1-F2 females, 3–5 d-old non–blood-fed, sugar-starved 24 h before assay.
[b] WHO-recommended diagnostic doses.
[c] CM, Chiang Mai strain; KAN, Kanchanaburi strain; and USDA, Gainesville, FL, USA strain.
[d] Number of mosquitoes tested in four replicates of 25 each.

Data Analysis. A Kaplan–Meier survival analysis method was used to analyze and interpret the behavioral response data (Kleinbaum 1995, Roberts et al. 1997). Survival analysis was used to estimate the probability of escape time (ET) and to compare differences in mosquito response among the three mosquito strains and two insecticides. For analysis, mosquitoes that escaped were treated as "dead" and those remaining in the exposure chambers considered "survivors" (Chareonviriyaphap et al. 1997). The ET_{25}, ET_{50}, and ET_{75}, time in minutes for 25, 50, and 75% of the mosquitoes to escape, respectively, were estimated from data collected at 1-min intervals. Patterns of escape response and statistical significance were determined using a log-rank method (Mantel and Haenzel 1959). Statistical significance for all tests was set at $P < 0.05$.

Results

Insecticide Resistance Characterization. Three test strains of *Ae. aegypti* were exposed to diagnostic doses of ACyp and DDT to characterize the susceptibility status to the compounds following WHO protocol (Table 1). All three test strains were susceptible to ACyp (98.0–100% mortality). However, the resistance level to DDT varied between KAN and CM strains, with degree of resistance of 2.0 and 37.1%, respectively. The USDA strain demonstrated complete susceptibility.

Mortality of *Ae. aegypti* to ACyp and DDT after contact and noncontact behavioral assays were observed 24-h postexposure (Tables 2 and 3). For ACyp, higher percentage of mortality was observed in contact trials than in noncontact trials, and greater percentage of mortality were observed in both treated chambers compared with respective controls. For all three strains, no mortality was seen in escaped mosquitoes in DDT contact and noncontact trials (Tables 2 and 3).

Behavioral Tests. The female mosquito escape pattern from contact and noncontact chambers during the observation period was calculated for ACyp and DDT (Table 4). Results represent combined data from

Table 2. Percentage of escape and 24-h mortality of *Ae. aegypti*[a] strains in contact irritancy trials by using 0.025 g/m^2 ACyp and 2 g/m^2 DDT in an excito-repellency test chamber system

Strain[b]	Chemical	No. mosquitoes[c]	% escaped (no. escaped)	% mortality	
				Escaped	Not escaped
CM	ACyp	59	86.4 (51)*	19.6	25.0
	Control	58	6.90 (4)	0	0
	DDT	59	44.1 (26)*	0	2.63
	Control	59	6.78 (4)	0	0
KAN	ACyp	60	73.3 (44)*	0	12.5
	Control	60	15.0 (9)	0	0
	DDT	60	18.6 (11)*	0	2.04
	Control	60	1.69 (1)	0	0
USDA	ACyp	59	49.2 (29)*	6.90	90.0
	Control	59	3.39 (2)	0	0
	DDT	60	55.0 (33)*	0	18.5
	Control	59	15.3 (9)	0	0

* Log-rank tests with statistically significant differences ($P < 0.05$) in escape pattern comparisons.

[a] F1-F2 females, 4–5-d-old, non–blood-fed, sugar-starved 24 h before assay.

[b] CM, Chiang Mai strain; KAN, Kanchanaburi strain; USDA Gainesville, FL, USA strain.

[c] Numbers of mosquitoes tested in four replicates of 15 each.

Table 4. Time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of three strains of *Ae. aegypti*[a] females to escape from contact and noncontact exposure chambers containing α-cypermethrin- or DDT-impregnated net material during a 30-min observation period

Strain[b]	ACyp (0.025 g/m^2)			DDT (2 g/m^2)		
	ET_{25}[c]	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact						
CM	1	5	12	6	—[d]	—
KAN	1	7	—	—	—	—
USDA	2	6	—	5	12	—
Noncontact						
CM	—	—	—	—	—	—
KAN	10	—	—	—	—	—
USDA	3	—	—	4	—	—

[a] F1-F2 females, 4–5-d-old, non–blood-fed, sugar-starved 24 h before assay.

[b] CM, Chiang Mai strain; KAN, Kanchanaburi strain; USDA Gainesville, FL, USA strain.

[c] ET_{25}, ET_{50}, and ET_{75} indicate time in minutes for 25, 50, and 75% of mosquitoes of each strain to escape from excito-repellency test chambers.

[d] Insufficient number escaped from exposure chambers to estimate ET_{25}, ET_{50}, and ET_{75} during 30-min exposure period.

the four replicates of each trial. Variation in escape response between individual replicate tests within each trial configuration was not significantly different. ET was defined as time required for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of a test strain to escape from the test chamber containing insecticide or solvent only (Chareonviriyaphap et al. 2002). In contact trials with ACyp, the ET_{25} values for all test strains ranged from 1 to 2 min, the ET_{50} ranged from 5 to 7 min, and the ET_{75} ranged from 12 min (for CM) to >30 min (KAN and USDA) (Table 4). Contact trials using DDT indicated slower escape response time compared with ACyp for all corresponding ET values, indicating DDT to be a weaker contact irritant, a condition also seen with the DDT-susceptible USDA strain (Table 4). As expected, the escape response was delayed during noncontact trials for each chemical and strain tested.

Multiple comparisons among the three test strains were performed by chemical and assay design (Tables 2 and 3). The patterns of escape responses examined using a log-rank method showed escape probabilities with ACyp and DDT in both contact and noncontact trials were significantly different from paired controls regardless of mosquito origin ($P < 0.05$). Marked differences in escape responses to DDT in contact trials were observed between KAN and both CM and USDA mosquitoes ($P < 0.05$). There was no significant difference in ACyp escape patterns in contact trials between KAN and CM ($P > 0.05$). Conversely, significant differences in escape patterns in noncontact trials using ACyp were found among all strains and their controls ($P < 0.05$).

The percentages of mosquitoes remaining in the contact chambers up to 30-min exposure for both ACyp and DDT are shown in Fig. 1A and B. There were significant differences in escape responses of CM and KAN in contact and noncontact exposure to DDT ($P < 0.05$). Both CM and KAN females demonstrated significant greater escape responses to ACyp compared with USDA mosquitoes (Fig. 1A). For DDT contact, a greater escape response was seen in USDA compared with CM and KAN field strains (Fig. 1B), similar to overall response to ACyp. Although KAN females did not show as great an escape response, the pattern of activity was still more pronounced compared with controls. In noncontact trials, significantly greater escape response to ACyp was observed in KAN compared with either CM or USDA (Fig. 2A). In contrast, with DDT a significantly weaker escape response was observed in CM and KAN compared with the USDA mosquitoes (Fig. 2B).

Table 3. Percentage of escape and 24-h mortality of *Ae. aegypti*[a] strains in noncontact trial by using 0.025 g/m^2 ACyp and 2 g/m^2 DDT in an excito-repellency test chamber system

Strain[b]	Chemical	No. mosquitoes[c]	% escaped (no. escaped)	% mortality	
				Escaped	Not escaped
CM	ACyp	60	8.33 (5)	0	3.64
	Control	59	8.47 (5)	0	1.85
	DDT	59	20.3 (12)*	0	0
	Control	60	3.33 (2)	0	0
KAN	ACyp	60	45.0 (27)*	0	3.03
	Control	60	6.67 (4)	0	0
	DDT	60	6.67 (4)*	0	1.79
	Control	60	0 (0)	0	0
USDA	ACyp	60	63.3 (38)*	0	4.55
	Control	60	5.00 (3)	0	0
	DDT	60	46.7 (28)*	0	0
	Control	60	15.0 (9)	0	0

* Log-rank tests with statistically significant differences ($P < 0.05$) escape pattern comparisons.

[a] F1-F2 females, 4–5-d-old, non–blood-fed, sugar-starved 24 h before assay.

[b] CM, Chiang Mai strain; KAN, Kanchanaburi strain; USDA Gainesville, FL, USA strain.

[c] Numbers of mosquitoes tested in four replicates of 15 each.

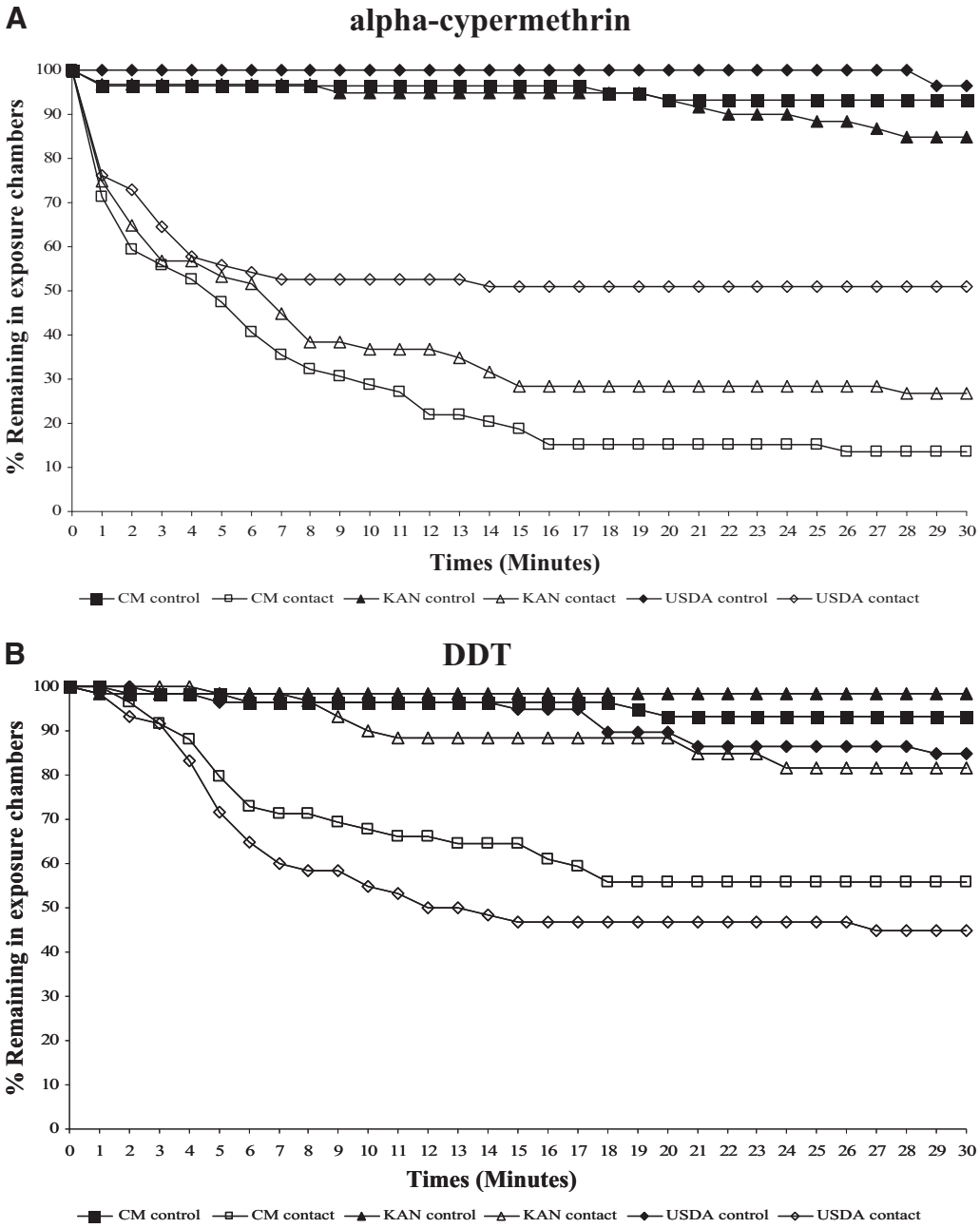

Fig. 1. Escape response of three strains of *Ae. aegypti* in contact irritancy trials with (A) α-cypermethrin at 0.025 g/m^2 and (B) DDT at 2 g/m^2.

Discussion

There are potentially three primary actions of how chemicals might protect humans from adult mosquito vectors of arthropod-borne diseases. These include contact irritancy, noncontact (spatial) repellency, and toxicity (Roberts et al. 2000, Grieco et al. 2007). Most research has focused on toxicity alone without evaluating the irritant/repellent action of the same chemicals use against disease vectors and the impact on pathogen transmission. The lack of a standardized test system for the study of mosquito behavior and the inherent difficulties in comparability of findings across different study designs has been a significant shortcoming to this information gap.

In 1997, a new behavioral test system was developed to quantitatively distinguish two types of behavioral responses: contact irritancy and noncontact repellency, collectively termed excito-repellency (Roberts

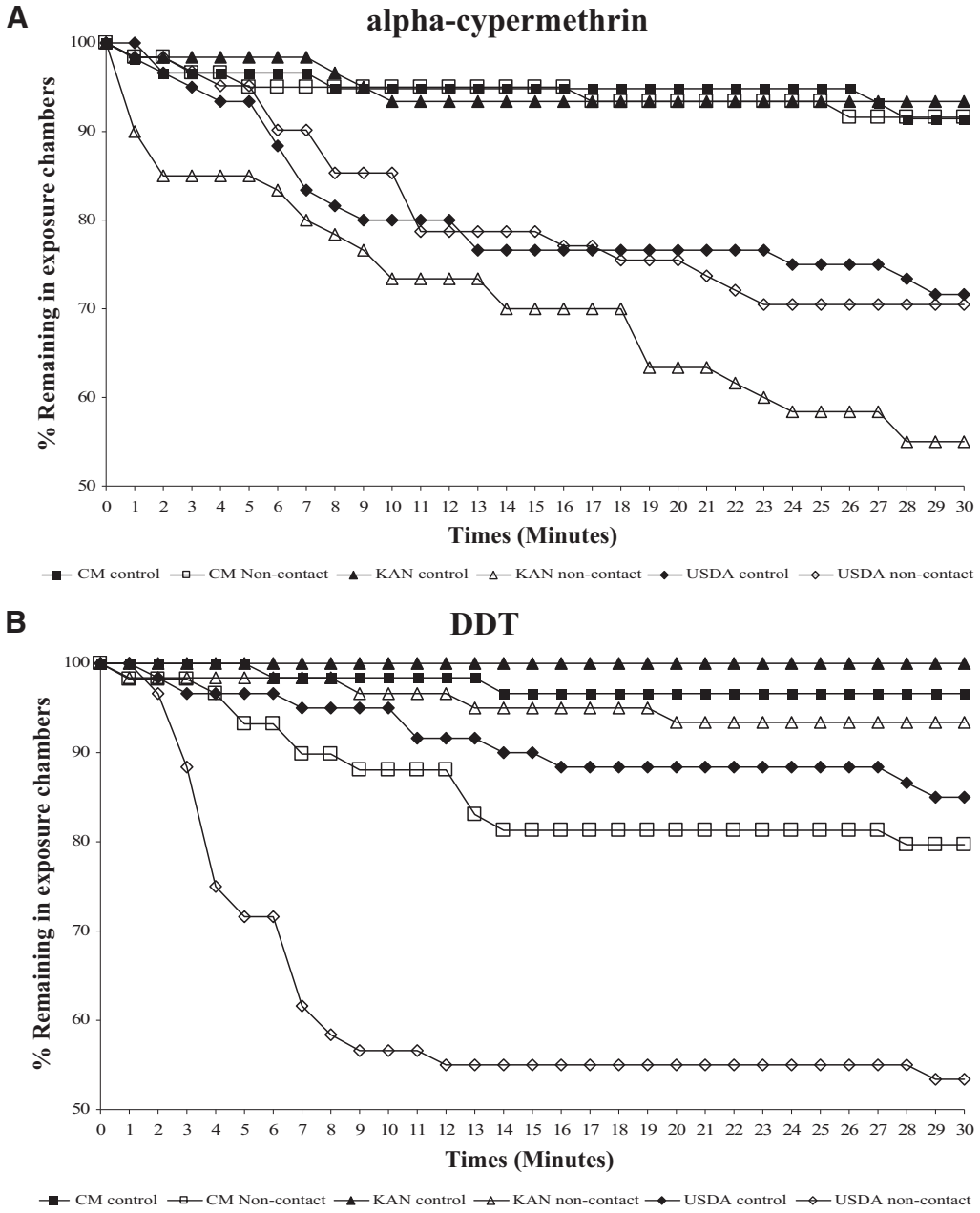

Fig. 2. Escape response of three strains of *Ae. aegypti* in noncontact repellency trials with (A) α-cypermethrin at 0.025 g/m^2 and (B) DDT at 2 g/m^2.

et al. 1997, Chareonviriyaphap et al. 1997). Recently, more field-friendly versions of the ER test system have been developed (Chareonviriyaphap et al. 2002, Tanasinchayakul et al. 2006). Using this system, behavioral responses of several major mosquito disease vectors have been evaluated against various public health insecticides (Chareonviriyaphap et al. 2002, 2004; Kongmee et al. 2004; Muenworn et al. 2006; Sathanatriphop et al. 2006; Polsomboon et al. 2008). A detailed literature base has gradually been developed for many of the pyrethroids, by far the most common and successful public health chemicals in use today, including permethrin, deltamethrin, and λ-cyhalothrin. In this study, we observed the irritant and repellent actions of ACyp and DDT, two successful residual insecticides used to control mosquito-borne diseases.

The three strains of *Ae. aegypti* showed varying degrees of escape response and mortality to both contact and noncontact assays. Our results indicate a strong contact irritant effect of ACyp in all three test

mosquitoes. In addition, the majority mosquitoes escaped from treatment chambers before receiving a lethal dose of insecticide. The contact irritancy response to ACyp seems attenuated in the USDA strain that had been in colony for >40 yr. However, a dramatic escape response was observed in the same colony for repellency trials using DDT. This indicates a possible association (either negative or positive) between long-term colonization and modified behavioral response and therefore further justification and need to evaluate field strains when conducting behavioral tests. Similar results were seen in previous studies comparing long-colonized (>20 yr) strains of *An. albimanus* Wiedemann from Panama and El Salvador and wild-caught field populations (Brown 1958, Chareonviriyaphap et al. 1997). A long-colonized strain of *Anopheles dirus* Payton & Harrison (>15 yr) also showed a poorer response to deltamethrin compared with recently colonized field strains in Thailand (Chareonviriyaphap et al. 2004).

Exposure to DDT elicited varying degrees of irritancy and repellency in all three test strains. However, the irritant action of DDT was significantly weaker than observed with ACyp. The strongest escape response in contact trials was seen with the USDA and CM strains. The KAN *Ae. aegypti* showed a more subdued behavioral reaction to DDT in both contact and noncontact exposures. This may be related or influenced by the high degree of resistance to DDT by this strain. The resistance status of KAN is probably a consequence of either selection pressure from past exposure to agricultural use of this chemical or some form of cross-resistance associated with another chemical sharing similar mode of action (Chareonviriyaphap et al. 1999, Somboon et al. 2003). However, it remains unclear whether the attenuated behavioral response of KAN to DDT is directly influenced by physiological resistance. Similar observations have been documented for *Ae. aegypti* (Cepu strain) from Java, Indonesia, which demonstrated partial resistance to deltamethrin with a weak excito-repellency response (Kongmee et al. 2004). The intermediate escape response of CM between the two extremes of high resistance (KAN) and a strain showing complete susceptibility (USDA) indicates that just higher tolerance to a chemical seems sufficient to effectively influence behavioral response on suppressing escape activity. The evidence seems suggestive that degree of resistance may influence both irritancy and repellency response to the same or similar compound.

However, it is important to note that the irritant and repellent escape response patterns between the DDT tolerant and the highly resistant strain was still significantly greater than in paired controls, indicating that insecticide resistance and behavior may influence one or the other but might otherwise have separate modes of action. Whereas our finding suggest a possible link, a recent review of other studies has indicated that toxic action seems to have no influence on the behavioral responses of mosquitoes; therefore, any association between the two phenomena requires further evaluation (Grieco et al. 2007).

As control of the *Aedes* vector remains the only viable means to combat dengue transmission, the combination of source reduction of preimaginal habitats and the selective application of insecticides will be needed into the foreseeable future. Increased advocacy for use of indoor application of residual insecticides (on surface of structures or materials such as window curtains) makes it imperative that we redouble out efforts to better understand the behavioral response of *Ae. aegypti* to chemical applications and the proximate effect on reducing virus transmission.

In conclusion, a better understanding of irritancy and repellency actions of chemicals that may interfere with normal behavioral host-seeking and blood-feeding patterns is essential when evaluating the full impact these compounds may have on both mosquitoes and disease transmission. A standardize approach and quantification of a chemical's primary and secondary mode of actions will help to drive an optimization of currently available public health tools and hopefully promote the development of newer chemicals and other innovative control methodologies.

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Genetic structure among Thai populations of *Aedes aegypti* mosquitoes

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ABSTRACT: Thirty-one field populations of *Aedes aegypti* (L.) were compared using isozyme starch gel electrophoresis to characterize genetic variation between populations. *Ae. aegypti* were collected from seven provinces in Thailand. Thirty-one isozyme encoding loci, including 19 polymorphic loci, were characterized. Only small levels of genetic differentiation were observed among the 31 district populations in the seven provinces. Isolation by distance among populations from the seven provinces showed no correlation between genetic variation and geographical distance. ***Journal of Vector Ecology* 34 (1): 43-49. 2009.**

Keyword Index: Aedes aegypti, genetic structure, starch gel electrophoresis.

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most common and important arboviral mosquito-borne diseases syndromes in the world (Gubler 1988). During the last decade, the number of dengue cases in Thailand has increased and disease transmission remains prevalent throughout the country. Each year, 50,000 to 200,000 dengue cases are reported annually in the country (Ministry of Public Health 2006). Currently, control of the mosquito vectors remains the most efficient method to prevent dengue virus transmission and avert dengue epidemics (Reiter and Gubler, 1997, World Health Organization 1999).

Aedes aegypti (L.), a predominately human-biting mosquito, is the primary vector world-wide and a common species throughout Thailand. It was first reported in Thailand in 1907 (Theobald, 1907) and is considered to be a polytypic species worldwide (Tabachnick 1991). Several population genetic studies of *Ae. aegypti* have been conducted in different regions of the world (Tabachnick and Powell 1979, Wallis et al. 1983, Apostol et al. 1996, Gorrochotegui-Escalante et al. 2000, Failloux et al. 2002, Mousson et al. 2002). Analyses of genetic variation among populations, established by comparing variability in isozyme encoding loci, have been used to estimate gene flow between different *Ae. aegypti* populations (Tabachnick 1991, Manguin et al. 1995). Relatively little is known about the genetic structure and gene flow among *Ae. aegypti* populations among cities in Thailand. Previous studies have shown that *Ae. aegypti* populations are genetically similar, with high levels of gene flow between them in the city of Bangkok, in central Thailand (Sukonthabhirom et al. 2005). In other regions, for example, Chareonviriyaphap and Lerdthusnee (2002) found no significant differentiation between *Ae. aegypti* mainland and Samui Island populations of the Surat Thani province in southern Thailand. Netthanomsak[6] reported low genetic variation of *Ae. aegypti* along the coastal area of Gulf of Thailand, covering six provinces. Mousson et al. (2002) confirmed high genetic differentiation among *Ae. aegypti* samples collected in different subdistricts from the Chiang Mai province in northern Thailand. Bosio et al. (2005) studied *Ae. aegypti* populations in Thailand from Chiang Mai in the north to the Songkhla province in the south and reported that *Ae. aegypti* in urban areas were relatively panmictic, while suburban / rural sites exhibited more restricted gene flow and there was no isolation by distance. The goal of the present study was to characterize the genetic structure of populations of *Ae. aegypti* in different provinces in Thailand using gene frequencies of isozyme encoding loci. Population genetic structure and the extent of gene flow between mosquito populations is fundamental information for developing more effective strategies to combat insecticide resistance and more effective and appropriate national programs to control *Ae. aegypti* in Thailand.

[6]Netthanomsak, S. 2004. Ecological significance and isozyme patterns of *Aedes aegypti* (Linneus), a vector of Dengue fever in Thailand. M.S. thesis, Kasetsart University. Bangkok, Thailand.

Figure 1. Map of *Aedes aegypti* collections from seven leading endemic areas of Thailand.

MATERIALS AND METHODS

Mosquito populations

Aedes aegypti were collected from seven provinces with high numbers of dengue cases (Figure 1). Four to five districts with a radius of 30 km were selected within each province, except Chiang Mai, in which only two districts were sampled. Collections were made from four villages within each district.

All *Ae. aegypti* samples were collected as larvae or pupae to obtain large samples. An average of 100-500 larvae was collected per village site in each district. The larvae from containers in each village in a certain district were pooled together as a single district population and were brought back and reared to adults in a protected insectary at the Department of Entomology at Kasetsart University. Strict segregation of field specimens was maintained in the insectary to prevent potential contamination from other *Ae. aegypti* colonies. Adult mosquitoes were identified and either tested immediately or shortly frozen (-20° C) before processing.

Starch gel electrophoresis

An average of 30-40 adults, consisting of an approximately equal number of males and females, from each district population were tested by electrophoresis. Starch gel electrophoresis was performed using 20 enzyme systems according to methods described by Harris and Hopkinson (1976), Manguin et al. (1995), and Lerdthusnee and Chareonviriyaphap (1999).

Data analysis

Analysis of allele frequencies, mean number of alleles per locus, observed and expected heterozygosity, conformity to the Hardy-Weinberg equilibrium, percentage of polymorphic loci, and fixation index (*FST*) were calculated using BIOSYS-1 (Swofford and Selander 1989). Differentiation among populations was determined by *F*-statistics (*FST*) where $FST > 0.25$ signifies very great genetic differentiation, $0.25 > FST > 0.15$ signifies moderately great genetic differentiation, $0.15 > FST > 0.05$ signifies small genetic differentiation, and $FST \leq 0.05$ signifies negligible genetic differentiation (Wright 1978). The statistical significance of the *Fst* values was determined using the GENEPOP and ARLEQUIN program (Rousset 1997, Excoffier et al. 2005). The effective migration rate (*Nem*) among populations per generation was estimated from the *FST* values using $Nem = (1- FST) / 4\, FST$ where N_e is the effective population size and *m* is the migration rate between populations (Wright 1978). An analysis of molecular variance (AMOVA) partitioned genetic variance into intra- and inter- populations, and within and among provinces by the ARLEQUIN program, version 3.1 (Excoffier et al. 2005). The XLSTAT, version 2007, was used to analyze isolation by distance between populations by the Mantel regression. This was measured by the relationship between pairwise estimates of *FST* and logarithms of geographical distance to determine whether geographical distance among populations serves as a barrier to gene flow.

RESULTS

Table 1 shows the 31 putative loci. Twelve loci were monomorphic, whereas 19 were polymorphic. Three loci, *IDH-1, MDH-1,* and *PGM-1*, were polymorphic in all populations. The allelic frequencies of all polymorphic loci in each of the 31 district populations were used for genetic differentiation analysis and are available upon request. *AKS-2* and *FUM-1* loci were polymorphic only in Bangkok populations. *ATA-1* showed allelic polymorphism in Bangkok and Surat Thani populations. *ATA-2* showed allelic polymorphism in Bangkok, Surat Thani, and Nakhon Sawan populations. *ACO-2* locus was polymorphic exclusively in Nakhon Sawan populations. *HAD-1* locus was polymorphic only in Bangkok and Chon Buri populations. *MDH-2* was polymorphic in Chon Buri, Surat Thani, Nakhon Sawan, and Nakhon Ratchasima populations, and *ME-1* was observed as polymorphic in Surat Thani and Nakhon Ratchasima populations.

Chi-square tests of 961 comparisons in allelic frequencies of 31 putative loci from 31 populations showed 58 significant deviations from the Hardy-Weinberg equilibrium ($P < 0.05$). The significant deviations from equilibrium occurred in 6% of the total number of comparison which

Table 1. Isozymes used in electrophoretic studies on adult *Aedes aegypti*.

Enzyme system	E.C. Number[1]	Gene symbol	No. loci[2]	Buffer[3]
Aconitase	4.2.1.3	*ACO*	2	TCss
Adenylate kinase	2.7.4.3	*AKS*	3	TCss
Aldehyde oxidase	1.2.3.1	*AOX*	1	LiOH
Arginine kinase	2.7.3.3	*ARK*	3	TCss
Aspartate transaminase	2.6.1.1	*ATA*	2	Morph
Fumarase	4.2.1.2	*FUM*	1	TCss
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6PD*	1	TCss
Glycerol dehydrogenase	1.1.1.72	*GCD*	1	TCss
α-glycerophosphate dehydrogenase	1.1.1.8	*GPD*	1	LiOH
Glucose phosphate isomerase	5.3.1.9	*GPI*	1	LiOH
Hydroxy acid dehydrogenase	1.1.1.30	*HAD*	1	Morph
Hexokinase	2.7.1.1	*HK*	3	Morph
Isocitrate dehydrogenase	1.1.1.42	*IDH*	1	Morph
Malate dehydrogenase	1.1.1.37	*MDH*	2	Morph
Malic enzyme	1.1.1.40	*ME*	1	LiOH
Manose-6-phosphate isomerase	5.3.1.8	*MPI*	1	Morph
Phosphogluconate dehydrogenase	1.1.1.43	*PGD*	1	TCss
Phosphoglucomutase	5.4.2.2	*PGM*	1	Morph
Pyruvate kinase	2.7.1.40	*PK*	3	TCss
Xanthine dehydrogenase	1.2.1.37	*XDH*	1	LiOH
Total			31	

[1] Enzyme Commission Number.
[2] Number of scorable loci per isozyme.
[3] Refers to electrophoresis buffer (see Materials and Methods).

was not significantly different from the expected deviations due purely to chance ($\chi^2 = 0.998$, df =1, p=0.318).

The expected heterozygosity (H_e) varied from 0.037 to 0.123 with an average ($\pm$SEM) of 0.071 ($\pm$) 0.026 (Table 2). H_e was greatest in Bangkok (Huai Khwang) with $H_e = 0.123\pm0.033$, whereas the lowest H_e was in a collection from Nakhon Ratchasima (Dan Khun Thot), $H_e = 0.037\pm0.014$ (Table 2). When all populations within a province were grouped together, the lowest expected heterozygosities were in Nakhon Ratchasima ($H_e = 0.051\pm0.021$) and the highest was in Bangkok ($H_e = 0.096\pm0.031$).

An analysis of isolation by distance on pairwise *Fst*/(1-*Fst*) among all populations was performed against geographical distance using the Mantel regression. The Mantel test indicated no correlation ($P > 0.05$) between genetic and geographical distance across all provincial populations and within provincial populations (Table 3).

Variation in populations was also tested at different hierarchical levels by AMOVA (Table 4). Most of the variation was found within populations (91.9% of the total variation). Significantly little variation was found among populations within a province (3.7% of the total variation). In addition there was also significantly little genetic differentiation among the seven provinces (4.4% of the total variation). There was only slightly lower genetic differentiation among populations within a province, an inference reinforced by the significantly lower estimate of *Fsc* (*Fsc* = 0.038, $P < 0.01$) within provinces compared to the higher estimate of *Fct* (*Fct* = 0.044, $P < 0.01$) between provinces (Table 4).

DISCUSSION

Aedes aegypti is one of the most important mosquito vectors of viral agents in the world, due to its role in transmitting dengue virus (DENV), and also the chikungunya virus (CHIKV) throughout most of Thailand (Thavara et al. 2001, Bosio et al. 2005). Progressive urbanization and human activities have decreased natural *Aedes* larval habitats in Thailand, replacing them with artificial ones, which has greatly assisted the expansion and prevalence of *Ae. aegypti* and displacement of native species (Pant et al. 1973, Thavara et al. 2001). The use of insecticides and the elimination of larval sites ("source reduction") for control of habitats in and around dwellings have had a demonstrable impact on the genetic structure and gene flow of *Ae aegypti* populations in several regions of the world

Table 2. Genetic variability at 31 loci of 31 populations of *Aedes aegypti* from seven provinces in Thailand.

Population	Average alleles per locus	% polymorphic loci[1]	Mean heterozygosity[2]	
			Hobs	Hexp[3]
Bangkok				
-Lak Si	1.6 ± 0.3	22.6	0.082 ± 0.031	0.088 ± 0.031
-Rat Burana	1.5 ± 0.2	25.8	0.071 ± 0.029	0.089 ± 0.032
-Lat Krabang	1.6 ± 0.2	22.6	0.060 ± 0.025	0.082 ± 0.031
-Huai Khwang	1.7 ± 0.2	35.5	0.116 ± 0.035	0.123 ± 0.033
-Bangkok Noi	1.6 ± 0.2	32.3	0.088 ± 0.030	0.099 ± 0.031
Chon Buri				
-Bang Lamung	1.3 ± 0.1	16.1	0.052 ± 0.025	0.047 ± 0.022
-Phanat Nikhom	1.3 ± 0.1	12.9	0.044 ± 0.023	0.045 ± 0.023
-Mueang Chon Buri	1.4 ± 0.1	12.9	0.059 ± 0.025	0.058 ± 0.025
-Si Racha	1.4 ± 0.1	16.1	0.069 ± 0.030	0.067 ± 0.026
Surat Thani				
-Chaiya	1.5 ± 0.2	19.4	0.084 ± 0.032	0.073 ± 0.027
-Ban Na Doem	1.5 ± 0.2	25.8	0.094 ± 0.034	0.082 ± 0.029
-Khian Sa	1.5 ± 0.2	22.6	0.077 ± 0.028	0.078 ± 0.028
-Don Sak	1.6 ± 0.2	19.4	0.076 ± 0.026	0.079 ± 0.028
-Mueang Surat Thani	1.5 ± 0.2	19.4	0.081 ± 0.029	0.072 ± 0.025
Nakhon Sawan				
-Mae Poen	1.4 ± 0.2	19.4	0.095 ± 0.036	0.082 ± 0.030
-Mae Wong	1.3 ± 0.1	19.4	0.085 ± 0.037	0.063 ± 0.025
-Mueang Nakhon Sawan	1.5 ± 0.2	22.6	0.100 ± 0.035	0.094 ± 0.032
-Krok Phra	1.4 ± 0.1	22.6	0.063 ± 0.024	0.064 ± 0.023
-Takhli	1.5 ± 0.2	25.8	0.086 ± 0.032	0.074 ± 0.026
Nakhon Ratchasima				
-Soeng Sang	1.3 ± 0.1	19.4	0.052 ± 0.023	0.052 ± 0.022
-Prathai	1.4 ± 0.2	12.9	0.057 ± 0.029	0.050 ± 0.024
-Kaeng Sanam Nang	1.4 ± 0.1	19.4	0.069 ± 0.027	0.064 ± 0.025
-Sikhio	1.4 ± 0.1	12.9	0.059 ± 0.025	0.052 ± 0.022
-Dan Khun Thot	1.2 ± 0.1	19.4	0.039 ± 0.015	0.037 ± 0.014
Songkhla				
-Hat Yai	1.5 ± 0.2	19.4	0.087 ± 0.035	0.086 ± 0.030
-Mueang Songkhla	1.4 ± 0.1	16.1	0.095 ± 0.039	0.075 ± 0.029
-Bang Klam	1.4 ± 0.2	16.1	0.100 ± 0.042	0.077 ± 0.031
-Singhanakhon	1.5 ± 0.2	22.6	0.088 ± 0.035	0.083 ± 0.032
-Chana	1.4 ± 0.2	16.1	0.070 ± 0.035	0.061 ± 0.027
Chiang Mai				
-Mae Taeng	1.3±0.1	25.8	0.105±0.038	0.091±0.032
-Mueang Chiang Mai	1.2±0.1	19.4	0.058±0.027	0.046±0.020

[1]A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95. [2]The observed and expected mean heterozygosities were not significantly different by the Student's *t*-test (*t* 0.025 = 0.863 ns). [3]Unbiased estimate and standard error (Nei 1978).

Table 3. F-statistics, effective migration rates and Mantel test of isolation by distance among populations of *Aedes aegypti* in Thailand.

Population	FST[1]	Nem[2]	Mantel regression	
			r	*p*-value
Among seven provinces	0.064**	3.76	0.299	0.182
Within Bangkok	0.052**	4.54	-0.285	0.419
Within Chon Buri	0.053**	4.47	0.179	0.726
Within Surat Thani	0.026**	9.37	-0.342	0.333
Within Nakhon Sawan	0.046**	5.18	0.135	0.732
Within Nakhon Ratchasima	0.051**	4.65	0.087	0.889
Within Songkhla	0.020**	12.25	0.111	0.796
Within Chiang Mai	0.068**	3.43	NA[4]	NA[4]

[1]FST = Degree of genetic differentiation among or within the province populations; levels of significance for FST > 0 are: *p <0.05; **p <0.01.
[2]Nem = Effective migration rates.
[3]The p-values have been calculated using the distribution of r(AB) estimated from 10,000 permutations. All the computed p-values are greater than the significant level, alpha = 0.05.
[4]NA = Not applicable.

(Wallis et al. 1984, Failloux et al. 1995, Lerdthusnee and Chareonviriyaphap 1999, Paupy et al. 2000). However, the reduced heterozygosity and lack of genetic differentiation in Thailand is likely not the result of an efficient nationwide control program using insecticides to control both adult and larval mosquitoes and widespread source reduction in dengue receptive areas. This practice would likely have resulted in resident small *Ae. aegypti* populations subject to founder effects, accompanying genetic drift, and more genetic differentiation within and between provinces. Thus, the results are consistent with the widespread historically low heterozygosity of Asian *Ae. aegypti*, coinciding with the introduction of *Ae. aegypti* to Asia noted by Tabachnick (1991).

We observed little genetic differentiation between populations within and among the seven Thailand provinces examined. An analysis showed no isolation by distance across the regions of Thailand in this study, although there were some differences in genetic variation between a few loci. Generally, *Ae. aegypti* populations in Thailand showed little genetic differentiation. Most of the variation was found within populations, indicating that genetic diversity in Thailand *Ae. aegypti* populations was significantly greater within populations than between.

The average expected heterozygosity in *Ae. aegypti* Thai populations (H_{exp} = 0.071) was seemingly not different than previously reported (Chareonviriyaphap and Lerdthusnee 2002, Sukonthabhirom et al. 2005). Heterozygosities have been reported for *Ae. aegypti* in East Africa[7], the Caribbean area (Wallis et al. 1983), Puerto Rico (Wallis et al. 1984), Houston, TX U.S.A. (Harrington et al. 1984), areas of Asia (Tabachnick 1991), and worldwide collections (Tabachnick and Powell 1979). Tabachnick (1991) reported lower heterozygosities in Asian *Ae. aegypti* compared to other regions of the world, consistent with a recent introduction of *Ae. aegypti* to Asia, and Thai populations are consistent with the lower heterozygosity and genetic diversity reported earlier.

The mean number of alleles per locus was lower than those reported from *Ae. aegypti* in six provinces along the coast of the Gulf of Thailand[6]. The Huai Khwang (Bangkok

[7]Munstermann, L.E. 1979. Isozymes of *Aedes aegypti*: phenotypes, linkage, and use in the genetic analysis of sympatric subspecies populations in East Africa. Ph.D. Dissertation, University of Notre Dame.

Table 4. Hierarchical analysis of molecular variance among *Ae. aegypti* populations in Thailand.

Source of variation	d.f.	Variation (%)	Fixation index 1	p-value
Among seven provinces	6	4.40	F_{CT} = 0.04403	*<0.01*
Among populations within a province	24	3.68	F_{SC} = 0.03845	*<0.01*
Within populations	2229	91.92	F_{ST} = 0.08079	*<0.01*

[1]F_{CT} = Fixation index among seven provinces; F_{SC} = Fixation index among populations within a province; F_{ST} = Fixation index within populations.

Province) population had the greatest percentage of polymorphic loci (35.5%) compared to other locations. However, the percent of polymorphic loci in the seven provinces were lower (12.9-35.5%) than reported elsewhere, both 37.5-55.6% from Brazil (Dinardo-Miranda and Contel 1996, Costa Fraga et al. 2003), 59% from Kenya (Tabachnick and Powell 1976), 27.3-63.6% from Argentina (Sousa et al. 2000), and 40-50% from along the coastal area of Gulf of Thailand[6]. The percent of polymorphic loci of all populations in Thailand were similar to reports from southern Thailand (24.2-36.4%) (Chareonviriyaphap and Lerdthusnee 2002). The greater level of polymorphism found in populations from Bangkok, especially from Huai Khwang and Bangkok Noi Districts, may be due to the greater population size or the result of multiple introductions of different populations from outside the city. Thai *Ae. aegypti* populations show lower levels of genetic variation than other regions of the world that have been examined.

From the AMOVA analysis, while *FCT* (fixation index among seven provinces) and *FSC* (fixation index among district populations within a province) were relatively minor sources of variation, they were statistically significant. Therefore, there is significant genetic structure at these levels also. A large proportion of variation within populations was drastic, indicated by higher *FST* (fixation index within populations) value. From our results, it was concluded that *Ae aegypti* populations in Thailand have no obvious genetic structure since we observed little genetic heterogeneity and the absence of small population size effects that would result in genetic drift and population differentiation. The results showing low heterozygosity, lower mean number of alleles per locus, and generally low *FST* are consistent with large effective population sizes and the low level of genetic diversity. Due to little genetic differentiation between the populations throughout this study, *Ae. aegypti* populations in Thailand appear to be panmictic with no significant barriers to gene flow between populations.

The rapid re-establishment of *Ae. aegypti* by migrants from nearby areas or rapid adaptation to new mosquito receptive areas likely plays a significant role in maintaining the large panmictic populations seen in our study. However, since Thai *Ae. aegypti* populations contain little genetic diversity to begin with, even founder events might not explain genetic differentiation since the founders are more likely to retain the genetic variants of the original population. These results are in concordance with the small genetic differentiation seen among *Ae. aegypti* populations in New Orleans in the United States, French Polynesia and Colombia (Tabachnick 1982, Failloux et al. 1995, Ocampo and Wesson 2004) and the low heterozygosity observed in *Ae. aegypti* populations in the southeastern United States (Tabachnick 1991).

Analysis showed no significant differences of isolation by distance between populations in Thailand ($P > 0.05$). This result was in agreement with Mousson et al. (2002) that investigated the relationship between rate of genetic exchanges and geographical distances of *Ae. aegypti* populations in Chiang Mai, Thailand. Likewise, these findings are in agreement with analysis of *Ae. aegypti* populations in Thailand using the NADH dehydrogenase subunit 4 mitochondrial DNA gene (Bosio et al. 2005). The results from this study expand and support previous studies that showed Thailand's *Ae. aegypti* to contain low levels of genetic diversity and genetically similar populations.

Understanding the local population dynamics and genetic exchanges between vector populations will provide information that will be useful in understanding their abilities to harbor and transmit dengue viruses as well as their ability to develop insecticide resistance. This knowledge can greatly assist predictive modeling, surveillance planning, and the most appropriate control strategies.

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Irritability and repellency of synthetic pyrethroids on an *Aedes aegypti* population from Thailand

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ABSTRACT: The main objective of this study was to find the optimal dosage of deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin that would elicit repellency and irritability responses of *Aedes aegypti*. The F1-F3 generations of field mosquitoes collected from Pu Teuy Village, Sai-Yok District, Kanchanaburi Province, Thailand, were tested with four pyrethroids to determine the LC_{25}, LC_{50}, and LC_{99}. These concentrations were 0.010%, 0.020%, and 0.055%, respectively, for deltamethrin; 0.113%, 0.167%, and 0.353%, respectively, for cyphenothrin; 2.091%, 2.770%, and 5.114%, respectively, for d-tetramethrin; and 2.377%, 4.251%, and 10.715%, respectively, for tetramethrin. All dosages were tested in the excito-repellency system. Survival analysis was used to compare each chamber of the test. It was found that cyphenothrin had a stronger repellent effect than the other pyrethroids, while the contact irritant effect was similar among compounds tested. The LC_{50} of each pyrethroid was found to be the optimal dose for repelling *Ae. aegypti*. There was no significant difference in LC_{99} values for either non-contact or contact trials for each pyrethroid. ***Journal of Vector Ecology* 34 (2): 217-224. 2009.**

Keyword Index: Aedes aegypti, deltamethrin, cyphenothrin, d-tetramethrin, tetramethrin, excito-repellency system, Thailand

INTRODUCTION

People in many areas of the world are at risk from a wide variety of vector-borne diseases, including dengue fever and dengue hemorrhagic fever (DF/DHF). It is estimated that 50-100 million people are infected with dengue viruses worldwide (Gubler 1997). This mosquito-borne disease results in considerable morbidity and mortality in humans annually. The virus is vectored primarily by *Aedes aegypti* (L.), a notoriously efficient vector mosquito that often resides in and near human dwellings (Gubler 1997, WHO 1997). Despite progress in vaccine development, no effective and acceptable multi-valent dengue vaccine is currently available. Prevention of this disease remains entirely dependent on vector reduction through the use of insecticides (Roberts et al.1997, Chareonviriyaphap et al. 2004, Grieco et al. 2007).

Previously, studies on how chemicals function have focused primarily on toxicity. Little is known about two types of behavioral avoidance responses elicited by these chemicals: irritability and repellency (Chareonviriyaphap et al. 1997, Grieco et al. 2007). Irritability occurs when an insect is stimulated to move away from an insecticide after making direct physical contact with the chemical residue, whereas repellency occurs when the insect detects chemicals from a distance, and is diverted out of the treated area without making physical contact with the chemical (Roberts et al. 1997). In the last decade, these types of responses have been documented in both field and laboratory mosquito populations. The outcome of either form of behavioral avoidance can be quantified using a specially designed excito-repellency test system (Roberts et al. 1997, Chareonviriyaphap et al. 1997, 2004, Sungvornyothin et al. 2001).

Many chemical compounds, including synthetic pyrethroids, have long been used in national vector control programs (Rieter and Gubler 1997). In Thailand, deltamethrin is frequently and widely used as an indoor residual spray for controlling household nuisance mosquitoes and disease vectors, including *Ae. aegypti* (Chareonviriyaphap et al. 1999, Somboon et al. 2003). Deltamethrin, applied as a space spray, has also been used in attempts to break the mosquito-virus transmission cycle in dengue active areas (Communicable Disease Control (CDC) 2006.). The impact of pyrethroids on disease vectors requires continued investigation and serves as a stimulus for future studies on the mode of action and epidemiological significance of avoidance behavior (Chareonviriyaphap et al. 2001).

Recent studies have reported the spread of deltamethrin resistance in several field *Ae. aegypti* populations from Thailand (Jirakanjanakit et al. 2007). The spread of resistance is raising awareness for the need of alternative insecticides or new methods of controlling mosquito vectors in Thailand. New, effective, and safe synthetic pyrethroids are readily available and are becoming more common for domestic

protection against indoor-biting mosquitoes and other arthropod pests. Therefore, it is important to investigate how these new synthetic pyrethroids might behaviorally impact *Ae. aegypti* populations before large scale use. Careful monitoring of the behavioral responses of *Ae. aegypti* to test compounds is extremely important and is facilitated by using an excito-repellency test system (Tanasinchayakul et al. 2006). Behavioral responses of wild-caught *Ae. aegypti* were compared using three different concentrations (LC_{25}, LC_{50}, and LC_{99}) of four synthetic pyrethroids; deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin, and with or without physical contact with insecticides.

MATERIALS AND METHODS

Mosquito population

A group of 300-400 larvae was collected from the Pu Teuy Village, Sai Yok District, Kanchanaburi Province (14^{O} 17' N, 99^{O} 11'E, 310 asl) in December, 2008. The colony was maintained at the Department of Entomology, Kasetsart University. Populations of F1-F3 *Ae. aegypti* mosquitoes were used for testing.

Mosquito rearing

All life stages of mosquito were maintained at 25±5° C and 80±10% relative humidity in the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University. Adults were provided with cotton pads soaked with 10% sugar solution from the day of emergence and adults were maintained in a 30 cm^3 screened cage. Female mosquitoes were permitted to feed on a mouse. Two days post-blood feeding, oviposition dishes were placed in the cages with the gravid females.

Insecticides

Four insecticides were used in behavioral tests:

1. Deltamethrin [(S)-α-cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate] was received from BASF company in January, 2007 (CAS# 52918-63-5, 67375-30-8 Bayer).

2. Cyphenothrin [(RS)-α-cyano-3-phenoxybenzyl (1RS)-cis-trans-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate] was received from TJC, Bangkok, Thailand, in January, 2007 (CAS#39515-40-7).

3. *D-Tetramethrin [(3,4,5,6-Tetrahydrophthalimidomethyl (1R)-cis-trans-chrysanthemate]* was received from TJC, Bangkok, Thailand, in January, 2007 (CAS#1166-46-7).

4. *Tetramethrin [(cyclohex-1-ene-1,2-dicarboximidomethyl (1RS)-cis-trans-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate]* was received from TJC, Bangkok, Thailand, in January, 2007 (CAS#1166-46-7).

Insecticide-treated papers

Based on the established LC_{25}, LC_{50}, and LC_{99} for all four insecticides on an *Ae. aegypti* field strain, test papers (15 cm x 17.5 cm) were impregnated with deltamethrin at 0.010%, 0.020%, and 0.055% with cyphenothrin at 0.113%, 0.167%, and 0.353%, with d-tetramethrin at 2.091%, 2.770%, and 5.114%, and with tetramethrin at 2.377%, 4.251%, and 10.715%, respectively. All papers were treated at the rate of 2.9 ml of insecticide solution per 262.5 cm^2.

Behavioral tests

Tests were carried out by using the excito-repellency test system improved by Tanasinchayakul et al. (2006). The complete system consisted of four chambers which are made from stainless steel. Each chamber is comprised of 1) a rear door cover, 2) a Plexiglas panel with an 11.5 cm diameter hole, 3) a Plexiglas holding frame, 4) an inner screen chamber that measures 22.5 x 19 cm^2, 5) an outer chamber that measures 23 x 23 cm^2, 6) a front door with an exit portal slot, and 7) an exit portal. A receiving box was constructed from a 6 x 6 x 6 cm^3 paper carton and was attached to the exit portal for collecting all escaping mosquitoes. Each treatment was paired with a matched control in the following configurations: a control non-contact chamber with a non-contact chamber, and a control contact chamber with a contact chamber (Chareonviriyaphap et al. 2002). Non-blood-fed, three to five-day-old females were used in the tests. Mosquitoes were deprived of a sugar meal for 24 h prior to testing, but were provided with water-soaked cotton pads. All tests were performed between 08:00 and 16:30. For each test, 15 female mosquitoes were introduced into each of four chambers via a hole in the Plexiglas panel, after which the rear of the outer chamber was shut. The mosquitoes were allowed to acclimate to the inside of the chamber for three min (Chareonviriyaphap et al. 1997). Subsequently, each exit portal slot was opened to begin testing. The numbers of mosquitoes that escaped from the exposure chamber into the receiving cage were recorded at one-min intervals for a period of 30 min (Chareonviriyaphap et al. 2002). All mosquitoes escaping during each one-min interval were transferred to holding cups.

Ambient temperatures and relative humidity were recorded during the experiment. All tests were performed during the day and each test series 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 small holding containers topped with cotton soaked with 10% sugar solution until 24 h mortalities were recorded.

Data analysis

The Kaplan-Meier survival analysis method was used to analyze and interpret the rates of escaping mosquitoes from each chamber of the excito-repellency system (Roberts et al. 1997, Chareonviriyaphap et al. 1997). Mosquitoes that escaped out of the test chamber were treated as "deaths," and those still remaining in the test chamber from one minute to the next were treated as "survivals." At the end of the test, the remaining mosquitoes in the exposure chambers were treated as "censored." Survival analysis was also used to estimate escape time (ET) when the percentages of escaping mosquitoes reached 25, 50, and 75% (ET_{25}, ET_{50}, and ET_{75}).

The log-rank method was used to compare patterns of escape behavior within the groups of testing chambers and between difference treatment groups (Mantel and Haenzel 1959). Statistical significances for all tests were determined at P <0.05. The SAS system for windows V. 6.12 analysis (SAS program package (SAS Release 6.12, SAS Institute, Cary, NC) was used in the analysis.

RESULTS

Data on dose-mortality relationships for all four synthetic pyrethroids, deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin against *Aedes aegypti* are given in Table 1. The LC_{25}, LC_{50}, and LC_{99} values of deltamethrin were lower when compared to those of the other three chemicals at the same levels. The slopes of the regression lines for test data from each chemical were computed. The highest slope was obtained from deltamethrin (24.587) and the lowest value was from tetramethrin (0.121) (Table 1). Results of susceptibility tests established from the single diagnostic doses (LC_{99}x2) of deltamethrin (2x0.055=0.111%), cyphenothrin (2x0.353=0.707%), d-tetramethrin (2x5.114=10.228%), and tetramethrin (2x10.715=21.430%) found that the *Ae. aegypti* test population was completely susceptible to all four insecticides (100% mortality: data not shown).

Mortalities of *Ae. aegypti* at three levels (LC_{25}, LC_{50}, and LC_{99}) of deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin were tested in contact and non-contact exposure chambers (Tables 2-5). In general, higher mortalities were observed in contact trials than in non-contact trials and in controls. Within treatment trials, higher mortalities were observed from non-escaped mosquitoes compared with those that were able to escape. A level of high mortality can be seen in non-escaped mosquitoes from the LC_{99} compared with the LC_{25} and LC_{50} values. Comparatively, low percent mortality was observed from escaping females in contact trials, ranging from 0 to 7.69% for deltamethrin, from 2-8% for cyphenothrin, from 2.13-2.27 % for d-tetramethrin, and from 4.17-8.70% for tetramethrin. A high mortality of non-escaped females from the treated chambers with cyphenothrin at LC_{99} (60%) and d-tetramethrin at LC_{99} (31.25%) was recorded. Mortalities in non-contact insecticide trials were low, ranging from 0-4.76% for escaped specimens and 0-7.69% for non-escaped specimens.

The percentage of escaping females in response to the four test chemicals and separated by contact irritancy and non-contact repellency is shown in Tables 2-5. In general, significantly greater escape responses were found in contact trials compared with non-contact trials (P<0.05). In the deltamethrin contact trials, higher escape responses were seen at LC_{50} (88.33%) and LC_{99} (86.67%) compared to LC_{25} (63.33%). Cyphenothrin produced a stronger escape response (80.36-83.33%) compared to d-tetramethrin (73.33%-79.66%) and tetramethrin (60.00-72.41%), regardless of test concentrations (Tables 2-5). Escape responses for insecticide non-contact trials were low, but significantly different from the controls for all cases (P <0.05).

Time in minutes for *Ae. aegypti* females to escape from the treated chamber with deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin at three different doses is listed in Table 6. The escape patterns from chambers treated with chemicals were defined as times for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of a test population to depart the treated chambers (Chareonviriyaphap et al. 1997). In contact trials, the ET_{25} value for all four chemicals was one min, except for deltamethrin at LC_{25} (four min). ET_{50} values for three concentrations of cyphenothrin and d-tetramethrin were recorded at < four min. In general, ET_{99} value for d-tetramethrin was somewhat high compared with deltamethrin and cyphenothrin. Due to insufficient numbers of escaping mosquitoes after 30 min, the ET_{99} value for tetramethrin could not be calculated. In non-contact trials, ET_{25} values for all four chemicals were comparatively high (>18 min). The ET_{50} and ET_{99} values for all chemicals in non-contact trials could not be estimated due to low escape numbers (Table 6).

Comparison between contact vs non-contact and control vs contact responses demonstrated significant differences in escape response of *Ae. aegypti* across all doses for all four insecticides (P <0.01). Significant differences in escape patterns were not observed between paired non-contact vs control trials, except the pairs of LC_{50} and LC_{99} values for cyphenothrin (P <0.05). Multiple comparisons among three different doses for all four chemicals in contact,

Table 1. Toxicity data for four insecticides tested against adult *Aedes aegypti.*

Parameters	Insecticides			
	deltamethrin	cyphenothrin	d-tetramethrin	tetramethrin
Slope ± SE	24.587 ± 0.001	3.113 ± 0.002	0.190 ± 0.002	0.121 ± 0.001
LC_{25} (%)	0.010	0.113	2.091	2.377
LC_{50} (%)	0.020	0.167	2.770	4.251
LC_{99} (%)	0.055	0.353	5.114	10.715

Table 2. Escape rate and mortality of *Ae. aegypti* in response to three lethal concentrations of deltamethrin, LC_{25}, LC_{50}, and LC_{99}.

								% Mortality at 24 h			
		Treatment			Control			Treatment		Control	
Test condition	LC	No. tested	% Escaped		No. tested	% Escaped		Escaped	Not escaped	Escaped	Not escaped
Contact	25	60	63.33	(38)	60	30.00	(18)	0	0	0	0
	50	60	88.33	(53)	60	16.67	(10)	1.89(1)	28.5(2)	0	0
	99	60	86.67	(52)	60	21.67	(13)	7.69(4)	12.5(1)	0	0
Non-contact	25	60	25.00	(15)	59	16.95	(10)	0	0	0	0
	50	60	13.33	(8)	60	6.67	(4)	0	0	0	0
	99	60	26.67	(16)	59	22.03	(13)	0	2.27(1)	0	0

Table 3. Escape rate and mortality of *Ae. aegypti* in response to three lethal concentrations of cyphenothrin, LC_{25}, LC_{50}, and LC_{99}.

								% Mortality at 24 h			
		Treatment			Control			Treatment		Control	
Test condition	LC	No. tested	% Escaped		No. tested	% Escaped		Escaped	Not escaped	Escaped	Not escaped
Contact	25	60	83.33	(50)	60	20.00	(12)	2.00(1)	0	0	0
	50	56	80.36	(45)	60	16.67	(10)	2.22(1)	0	0	0
	99	60	83.33	(50)	60	28.33	(17)	8.00(4)	60.00(6)	0	0
Non-contact	25	60	18.33	(11)	60	15.00	(9)	0	2.04(1)	0	0
	50	60	23.33	(14)	59	8.47	(5)	0	0	0	0
	99	60	35	(21)	60	16.67	(10)	4.76(1)	7.69(3)	0	0

Table 4. Escape rate and mortality of *Ae. aegypti* in response to three lethal concentrations of d-tetramethrin, LC_{25}, LC_{50}, and LC_{99}.

								% Mortality at 24 h			
		Treatment			Control			Treatment		Control	
Test condition	LC	No. tested	% Escaped		No. tested	% Escaped		Escaped	Not escaped	Escaped	Not escaped
Contact	25	59	76.27	(45)	60	26.67	(16)	2.22(1)	21.43(3)	0	2.27(1)
	50	59	79.66	(47)	60	26.67	(16)	2.13(1)	0	(1)	0
	99	60	73.33	(44)	59	25.42	(15)	2.27(1)	31.25(5)	0	0
Non-contact	25	60	23.33	(14)	59	15.25	(9)	0	2.17(1)	0	0
	50	59	23.73	(14)	58	15.52	(9)	0	2.22(1)	0	2.04(1)
	99	59	20.34	(12)	60	13.33	(8)	0	4.26(2)	0	0

Table 5. Escape rate and mortality of *Ae. aegypti* females in response to three lethal concentrations of tetramethrin, LC_{25}, LC_{50}, and LC_{99}.

								% Mortality at 24 h			
		Treatment			Control			Treatment		Control	
Test condition	LC	No. tested	% Escaped		No. tested	% Escaped		Escaped	Not escaped	Escaped	Not escaped
Contact	25	60	60.00	(36)	60	28.33	(17)	0	4.17(1)	0	0
	50	59	61.02	(36)	60	20.00	(12)	0	8.70(2)	0	0
	99	58	72.41	(42)	60	23.33	(14)	0	6.25(1)	0	0
Non-contact	25	60	28.33	(17)	60	18.33	(11)	0	0	0	0
	50	59	20.34	(12)	60	15.00	(9)	0	0	0	0
	99	60	30.00	(18)	60	18.33	(11)	0	2.38(1)	0	0

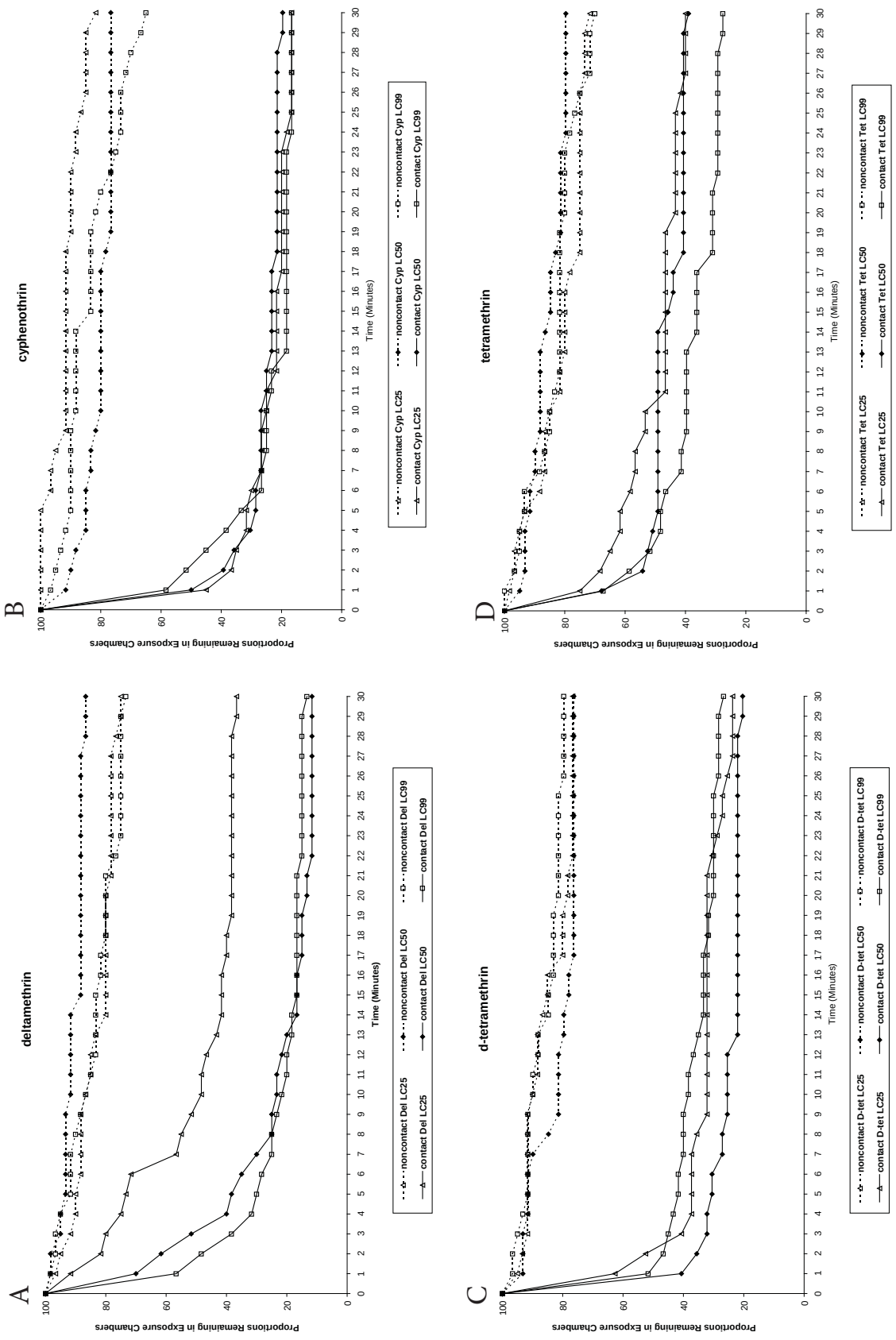

Figure 1. Escape rate of *Aedes aegypti* exposed to deltamethrin (A), cyphenothrin (B), d-tetramethrin (C), and tetramethrin (D) at LC_{25}, LC_{50} and LC_{90} levels for contact and non-contact trials.

Table 6. Time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of *Aedes aegypti* females to escape from non-contact and contact chambers treated with three lethal concentrations (LC_{25}, LC_{50}, and LC_{99}) of deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin.

	Non-contact (min)			Contact (min)		
Insecticides	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
deltamethrin, LC_{25}	29	-	-	4	9	-
deltamethrin, LC_{50}	-	-	-	1	3	8
deltamethrin, LC_{99}	23	-	-	1	2	7
cyphenothrin, LC_{25}	-	-	-	1	1	10
cyphenothrin, LC_{50}	-	-	-	1	1	11
cyphenothrin, LC_{99}	23	-	-	1	1	8
d-tetramethrin, LC_{25}	-	-	-	1	2	26
d-tetramethrin, LC_{50}	-	-	-	1	1	12
d-tetramethrin, LC_{99}	-	-	-	1	2	-
tetramethrin, LC_{25}	18	-	-	1	10	-
tetramethrin, LC_{50}	-	-	-	1	5	-
tetramethrin, LC_{99}	26	-	-	1	4	-

non-contact, and control trials were evaluated. The pattern of escape response was analyzed with the log-rank method, and statistical significance was established at the 0.05 level of probability. No significant differences in escape patterns between any two doses from contact and non-contact trials were found, except in two comparisons of deltamethrin in contact trials (LC_{25} vs LC_{50} and LC_{25} vs LC_{99}) and in one comparison of cyphenothrin in non-contact trials (LC_{25} vs LC_{99}).

Figure 1 demonstrated the proportions of mosquitoes remaining in the insecticide treated chambers under different test conditions. These proportions are used to demonstrate patterns of escape rate. These patterns are indicative of escape probabilities of *Ae. aegypti* females between contact and non-contact trials with three doses of deltamethrin (Figure 1A), cyphenothrin (Figure 1B), d-tetramethrin (Figure 1C), and tetramethrin (Figure 1D). Significant differences in escape patterns were seen when contact trials were compared to non-contact trials for all tests (P <0.01).

DISCUSSION

Behavioral responses to chemicals by mosquitoes have long been recognized (Roberts and Andre 1994). In the past, responses to chemicals by mosquitoes were often ignored when selecting compounds for vector control programs. Most work focused on the toxic action (killing) of test chemicals on insect populations. Relatively little has been done on the behavioral responses of mosquitoes to chemicals. We believe that at least two different categories of behavioral responses in mosquito vectors exist: contact irritancy and non-contact spatial repellency (Rawlings and Davidson 1982, Roberts and Andre 1994, Chareonviriyaphap et al. 1997). Irritability occurs when an insect is stimulated to move away from an insecticide-treated surface after making direct physical contact with the insecticide residue. In contrast, spatial repellency takes place when the insect detects and avoids a treated surface without making physical contact (Roberts and Andre 1994, Chareonviriyaphap et al. 1997).

Both types of behavioral response can be experimentally differentiated by using the excito-repellency (ER) test system (Chareonviriyaphap et al. 1997, 2002). In 2000, Roberts et al. (2000) proposed a mathematical model for better understanding the repellent, irritant, and toxic functions of insecticides to control vector-borne disease. Recently, a modular, high-throughput laboratory-based assay system for rapid detecting the three actions, irritancy, repellency, and toxicity, of insecticides was developed (Grieco et al. 2007). Since the development of the two systems for evaluating the behavioral response in mosquitoes and with the development of a quantified mathematical framework, reports on behavioral responses by mosquitoes to public health insecticides has been progressively increasing (Chareonviriyaphap et al. 2001, 2004, Grieco et al. 2007, Polsomboon et al. 2008). Most of these studies have focused on the behavioral response of *Anopheles* species to insecticides, whereas comparatively little has been published on the avoidance behavior of *Ae. aegypti* exposed to test chemicals (Kennedy 1947, Kongmee et al. 2004, Grieco et al. 2007, Poolsomboon et al. 2008).

In the past, we observed the clear insecticide responses of several *Anopheles* mosquitoes (Chareonviriyaphap et al. 1997, 2001, 2004, Sungvornyothrin et al. 2001), *Aedes* mosquitoes (Kongmee et al. 2004, Grieco et al. 2007), and *Culex* mosquitoes (Sathantriphop et al. 2006). In this study, two different types of behavioral responses of *Ae. aegypti* to four synthetic pyrethroids, deltamethrin, cyphenothrin, d-tetramethrin, and tetramethrin were determined. We found that contact irritancy was the primary behavioral response elicited by these compounds. However, we did find that there was a low but statistically significant non-contact spatial repellent escape response in the pairs of non-contact vs control trials of LC_{50} and LC_{99} values for cyphenothrin. Significant behavioral avoidance responses were observed in all contact trials when compared with their paired controls, regardless of insecticide or concentration used. Greater escape responses after physical contact were observed from deltamethrin at LC_{50} and LC_{99} compared with the other three chemicals. In general, no significant differences in escape responses to the three doses of four different chemicals in *Ae. aegypti* females were found, suggesting that using insecticides at sub-lethal doses may be appropriate in controlling the disease vectors. Applying the minimal dose to elicit a behavioral response can also help to prevent or delay the resistance in insect populations (Grieco et al. 2007).

Deltamethrin is currently one of the most commonly used insecticides for public health and has been the mainstay for the emergency control of *Ae. aegypti* adults in Thailand since 1994 (Chareonviriyaphap et al. 1999, Kongmee et al. 2004). In addition to deltamethrin, several other synthetic pyrethroids, i.e., cyphenothrin and tetramethrin, are commonly used by home owners to control household mosquitoes and other arthropod pests (Sathantrihop et al. 2006). The continued demand for synthetic pyrethroids serves as the stimulus for further studies to evaluate the avoidance behavior of pyrethroids to *Ae. aegypti* mosquitoes. In addition, little is known about the role of irritant and repellent actions of pyrethroids on *Ae. aegypti* and how they function to break disease transmission (Kongmee et al. 2004).

We now have a laboratory-based system to evaluate these two behavioral actions, irritancy and repellency (Chareonviriyaphap et al. 2002, Grieco et al. 2005). A mathematical framework for understanding the true function of chemicals in controlling disease transmission has also been developed (Roberts et al. 2000). Grieco et al. (2007) have demonstrated the three actions of chemicals using an experimental hut study. However, this model must be evaluated using different mosquito species from endemic areas, as well as other chemicals, in order to gain a better understanding of how to improve our vector control strategies. In the future, a greater focus should be placed on the role of excito-repellency in the control of dengue.

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SEASONAL ABUNDANCE AND BLOODFEEDING ACTIVITY OF *ANOPHELES DIRUS* SENSU LATO IN WESTERN THAILAND

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ABSTRACT. Bloodfeeding activity, host preference, and seasonal abundance of the *Anopheles dirus* complex in relation to environmental factors were measured during a 2-yr period at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand. *Anopheles dirus* s.l. was found more abundant during the wet season compared with the dry and hot seasons. Bloodfeeding by *An. dirus* s.l. commenced immediately after sunset with a distinct peak of activity at 2000 h. *Anopheles dirus* s.l. fed more readily on cattle than on humans, demonstrating a significant but slightly stronger zoophilic behavior ($P = 0.02$). The biting activity of *An. dirus* s.l. in Pu Teuy appears to differ from other localities in Thailand, suggesting the existence of significant biological variability within the species complex depending on the local circumstances.

KEY WORDS *Anopheles dirus*, biting frequency, abundance, Thailand

INTRODUCTION

Several vector-borne diseases are widely distributed in Thailand, including malaria, dengue, and lymphatic filariasis. Among these, malaria remains one of the most important infectious diseases, primarily concentrated in forested areas along the western border with Myanmar, the eastern border with Cambodia, and the southern border with Malaysia (Chareonviriyaphap et al. 2000, Ministry of Public Health [MOPH] 2006). Approximately 70% of the country's malaria cases are documented from the border areas with Myanmar, where the combination of malaria-infected migrants and efficient malaria vectors such as *Anopheles dirus* Peyton and Harrison complex species commonly coexist (MOPH 2006). For example, the border province of Tak has reported between 3,000 and 25,000 cases annually over the last 10 yr. The *An. dirus* complex is primarily a collection of forest and forest-fringe inhabiting mosquitoes, some of which are considered highly endophagic and anthropophilic, with sufficiently high malarial infectivity rates to maintain transmission year-round (Ismail et al. 1974, Rosenberg and Maheswary 1982, Baimai 1988, Baimai et al. 1988). Early recognition of this species' (known until 1979 as *Anopheles balabacensis* Baisas) importance in transmitting malaria in Thailand subsequently led to many field and laboratory studies to discern adult behavioral patterns in relation to transmission and response to control measures (Scanlon and Sandhinand 1965; Wilkinson et al. 1970; Ismail et al. 1974, 1975; Kitthawee et al. 1990; Suwonkerd et al. 1990; Rattanarithikul et al. 1996a, 1996b; Chareonviriyaphap et al. 2004).

Two complexes within the Leucosphyrus group are recognized, including 7 species in the Dirus complex (*An. baimaii* Sallum and Peyton, *An. cracens* Sallum and Peyton, *An. dirus*, *An. elegans* (James), *An. nemophilous* Peyton and Ramalinham, *An. scanloni* Sallum and Peyton, and *An. takasagoensis* Morishita) and 4 species in the Leucosphyrus complex (*An. balabacensis* Baisas, *An. introlatus* Colless, *An. latens* Sallum and Peyton, and *An. leucosphyrus* Doenitz (Peyton 1989, Sallum et al. 2007, Manguin et al. 2008). Unlike most species in the group that have infrequent contact with humans, a few members are regarded as excellent malaria vectors because of their highly endophagic and anthropophilic behavior. Five species within the Dirus complex are present in Thailand; 4 of them, *An. dirus* s.s. (former *An. dirus* A), *An. cracens* (species B), *An. scanloni* (species C), and *An. baimaii* (species D), are regarded as malaria vectors with sporozoite rates of up to 10% (Peyton 1989, Sallum et al. 2005). However, only *An. baimaii* and *An. dirus* are considered to be among the most important primary malaria vectors in Thailand (Rattanarithikul et al. 2006, Manguin et al. 2008). Unpublished field data in western Thailand have shown *An. dirus* and *An. baimaii* are closely associated with increased rainfall and high relative humidity, and the most preferred breeding habitats are shaded animal footprints, wheel-tracks, and temporary ground pools, thus making

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environmental control of this species particularly difficult (Suwonkerd, personal communication). *Anopheles dirus* larvae are occasionally found along the margins of slow running water (Chareonviriyaphap et al. 2003), water jars, cut tree stumps, and rot holes (Rosenberg 1982, Rattanarithikul et al. 2006) adding to the problem of detection and control.

Knowledge of mosquito behavior is of paramount importance to understand the epidemiology of disease transmission and vector control. Detailed information on feeding and host-seeking behavior helps to define a species' capacity to acquire and transmit malaria and its contribution to the relative risk for disease transmission in the human population; it also assists in the design and implementation of appropriate vector prevention and control strategies. In recent decades, studies on vectors responsible for malaria transmission have allowed recognition of additional *Anopheles* species and species complexes in Thailand (Baimai et al. 1984, 1988; Baimai 1989; Rattanarithikul et al. 2006). In spite of increased agricultural development, housing, and facility-based tourism (i.e., campgrounds, resort hotels) in forest and forest-fringe areas, many malaria vectors have apparently been little affected by these environmental changes. Modified environmental conditions require renewed investigation on the impact of these changes on mosquito vectors and malaria transmission, such as population densities and blood-feeding activity and transformed ecological relationships for pathogen transmission, especially in areas where sympatric sibling species coexist (Manguin, personal communication). Therefore, a study on *An. dirus* adult density fluctuations and blood-feeding behavior due to seasonally influenced environmental factors was carried out at Pu Teuy Village, Kanchanaburi Province, western Thailand, to provide updated vector ecological information in relation to malaria transmission.

MATERIALS AND METHODS

Study area: The study was conducted at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14°17′N, 99°11′E), approximately 150 km northwest of Bangkok. The rural village sits astride a protected forest reserve with a scattered population of approximately 1,400, whose major occupation is forest or fruit-and-vegetable agricultural-based pursuits. The study site is located at the base of steep mountainous karstic terrain and surrounded by natural deep forest, approximately 800 m from the nearest occupied house at Pu Teuy Village. During the study, there were a variety of potential breeding sites for *An. dirus*, particularly scattered animal footprints and small, shaded, freshwater pools.

Adult mosquito collections: Adult female mosquitoes were collected during 3 consecutive nights each month for 2 yr, beginning February 2005 to January 2007. Collection methods included indoor human-landing (HLI), outdoor human-landing (HLO), and cattle-bait captures (CBC). Collections were performed in a local house with all windows closed and 1 door remaining open throughout the collection period. The indoor/outdoor human-landing collectors were divided into 2 teams of 4 persons each. Two persons performed landing collections inside the house, with 2 persons outside at a distance of 100 m from the house. The first team began collecting at 1800 to 2400 h, followed by the second team starting at midnight and ending at 0600 h. The 2 teams were rotated between the first and second halves of the evening each subsequent collection night to avoid the potential collector bias. Team members were also rotated each night between indoor and outdoor collection stations. Human-landing collections occurred uninterrupted for 45 min each hour. Further details on human-landing collection methods are published elsewhere (Chareonviriyaphap et al. 2003). Formal human-use approval was granted by the Ethical Research Committee convened by the Research and Development Institute, Kasetsart University. Cattle-baited collections involved placing a single cow under a cotton bed net measuring 3.6 m (length) × 3.3 m (width) × 2.0 m (high) with the net suspended 30 cm above ground level to allow mosquitoes access inside. Collections were conducted by a separate team of 2 collectors for 15 min at the end of each collection period following 45 min of uninterrupted exposure of the net-enclosed cow to outdoor mosquitoes. Mosquitoes either resting inside the net or on the cow were collected with a mouth aspirator and placed in labeled cups. The same cow was used throughout the study and was placed at least 50 m from the nearest outdoor human-landing collection and at equal distances from forest fringe to avoid potential bias in attracting mosquitoes. All collected anophelines were retained in plastic cups and labeled by hour and type of collection (indoor/outdoor/cow bait). Each cup was covered with fine-mesh netting and cotton soaked with a 10% sugar solution. Mosquitoes were returned to the laboratory the same morning for morphological identification (Rattanarithikul and Panthusiri 1994). Hourly ambient indoor and outdoor air temperatures and relative humidity were recorded by teams during the collection periods with a manual thermohygrometer (BARIGO GmbH, Villingen-Schwenningen, Germany). Daily maximum and minimum air temperatures were collected on site, and rainfall data were obtained from the Sai Yok District meteorological station located approximately 10 km from the study site.

Table 1. Total monthly 12-h human-landing and cow-baited collections of female *Anopheles dirus* s.l. indoors and outdoors at Pu Teuy, Kanchanaburi Province.

Month	Year 1						Year 2						Total no. of mosquitoes
	In	Out	Cow	Mean Temp.	RH[1]	Rain[2]	In	Out	Cow	Mean Temp.	RH	Rainfall	
February	1	0	1	24.17	76.50	24.10	0	0	0	23.92	73.59	0.00	2
March	0	0	0	26.33	73.58	50.50	0	0	0	26.50	73.17	51.50	0
April	1	0	2	26.00	73.17	89.30	0	0	1	26.17	81.17	125.90	4
May	21	22	26	25.75	81.17	434.90	1	1	3	25.42	79.83	123.10	74
June	19	48	120	25.50	79.83	201.10	9	32	22	25.33	78.91	100.20	250
July	7	15	35	24.42	8.92	126.00	16	7	24	24.67	77.25	276.70	104
August	14	20	24	29.08	77.25	107.70	22	12	45	25.08	77.83	183.20	137
September	14	19	33	24.83	77.83	168.40	14	14	46	24.53	81.17	468.50	140
October	1	3	7	24.58	78.33	30.70	13	20	40	24.58	73.42	212.40	84
November	1	0	1	24.92	78.42	0.00	1	0	0	24.92	80.58	37.60	3
December	0	0	0	23.08	80.58	0.00	0	0	0	22.92	79.42	6.80	0
January	0	0	0	23.08	79.42	24.80	0	0	0	22.33	76.50	1.00	0
Total	79	127	249				76	86	181				798

[1] RH, relative humidity.
[2] Precipitation data collected at government meteorological station approximately 10 km from study site.

Data analysis: Three main study variables (factors) were selected for analysis, including time of year (season), time period during the evening, and collection category (method). Seasons were identified as wet (June to November), dry (December to February), and hot (March to May). Evening time periods were divided into quarters: early evening (1800–2100 h), late evening (2100–2400 h), predawn (0000–0300 h), and dawn (0300–0600 h). Collection categories included indoor, outdoor, and cow-baited captures.

The nocturnal biting cycles of *An. dirus* were tabulated by average number landing per human by hour for indoor and outdoor collections during the 2-yr period. The differences in average number of landing mosquitoes were analyzed by 3-way analysis of variance (ANOVA), with year as a blocked factor and differences among groups (times and types of collection) and Duncan's multiple-range test (Duncan 1955). The accepted level of significance was set at 0.05% ($P < 0.05$). All data were analyzed with SAS program package (SAS Release 6.01, SAS Institute, Cary, NC, USA).

RESULTS

Anopheles dirus s.l. collection results from February 2005 to January 2007 are summarized in Table 1. A combined 2-yr total of 798 *Anopheles dirus* s.l. were collected. These mosquitoes were found to be more abundant in the rainy periods of the year (May–September for the first year and June–October for the second year) than during the other 2 marked seasonal periods of dry or hot. The earlier-than-expected increase in rainfall (April and May) during the second year did not result an increase in mosquitoes until June, whereas the dramatic increase in precipitation in May of year 1 saw an immediate increase in biting densities. Seasonal abundance of *An. dirus* peaked in June for the combined monthly 2-yr observations. Comparatively lower numbers of mosquitoes were collected during the observed dry and hot seasons in this study (Table 1). In general, cow-baited collections exceeded human-bait collections (outdoor alone and combined indoor and outdoor collections). Of the 798 *An. dirus* collected, 430 (53.9%) were collected from cow bait, 213 (26.9%) from outdoor collections, and 155 (19.4%) from indoor collection efforts. Total mosquito biting frequencies by hour and collection method are given in Fig. 1. Peak patterns of outdoor and indoor human-baited collections are similar, with a maximum landing density (bites per person/h) between 2000 and 2100 h (Fig. 1). Outdoor cattle-bait catches demonstrated a more prolonged early-to-late evening peak compared with indoor and outdoor human collections (Fig. 1).

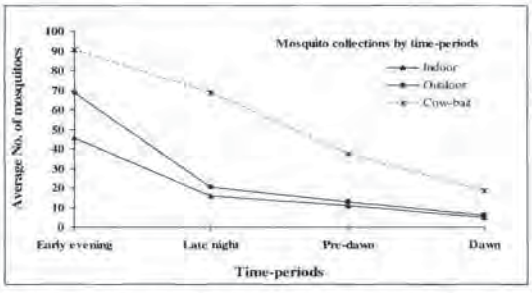

Fig. 1. Temporal patterns of *An. dirus* s.l. blood-feeding activity for indoor and outdoor human-landing collections and outdoor cow-baited collection.

Table 2. Three-way ANOVA of total number landing mosquitoes/h, 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.[1]

Source	df	Sum of squares	Mean square	F	Significance
Intercept	1	1,058.513	1,058.513	25.963	0.104
		45.710	40.769		
Year	2	42.781	42.781	1.888	0.171
		5,688.469	22.663		
Season	2	1,457.892	728.946	32.164	0.000
		5,688.469	22.663		
Time period	3	426.915	142.305	6.279	0.000
		5,688.469	22.663		
Types of collection	2	181.075	90.538	3.995	0.020
		5,688.469	22.663		
Season × time period	6	618.622	103.104	4.549	0.000
		5,688.469	22.663		
Season × collection methods	4	380.764	95.191	4.200	0.003
		5,688.469	22.663		
Time period × collection methods	6	50.581	8.430	0.372	0.896
		5,688.469	22.663		
Season × time period × collection methods	12	104.014	8.668	0.382	0.969
Error	251	5,688.469	22.663		
Total	287	9,728.469			

[1] Year = year 1 and 2; season = dry, hot, wet; time period = early evening, late evening, predawn, dawn; types of collection = indoor, outdoor, cow.

Total number of landing mosquitoes/h was used in a 3-way ANOVA, 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 (Table 2). There were no significant differences in the number of mosquitoes collected by hour from the 3 collection methods and between the 2 annual collection periods ($F = 1.888$; df = 1, 251; $P = 0.171$). Statistical significance among 3 collection methods was observed in the first year ($F = 3.4881$; df = 2, 241; $P = 0.033$) and in the second year ($F = 3.300$; df = 2, 141; $P = 0.040$). Significant differences in number of captured mosquitoes were found between seasons ($F = 32.164$; df = 2, 251; $P < 0.0001$), between human and cattle collections ($F = 3.995$; df = 2, 251; $P = 0.02$), and between the 4 quarterly evening time intervals ($F = 6.279$; df = 3, 251; $P = 0.0002$). Strong interactions between season and human and cattle bait collection ($F = 4.2000$; df = 4, 251; $P = 0.003$) and between season and collection-time intervals ($F = 4.549$; df = 6, 251; $P < 0.001$) were observed. There was no apparent interaction between collection-time periods and the 3 methods of collection (indoors, outdoor, cow bait; $F = 0.372$; df = 6, 251; $P = 0.896$), or among season, time period, and method of collection ($F = 0.382$; df = 12, 251; $P = 0.969$).

Data from all collection methods were pooled to determine the correlation between mosquito abundance and environmental variables (Table 1). *Anopheles dirus* densities were correlated with increase in total rainfall and relative humidity ($P < 0.05$) but not with minimum and maximum ambient air temperatures ($P > 0.05$).

DISCUSSION

In Pu Tuey, we observed the biting activity of *An. dirus* s.l. females for 3 consecutive evenings during the first week of each month for a period of 2 yr. Along the western border with Myanmar, *An. dirus*, *An. baimaii*, *An. nemophilous*, and *An. scanloni* have been identified (Rattanarithikul et al. 2006). Unfortunately, molecular techniques to differentiate cryptic species within the Dirus complex were not used in this study; therefore all collections are regarded as *Anopheles dirus* s.l., which may include both vector and nonvector species (Rattanarithikul et al. 2006, Manguin et al. 2008). Based on preliminary investigations, the majority of species in this study may likely have represented *An. dirus* and *An. baimaii* (Manguin, personal communication). In Thailand, *An. dirus* and *An. baimaii* have been found in sympatry and strongly associated with forested, forest fringe, and hilly environments along western areas of the Thai–Myanmar border, especially in Kanchanaburi and Tak Provinces (Baimai et al. 1988; Rattanarithikul et al. 1995, 2006). Both species are considered very anthropophilic in blood-feeding preference and demonstrate both exophagic and endophagic behavior (Rosenberg and Maheswary 1982), and in some cases a generally greater tendency toward exophily (Baimai et al. 1988; Manguin et al. 2008; Chareonviriyaphap, unpublished data).

Kanchanaburi Province is a malaria endemic area with overall incidence rates in excess of 87/100,000 population (MOPH 2008). Although malaria cases were relatively low in Pu Teuy compared with many other surrounding villages in the province, efficient malaria vectors like *An. dirus* s.l. have been commonly found in abundance, particularly during the rainy periods of the year (Sungvornyothin et al. 2006). In addition, the Pu Teuy settlement is located in close proximity to intact forest and forest-fringe zones that are ideal habitats for *An. dirus* s.l. (Rattanarithikul et al. 2006), which are plentiful during the wet season.

In this study, the all-evening biting cycle, host preference, and seasonal variation in adult densities of *An. dirus* s.l. were recorded. The collection of large numbers of anthropophilic *An. dirus* s.l. during the wet months suggests that this species can likely play a significant role in malaria transmission during this period. However, a larger relative number (54% of total) of *An. dirus* s.l. were consistently captured from the cow-baited collection compared to either human-bait collections, indicating that feeding habits of *An. dirus* s.l. in Pu Teuy show a slightly greater preference for nonhuman domestic animal hosts (in this case, cattle). Additionally, the differences in biting preference of *An. dirus*, s.l. in our study site suggest the existence of site-specific populations of *An. dirus*, demonstrating different host-seeking behaviors than what is typically associated with this species in Thailand (Ismail et al. 1974, Baimai et al. 1988, Manguin et al. 2008). Our conclusion is complicated by the fact we were not able to identify the exact numbers (or proportions) of all members of the complex that may have been present and captured in the study site.

Anopheles dirus s.l. showed a slightly greater predilection to biting (blood feeding) on humans outdoors rather than inside the house (58% of total-human landing samples) by a ratio of 1:1.3. The peak host-seeking activity of *An. dirus* s.l. in our study site was found to differ from most previous studies. In central Thailand, *An. dirus* s.l. demonstrated a delayed and more prolonged peak feeding activity between 2000 and 2400 h (Wilkinson et al. 1970). In southern Thailand, Scanlon and Sandhinand (1965) reported that peak biting occurred activity between 2400 and 0300 h, Rosenberg et al. (1990) observed peak biting occurring between 2200 and 0100 h, and Rattanarithikul et al. (1996b) reported a single early-evening biting peak between 2000 and 2200 h. In Bangladesh, during a 21-mo study, this species displayed different peaks depending on time period, phase of the moon, and site of collection; indoor peak activity was observed between 2200 and 0100 h (and up to 0200 h for year 2), with outdoor peaks ranging from 2200 to 2400 h (Rosenberg and Maheswary 1982). In our study, 1 short peak was seen in both indoor and outdoor collections (1900 and 2100 h), and a more prolonged peak was observed in the outdoor cow-baited catch (2000–2300 h), across all seasonal climatic differences. The reason for the differences in feeding patterns compared to other localities in Thailand and elsewhere is unclear, but illustrates the biological variability between populations of the same (or closely related) species.

Anopheles dirus abundance was found positively correlated with increased rainfall and humidity, but was negatively associated with higher mean ambient temperatures. A very similar rainfall-dependent pattern was observed in Bangladesh (Rosenberg and Maheswary 1982). The exact opposite was observed with *Anopheles minimus* Theobald in the same locality (Sungvornyothin et al. 2006). The preferred larval habitats of these 2 species differ considerably, with *An. dirus* s.l. preferring temporary breeding habitats such as animal footprints, wheel tracks, and temporary ground pools common during the wet season (Rattanarithikul et al. 1996b), whereas *An. minimus* s.l. prefer the edges of slow-running streams (Chareonviriyaphap et al. 2003, Sungvornyothin et al. 2006) and are less dependent on rainfall.

There are several weaknesses to this study and caveats that may have influenced monthly comparisons of biting densities. First, we did not use molecular methods to differentiate the different members of the *An. dirus* complex captured; thus our conclusions may likely reflect a mix of several sympatric species. Rainfall was recorded from a locality 10 km from the study site, so precipitation data served as only a temporal approximation of seasonality. Moon phase was not recorded or considered in determining time of collections (first of each month) and may have had a marked effect on overall mosquito flight and host-seeking behavior (Bidlingmayer 1967) or peak biting periods during the evening hours (Rosenberg and Maheswary 1982). There are also complicating factors making direct comparisons between human and cow-landing data that involved different collection methods and the inherent limitations associated with both procedures. For example, the use of animal-baited bed nets may have biased sampling by favoring some species over others, resulting in fewer individuals than can be caught in direct bait catches (Service 1993).

This study found the biting activity of *An. dirus* s.l. in Pu Teuy differed from other localities in Thailand, suggesting the existence of significant biological variability within the species complex depending on the local circumstances. Additional studies are planned to identify the exact species involved and their response to various control strategies.

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Effects of environmental conditions on the movement patterns of *Aedes aegypti* (Diptera: Culicidae) into and out of experimental huts in Thailand

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ABSTRACT: Mark-release-recapture experiments with *Aedes aegypti* were performed using experimental huts equipped with entrance and exit traps to evaluate their movement patterns during a two-year period in Thailand. Results indicate no significant differences in the patterns of movement between the two years of observation. Movement into the huts occurred during the early morning period (06:00-11:00) with a peak at 07:00 in the summer and rainy season and 09:00 in the winter. In contrast, the exit pattern was observed during the late morning (09:00-12:00) and early afternoon (12:00-16:00), with a peak at 16:00 in the winter, 11:00 in the summer, and 14:00 in the rainy season. Multiple regression analysis indicated that movements of *Ae. aegypti* females into and out of the huts were impacted by humidity and temperature during the day. ***Journal of Vector Ecology* 34 (2): 267-275. 2009.**

Keyword Index: *Aedes aegypti*, movement patterns, behavior, experimental hut, Thailand.

INTRODUCTION

Dengue viruses are transmitted from human to human by the bite of infected *Aedes aegypti* (L), a day-biting mosquito that breeds in or near human dwellings and preferentially feeds on humans even when other warm-blooded animals are freely available (Edman et al. 1992, Xue et al. 1995, WHO 1999). Because no commercial vaccine or successful drug therapy is yet available for the prevention and treatment of dengue infection, the control of the vector remains the most effective method for preventing dengue virus transmission and averting epidemics (Reiter and Gubler 1997, Perich et al. 2001). Control of the mosquito vector by chemical means remains the most successful method for reducing disease transmission and preventing human vector contact (Roberts et al. 1997, WHO 1999).

Chemicals may protect humans from the bite of insects through three different actions; irritancy, repellency, or toxicity (Grieco et al. 2007). Recently, it has been proposed that some chemicals, such as DEET, elicit a fourth action by effectively masking the presence of a host through the inhibition of odor-activated receptors (Ditzen et al. 2008). Most studies have concentrated on the toxic actions of these chemicals, whereas little focus has been placed on non-toxic properties of these compounds. Non-toxic actions can be placed into two different categories, contact irritancy and non-contact repellency (Roberts et al. 1997, Chareonviriyaphap et al. 1997). These two types of behavioral response can be evaluated through the use of laboratory and field assay systems (Roberts et al. 1997, Smith 1965, Chareonviriyaphap et al. 2005, Grieco et al. 2007). In order to truly quantify these behaviors in response to chemical treatments, a baseline of natural behaviors and movement patterns must first be established in the absence of the chemical. Recently, the effect of host types on movement patterns of *Ae. aegypti* using experimental huts was conducted in Thailand (Suwonkerd et al. 2006). These same experimental huts were also used to investigate the three actions of chemicals against *Ae. aegypti* (Grieco et al. 2007). As seen with these previous studies, most experimental hut work evaluates the impact of some type of intervention on mosquito behavior but little has been done to evaluate the movement patterns of *Ae. aegypti* in response to monthly changes in environmental factors. This study was the first attempt to investigate the ingress and egress movements of *Ae. aegypti* in response to changes in seasonal environmental parameters, i.e., relative humidity and ambient temperature, during a two-year period using experimental huts fitted with entrance and exit traps.

MATERIALS AND METHODS

Study site

This study was conducted at Pu Teuy Village, Sai Yok District, Kanchanaburi Province (14º 20'N, 98º 59'E, 304 m asl), western Thailand, approximately 150 km northwest of Bangkok. The site is located in a hilly area and is largely surrounded by primary dense forest. The study site belongs to the Armed Forces Development Command in the Ministry of Armed Forces. The nearest home is approximately 800 m away from the field site where the experimental huts were located.

Mosquito population

An experimental release population of *Ae. aegypti* was established from immature stages collected from Pu Teuy Village, Sai Yok District, Kanchanaburi Province. Approximately 200-300 pupae and larvae were brought back to the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Morphological identification was made by using the conventional key of Rattanarithikul and Panthusiri (1994). Subsequent colonization was performed at the same insectary, following the method of Kongmee et al. (2004).

Mosquito rearing

All life stages of *Ae. aegypti* were maintained under insectary controlled conditions. Larvae and adults were reared under a 12:12 h light: dark photophase regime, a 25±5° C controlled temperature and a 80±10% controlled relative humidity. Upon emergence, all adults were provided cotton pads soaked with a 10% sucrose solution. Female mosquitoes were starved for at least 12 h prior to testing.

Experimental huts

The experimental huts used in this study were previously described (Chareonviriyaphap et al. 2005, Grieco et al. 2007). Two identical huts were constructed in an isolated area adjacent to Pu Teuy village. Each hut measured 4 m wide x 5 m long x 3.5 m high, and had three windows and one door. The dimensions of the windows and door were 1.125 m x 1.175 m and 0.8 m x 2 m, respectively. Each portal was constructed in such a way as to allow them to be affixed with entrance and exit traps. Huts were built of similar materials and in a similar fashion to the indigenous Thai homes. Huts were constructed from pieces of 1 m x 2.5 m untreated wood and pieces of 0.75 m x 3 m zinc roofing. Hut frames used to support the walls were made from galvanized iron pipe measuring 1 m x 2.5 m in length and were custom-welded to accommodate each wall. The apex of the angled roof measured 3.5 m from leveled ground. The hut had three windows, one on each of three sides, and a northward-facing door, and all were affixed with either entrance or exit traps.

The dimensions of the window traps were 0.84 m long, 1.1 m wide, and 1.1 m high and were constructed using an iron frame. Louvers made of 9.5 mm non-treated plywood and fixed vertically at 60° angles were placed over the front opening of each of the three window traps, 1.1 m x 1.1 m, with a horizontal row of 10-cm wide slit openings made of 9.5 mm non-treated plywood fixed vertically to 60°. The louvers were placed in the open position producing a series of horizontal, 10-cm wide openings through which mosquitoes could enter. A door trap, measuring 1.2 m long x 0.85 m wide x 2.1 m high, was fixed to the door opening. Twenty plywood louvers identical to those used in the window traps were installed over the front opening and were again fixed at 60° angles to the vertical. These were arranged to facilitate the movement of mosquitoes from the hut into the trap. Both trap types were covered by nylon insect netting. Cotton sleeve material was sewn over several holes in both types of traps to facilitate the removal of mosquitoes. Additional details pertaining to the experimental huts were given in Suwonkerd et al. (2006) and Grieco et al. (2007).

Mosquito marking and release technique

Only the F1 adult generation was used in this study. Two groups of three- to five-day-old, non-bloodfed *Ae. aegypti* females were marked with luminous marking powder (BioQuip Products, Rancho Dominquez, CA) following the method of Achee et al. (2005). Combined, both groups numbered 125 females. This consisted of one population of 100 females that was used as a release population and 25 females that were used as controls. Marked mosquitoes were sugar starved for 24 h, placed in a humidified chamber that was kept moist using water soaked towels, and provided with water soaked cotton pads until the time of release.

For the entrance experiment, 100 marked mosquitoes were released 10 m outside of each hut. For the exit collections, 100 marked mosquitoes were released inside of each hut. The released time was set at 05:00, approximately one h before the start of the collection.

All experiments were replicated two times in both huts and in each month. Human hosts were covered by mosquito nets to protect them from being bitten during the study. Entrance and exit traps were sampled every 20 min between 06:00-18:00. The collections were made by two collectors per hut. Collectors were alternated between huts every 20 min to control for collectors bias. All mosquitoes from the traps were examined for fluorescent powder using a UV light and a stereomicroscope. The ambient temperature and relative humidity were recorded by the collector inside the hut every 20 min.

Data analysis

Differences in the number of mosquitoes recaptured from entrance and exit traps over four hourly intervals (06:00-09:00, 09:00-12:00, 12:00-15:00, and 15:00-18:00) were analyzed using regression analysis that included the following independent variables: ambient temperature, relative humidity, and precipitation. Multiple regression was performed to investigate the association between the two types of movement behaviors (hut entry and exit) of *Ae. aegypti* and the environmental variables of temperature and relative humidity. All data were analyzed using the SAS program package (SAS Release 6.10, SAS Institute, Cary, NC). The discriminating level for all tests was set at 0.05%.

RESULTS

The average number of *Ae. aegypti* females recaptured from entrance and exit traps during a two-year period is given in Table 1. A comparison of the number of mosquitoes recaptured from entrance and exit traps in the same months between the first and second year was performed. Results reveal that the number of *Ae. aegypti* females recaptured from entrance traps in the month of April was the only statistical different sample period during the two-year collection (T = -6.602, P = 0.007) (Table 1). However,

Table 1. Average numbers of *Ae. aegypti* recaptured in traps during the two-year study period.

Month	Average number of *Ae. aegypti* recaptured from entrance traps		Average number of *Ae. aegypti* recaptured from exit traps	
	Year 1	Year 2	Year 1	Year 2
January	8.25 ± 2.87	27.25 ± 10.87	70.50 ± 9.47	44.25 ± 10.31
February	21.00 ± 2.94	16.25 ± 8.30	30.50 ± 3.79*	65.00 ± 3.83*
March	36.25 ± 18.26	19.25 ± 3.86	18.75 ± 4.03*	58.75 ± 7.41*
April	3.75 ± 2.06*	39.25 ± 9.95*	72.75 ± 7.27	71.75 ± 15.41
May	35.00 ± 13.29	29.25 ± 2.87	47.50 ± 8.19	55.50 ± 10.28
June	45.00 ± 5.35	29.25 ± 5.85	67.50 ± 7.14*	37.75 ± 12.92*
July	18.75 ± 12.87	24.75 ± 9.43	53.50 ± 14.64*	35.75 ± 12.82*
August	32.50 ± 14.20	24.25 ± 9.32	69.25 ± 16.15*	25.25 ± 7.93*
September	26.75 ± 7.04	22.50 ± 7.94	48.00 ± 26.34	54.50 ± 6.61
October	15.00 ± 7.02	26.50 ± 12.87	69.75 ± 4.99	66.75 ± 14.64
November	41.75 ± 24.35	11.50 ± 0.58	53.00 ± 11.14	56.50 ± 5.54
December	22.00 ± 7.30	21.75 ± 9.88	40.50 ± 25.16	53.00 ± 6.93
Total	306.00 ± 13.00	291.75 ± 7.07	641.5 ± 17.42	624.75 ± 13.83

*Statistical differences in average number of *Ae. aegypti* recaptured in traps between year 1 and year 2 of the study ($P < 0.05$).

there was no significant difference in the total number of mosquitoes recaptured from entrance traps during the two- year period ($T = 0.235$, $P = 0.818$). For the exit regime, statistical differences in the average number of *Ae. aegypti* recaptured during the first two years of the study were found for the months of February ($T = -13.118$, $P = 0.001$), March ($T = -20.000$, $P = 0.000$), June ($T = 4.078$, $P = 0.027$), July ($T = 3.211$, $P = 0.049$), and August ($T = 4.422$, $P = 0.021$). No statistical difference in the total number of exit specimens recaptured between the first and second years was found ($T = 0.196$, $P = 0.848$).

The average number of *Ae. aegypti* recaptured in traps tabulated by time of collection and season is shown in Table 2. The average temperature and relative humidity for the three seasons are provided in Table 3. Overall, the highest proportion of *Ae. aegypti* females were recaptured from both entrance and exit traps (29.63% from entrance and 53.78% from exit traps) during the summer months. For the entrance experiment, the highest total number *of Ae. aegypti* were recaptured in the summer (29.63%), whereas the lowest number was recorded during the winter (21.22%). There was no significant difference in the number of *Ae. aegypti* recaptured during a single day's collections conducted during the winter and rainy seasons ($P > 0.05$). Significantly higher numbers of *Ae. aegypti* females were recaptured in traps during the summer as compared to the other seasons ($P = 0.003$ and 0.036, respectively). During the summer, a higher proportion of mosquitoes was recaptured between 06:00-09:00 compared to any other time period during the day ($P = 0.000$).

Table 2. Average number of *Ae. aegypti* recaptured in traps during a 12-h collection in all seasons.

Experiment	Times				Total
	06:00 – 09:00	09:00 – 12:00	12:00 – 15:00	15:00 – 18:00	
Entrance					
Winter	10.53 ± 7.81	8.06 ± 5.93	1.56 ± 1.24	1.06 ± 0.53	21.22 ± 10.32
Summer	24.19 ± 11.44	3.44 ± 1.95	1.34 ± 1.08	0.66 ± 0.60	29.63 ± 12.98
Rainy season	15.69 ± 5.19	5.00 ± 2.62	1.84 ± 0.79	1.34 ± 0.78	23.88 ± 5.31
Exit					
Winter	4.84 ± 3.03	15.84 ± 10.05	16.09 ± 6.18	14.88 ± 5.92	51.66 ± 13.02
Summer	11.78 ± 8.90	24.47 ± 11.45	10.28 ± 5.41	7.25 ± 2.33	53.78 ± 18.60
Rainy season	7.06 ± 6.41	20.13 ± 8.44	18.59 ± 5.43	7.06 ± 2.98	52.84 ± 16.16

Table 3. The average temperature and relative humidity by period during a 12 h collection in all seasons.

Factors	Seasons		
	Winter	Summer	Rainy season
Inside Temperature (°C)			
06:00 – 09:00	21.2 ± 2.84	24.9 ± 1.65	24.7 ± 0.98
09:00 – 12:00	26.4 ± 2.35	28.4 ± 1.59	27.2 ± 1.10
12:00 – 15:00	29.8 ± 2.15	29.9 ± 2.65	28.6 ± 1.23
15:00 – 18:00	28.9 ± 2.64	28.9 ± 2.99	27.3 ± 1.40
Min	12.8	19.0	22.3
Max	34.5	36.7	31.8
Outside Temperature (°C)			
06:00 – 09:00	20.1 ± 3.43	24.8 ± 2.38	25.0 ± 1.86
0900 – 12:00	29.7 ± 3.19	31.6 ± 2.62	30.0 ± 1.97
12:00 – 15:00	34.2 ± 3.01	32.7 ± 4.12	31.4 ± 2.23
15:00 – 18:00	30.1 ± 3.80	29.8 ± 4.56	27.7 ± 2.54
Min	12.0	18.0	20.7
Max	41.0	43.0	35.7
Relative Humidity (%)			
06:00 – 09:00	79.7 ± 5.28	80.6 ± 5.59	79.1 ± 3.79
09:00 – 12:00	59.2 ± 9.15	66.8 ± 9.47	67.2 ± 6.37
12:00 – 15:00	47.2 ± 9.20	62.5 ± 13.47	62.9 ± 6.77
15:00 – 18:00	54.0 ± 10.25	67.3 ± 13.91	68.6 ± 7.23
Min	28.3	28.0	40.0
Max	89.0	96.0	88.7

For the exit experiment, the total number of *Ae. aegypti* recaptured from the traps during all three seasons was quite similar, ranging from 51.7% in the winter to 53.8% in the summer (Table 2). In general, higher numbers of *Ae. aegypti* females were recaptured during the late morning (09:00-12:00), with the exception of winter when a higher proportion of specimens was recaptured between 12:00-15:00 (16.1%). Specifically, the proportion of *Ae. aegypti* recaptured in exit traps from 09:00-12:00 and 12:00-15:00 was significantly different from those of the other two periods (06:00-09:00 and 15:00-18:00) ($P < 0.05$) (Table 2).

Time trends for entering and exiting of *Ae. aegypti* females were also recorded in the three different seasons (Figures 1A, 1B). For the entrance experiment, the majority of entering behavior was seen during the morning period (07:00-11:00) with a peak at 07:00 in summer, at 09:00 in the winter, and a prolonged peak from 07:00 to 09:00 in the rainy seasons. A very distinct peak was seen in the summer as compared to what was observed in either the winter or rainy seasons. Very few mosquitoes tended to enter the hut during the afternoon period, regardless of season, with less than one mosquito entering each hour (Figure 1A). During exit collections, a very distinct exiting period was observed in the winter (12:00-17:00) with a peak in activity occurring at 16:00. The duration of exiting was considerably longer during the summer and rainy season (08:00-16:00), with a peak at 11:00 in the summer and 14:00 in the rainy season (Figure 1B).

Entrance and exit behaviors of *Ae. aegypti* into and out of experimental huts were evaluated in relation to ambient temperature and relative humidity using regression analysis. Entrance movement of *Ae. aegypti* was found to be negatively associated with temperature but positively correlated with relative humidity (Figures 2A, 2B, 2C). The average number of *Ae. aegypti* specimens recaptured in traps in response to the ambient temperature and relative humidity are shown in Tables 2 and 3, respectively. The results of the multiple regression analysis on the number of *Ae. aegypti* recaptured in both traps in relation to temperature and relative humidity indicate that these two variables strongly influence the entrance and exit patterns of this mosquito species ($R^2 = 0.585$; $F = 25.391$; $P = 0.000$).

The exiting patterns of *Ae. aegypti* from the experimental hut were found to be positively associated with temperature but negatively correlated with relative humidity (Figures 3A, 3B, 3C). Results of the multiple regression analysis on the number of *Ae. aegypti* recaptured in traps suggested that these two environmental factors also have a strong impact on the movement patterns of *Ae. aegypti* females into and out of the hut ($R^2 = 0.335$; $F = 9.077$; $P = 0.000$).

DISCUSSION

This study is the first attempt to evaluate the normal movement patterns of *Ae. aegypti* females, a day-biting mosquito, in response to changes in seasonal environmen-

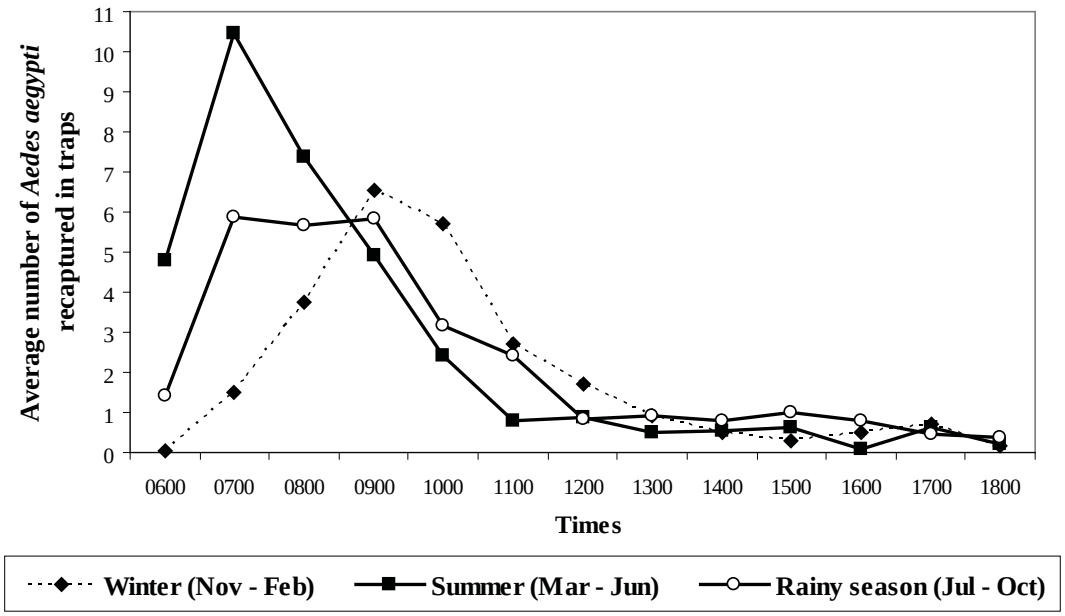

Figure 1A. Time of entry of *Ae. aegypti* into traps during a 12-h collection.

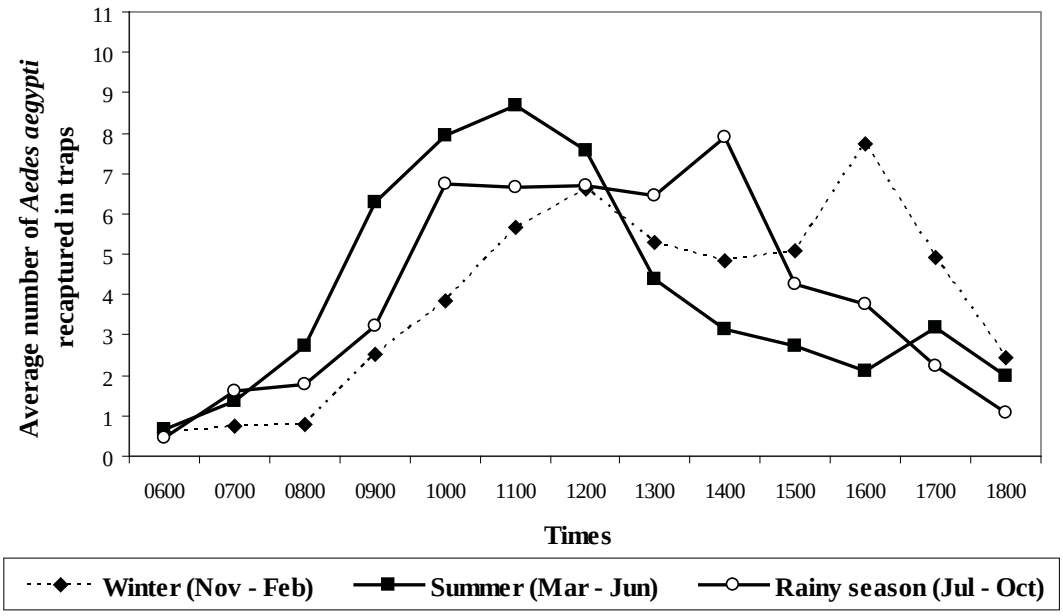

Figure 1B. Time of exit of *Ae. aegypti* into traps during a 12-h collection.

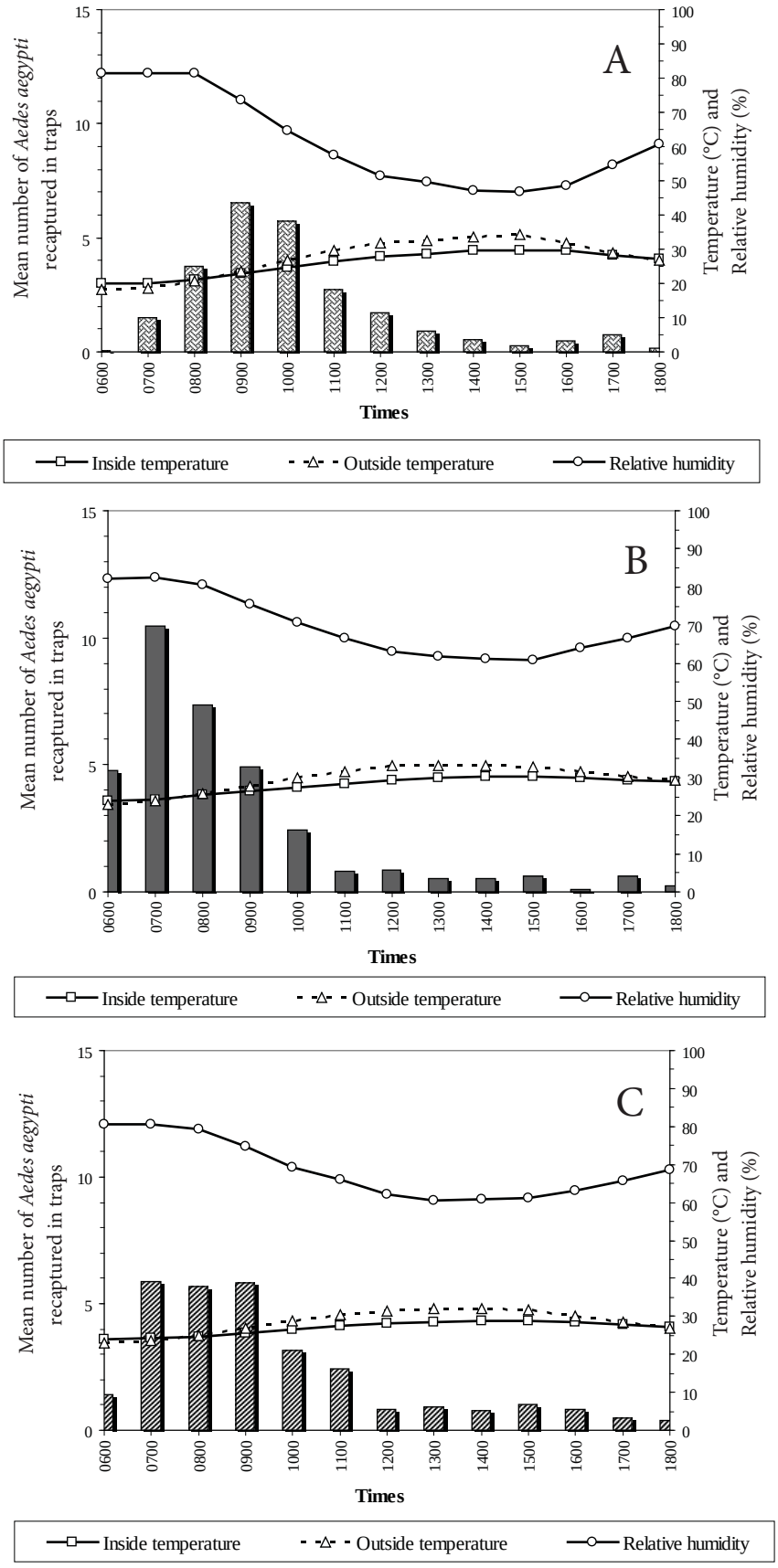

Figure 2. Average number of *Ae. aegypti* collected in entrance traps compared to temperature and relative humidity during a 12-h collection in winter (A), summer (B), and rainy season (C).

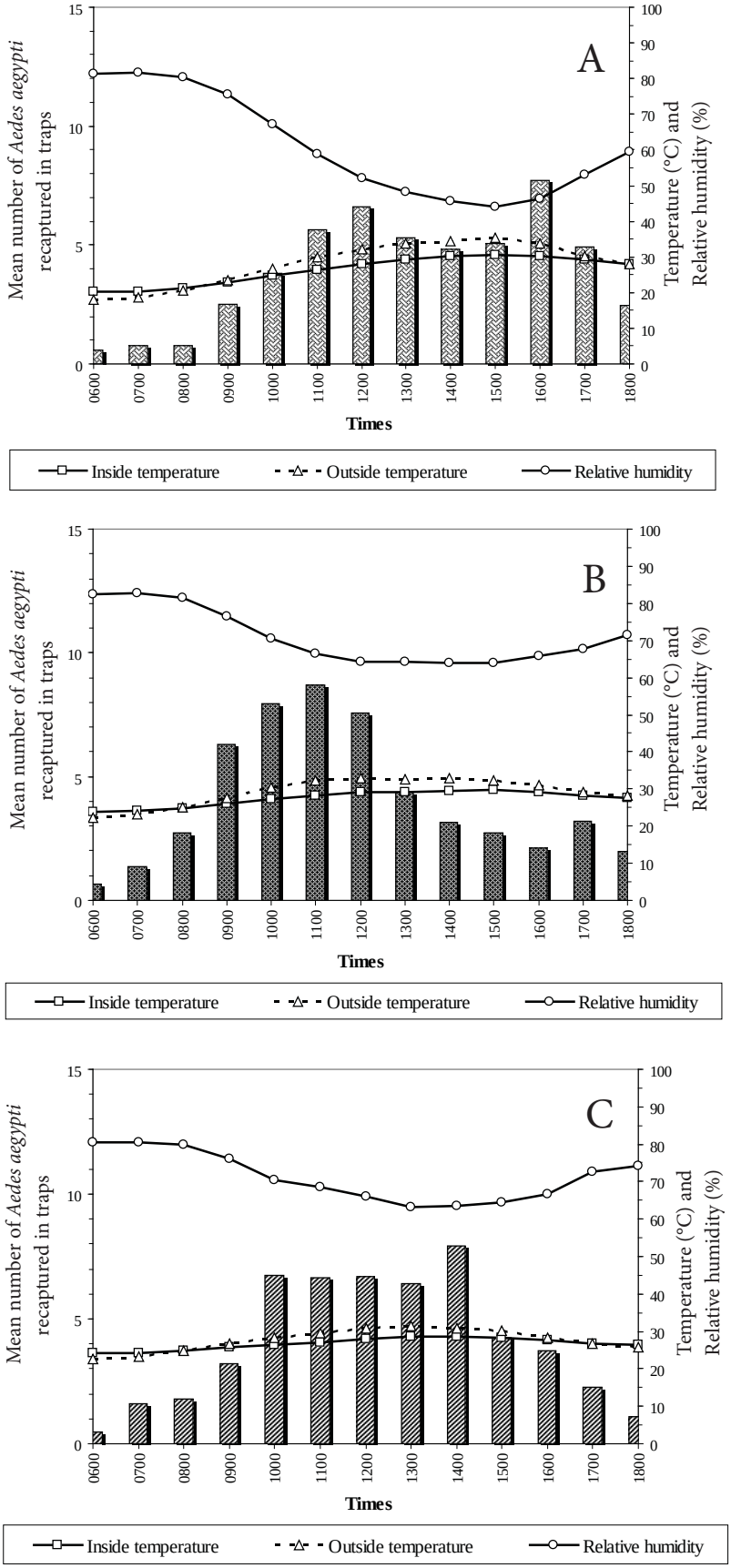

Figure 3. Average number of *Ae. aegypti* collected from exit traps compared to temperature and relative humidity during a 12-h collection in winter (A), summer (B) and rainy season (C).

tal parameters into and out of experimental huts in Thailand. Such information helps to define the vector capacity and relative risk for disease transmission, as well as supports the appropriate vector prevention and control strategies. A better understanding of vector biology and behavior is needed to guide vector control programs and evaluate chemical control strategies. In this study, we utilized the mark–release–recapture technique to evaluate the movement patterns of *Ae. aegypti* into and out of the experimental huts fitted with entrance and exit traps.

Many studies conducted on the host preference of *Ae. aegypti* suggest that this species has a high propensity for feeding on humans inside houses, thus characterizing it as strongly anthropophagic (Gubler and Kuno 1997, Harrington et al. 2001, Polawat and Harrington 2005). It would seem that an endophagic and endophilic species such as *Ae. aegypti* would be congregated in distinct locations and thus make it easier for vector control personnel to control them. However, various internal and external factors seem to impede the success of controlling *Ae. aegypti* inside homes. Mosquito behavior is one of those factors that plays a significant role, and it in turn is generally affected by changes in the environmental and biological conditions. The natural environment imposes a number of pressures that will impact mosquito behavior, including ambient temperature and relative humidity (Kennedy 1946, Busvine 1964, Drobozina et al. 1984). This study has increased our knowledge of how temperature and humidity affect the movement patterns of *Ae. aegypti* into and out of homes.

Previous reports suggest that entering behavior was much stronger when a human host was present in the hut compared with a dog or no host at all (Suwondkerd et al. 2006). In addition, the movement patterns of *Ae. aegypti* females vary according to outdoor temperature as reported by Chareonviriyaphap et al. (2005) and Suwonkerd et al. (2006). Vector control professionals need to carefully design and improve methods to effectively reduce mosquito populations and risk of disease transmission. In this study, two years of observations on the normal movement patterns of *Ae. aegypti* females were evaluated and it was found that temperature and relative humidity play a major role in altering these patterns.

This study showed that the entering and exiting patterns of *Ae. aegypti* followed unimodal periodicities with the peak of entering taking place between 09:00 and 12:00 in winter and between 06:00 to 09:00 in both the summer and the rainy season. These results are quite similar to those reported by Chareonviriyaphap et al. (2005) and Suwonkerd et al. (2006). In contrast, *Ae. aegypti* human landing collections result in either a bimodal or trimodal periodicity (Corbet and Smith 1974, Chadee 1988, Thavara et al. 2001, Atmosoedjono et al. 1972, Chadee and Martinez 2000).

The time of entering in winter was delayed compared to what was found in either the summer or the rainy season. This delay is most likely the result of the colder temperatures that occur during the winter which have been found to negatively impact the flight activity of *Ae. aegypti* (Clements 1999, Rowley and Graham 1968). In this study, it is quite clear that lower temperatures (as low as 12° C) and lower relative humidity in the morning impact flight behavior. Therefore, the peak of entering the huts during the winter was shifted to later in the morning. The peak of exiting the hut in this study occurred between 10:00 and 14:00 for all seasons. This result is in agreement with those reported by Chareonviriyaphap et al. (2005) and Suwonkerd et al. (2006). The extreme temperature and decreased relative humidity inside the hut in the afternoon appears to force the *Ae. aegypti* females to seek suitable outdoor resting sites.

Despite considerable progress in our understanding of vector biology, there remains much to understand about the biology and behavior of *Ae. aegypti* and how external factors influence disease transmission. The behavior of mosquitoes is impacted by several factors including climatic, environmental, and physiological factors. This study demonstrates how the entering and exiting behaviors of *Ae. aegypti* are affected by ambient environmental factors such as temperature and humidity. It is clear from this study that conditions in the peridomestic environment play a critical role in where mosquitoes will be found throughout the day. If the conditions are unsuitable inside the house (high temperature and low humidity), *Ae. aegypti* will seek more conducive resting sites outdoors. Likewise, the outdoor conditions may delay movement inside if temperatures are too cold for flight. Knowing where the mosquitoes are likely to be in the peridomestic environment based on environmental conditions may aid in control efforts by more precisely directing particular interventions. This information on how climatic factors influence mosquito behavior will also be useful when designing future studies and serve as a baseline for the natural movement patterns of *Ae. aegypti* when evaluating the response of this vector to test compounds.

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Scientific Note

An improved experimental hut design for the study of *Aedes aegypti* (Diptera: Culicidae) movement patterns in Thailand

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Knowledge of the behavioral responses of mosquito vectors to chemical insecticides is of paramount importance to understanding the epidemiology of disease transmission and optimum strategies for vector control. Several studies have employed the use of experimental huts to evaluate changes in mosquito behavioral responses that are relevant to disease transmission (i.e., disruption of human-vector contact) when they are exposed to insecticides, as compared to chemical-free conditions, including measurement of time and density of house entry, exit, and indoor resting, with attention concentrated mainly on *Anopheles* species (Smith 1965, Roberts et al. 1984, Grieco et al. 2000, Pates and Curtis 2005). Relatively few attempts have been made, however, to describe the responses incited by insecticides on other mosquito species using experimental huts (Symes et al. 1942, Kennedy 1947, Brown 1964, Lal et al. 1965, Moore 1977, Suwannachote et al. 2009).

Our previous research describing the movement patterns of *Ae. aegpyti* to identify chemical modes of action demonstrated that a portable hut based on the design of Achee et al. (2005) served as a successful tool for such studies (Suwonkerd et al. 2006, Chareonviriyaphap et al. 2005, Grieco et al. 2007). Although these huts mimicked indigenous Thai homes and produced consistent and reliable results, structural adjustments were identified that could increase hut longevity and improve mosquito sampling efficiency. These modifications included: 1) a raised platform to prevent structural damage from termites and soil moisture; 2) cement ant traps placed underneath the raised platform to prevent predation on knock-down mosquitoes during chemical trials; 3) a walkway around the perimeter of the hut to facilitate mosquito removal from window and door traps and; 4) increased airflow between the ceiling and exterior roof to aide in indoor heat dissipation.

As part of a larger proof-of-concept research program evaluating a Push-Pull vector control strategy to reduce host-seeking *Ae. aegypti* from inside homes and the peridomestic environment using minimal chemical dose and treatment coverage of standard vector control compounds, additional experimental huts have been constructed based on these design modifications (Figure 1). This report describes these huts, interception traps, and baseline studies, that were generated without chemical intervention to determine if changes in hut design would negatively affect mosquito movement patterns as compared to previous findings.

The dimensions of the modified huts are 4 m wide x 5 m long x 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 traps, respectively (Figure 2). Hut frames are made of iron pipe, untreated wood planks are used for walls and flooring, with the roof constructed of zinc panels. The dimensions of the window traps are 0.60 m long x 0.90 m wide x 0.60 m high (Figure 3A). Louvers made of 1.6 cm non-treated hard wood were placed over the front opening of each trap with a hinged mesh flap used to cover the bevel opening during removal of trapped mosquitoes. The door trap is separated into two equal portions with each measuring 0.73 m wide × 0.56 m high × 0.93 m long (Figure 3B). Louvers and mesh flaps are integrated into each portion as described for window traps. The frames of both upper and lower traps are fixed to the hut door but the lower door trap can be opened independently and easily disassembled during experimental trials to allow collectors to exit and enter the huts during host rotation periods. Both window and door traps have three collection portals through which collectors insert manual aspirators for removal of trapped mosquitoes (depicted in Figure 3B).

To validate the modified experimental huts, entry and exit movement patterns of *Ae. aegypti* populations from Kanchanaburi Province, Thailand, were evaluated under chemical-free conditions on four consecutive days for entrance trials and four consecutive days for exit trials following previously described methodologies (Grieco et al. 2007). Briefly, each test population consisted of 100 five-to-seven-day-old, female mosquitoes marked

Figure 1. Experimental hut fitted with window and door interception traps for the evaluation of mosquito behavior patterns.

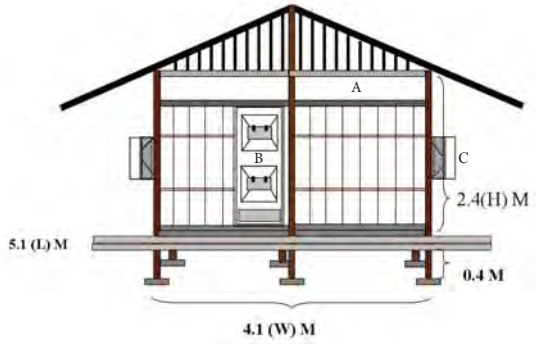

Figure 2. Graphic display of the modified hut with dimensions (A) ventilation space; (B) window trap; (C) door trap.

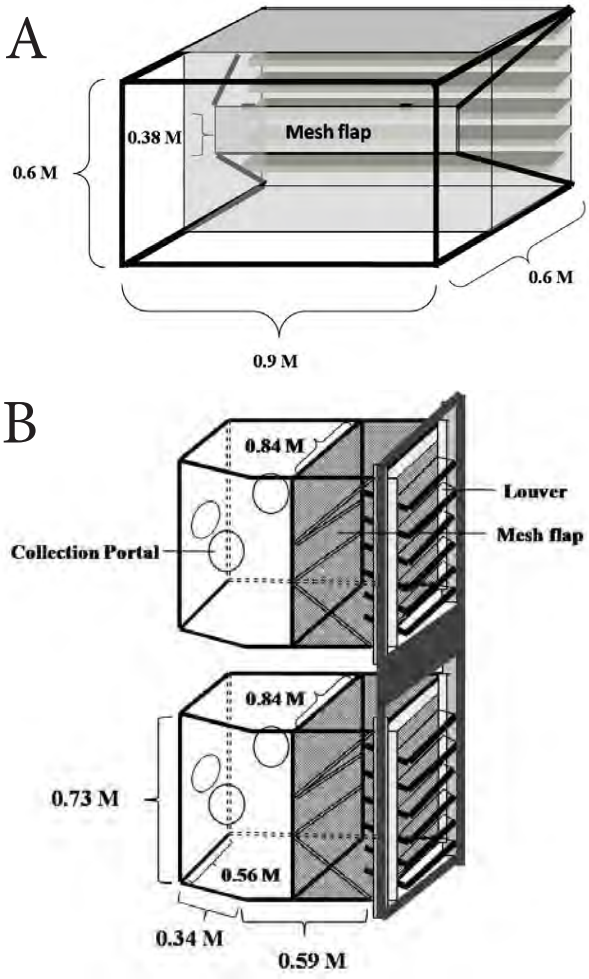

Figure 3. Window (A) and door (B) intercept trap design with dimensions.

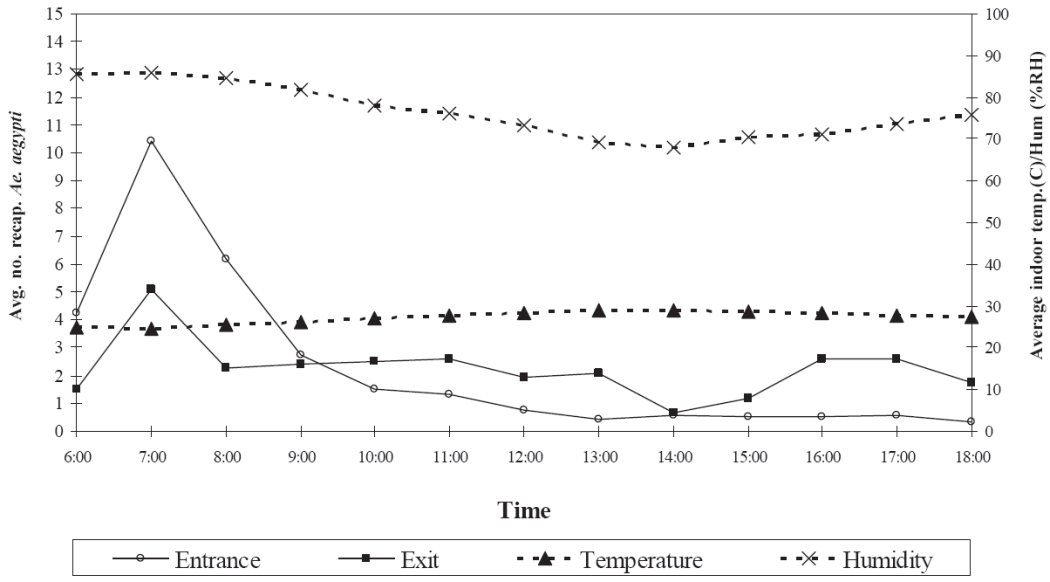

Figure 4. Average number of *Aedes aegypti* females collected from entrance and exit traps fitted to experimental huts during validation studies (without chemical intervention).

Table 1. Entrance and exit movements of *Aedes aegypti* to three experimental huts in Kanchanaburi Province,Thailand.

Experiment	Hut	Total released	Recapture in traps (marked mosquitoes)	Total recapture in trap[1]	Total recapture inside hut[2]	% KD[3] on floor	Avg[4] indoor temp (°C)	Avg indoor humidity (%RH)
Entrance	A	400	196	205	n/p	n/p	26.5	78.6
	B	400	129	133	n/p	n/p	27.4	73.6
	C	400	123	126	n/p	n/p	27.5	74.8
Exit	A	400	92	97	231	0	26.7	80.4
	B	400	52	56	253	1	27.7	75.7
	C	400	73	81	261	0	27.8	75.4

[1]Includes mosquitoes marked from previous days.
[2]Collected using a back-pack aspirator following last sampling period (18:00).
[3]KD= knockdown specimens removed from hut floor (n/p = not performed).
[4]Average 12-h reading from four replicate trials for a given hut.

with fluorescent powder (in separate colors) using the methods of Suwonkerd et al. (2006) and Tsuda et al. (2001). Test populations were released 30 min before sunrise (approx.05:30) and collections were made from traps at 20 min intervals from 06:00-18:00. For each entrance experiment, three mosquito test populations (n = 100/ release population) were released at a marked point 10 m NW from the door of each corresponding hut. Interception traps were positioned inside the hut (to capture entering mosquitoes) and two people were indoors to generate host cues and serve as collectors. For exit trials, one test population (n=100) was released at the inside center of each hut and traps were fixed to exterior hut walls (to capture exiting mosquitoes) while three, 2-person teams removed mosquitoes from traps using the same sampling scheme described for entrance experiments (i.e., 06:00-18:00). One volunteer occupied each hut during exit trials to generate host cues and was positioned underneath an untreated bed net for bite protection.

Results of movement pattern studies of *Ae. aegypti* females into and out of the modified huts are given in Table 1. Among the three huts, there were no statistical differences in the total number of recaptured mosquitoes entering (ANOVA; F=1.979, df =2, 9 , $P = 0.118$) or exiting (F=0.783, df =2, $P = 0.574$). Average time trends for hut entry and exit indicate similar peaks at 07:00 for both experiment al types with exit behavior continuing throughout the day (Figure 4). These patterns are similar to those described from previous findings of *Ae. aegypti* responses without chemical intervention during the same seasonal period (Suwonkerd et al. 2006, Grieco et al. 2007, Suwannachote et al. 2009), thereby indicating no negative effects from design modifications.

In summary, the experimental hut design reported here has shown to be successful in measuring expected movement patterns of *Ae. aegypti* populations under chemical-free conditions. These results validate the huts as a standard tool for evaluating mosquito exiting and entering behaviors as part of the development of a Push-Pull strategy within the larger research program.

Acknowledgments

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A High Throughput Screening System for Determining the Three Actions of Insecticides Against *Aedes aegypti* (Diptera: Culicidae) Populations in Thailand

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ABSTRACT Chemicals can protect humans from the bites of hemophagous arthropods through three different primary actions: irritancy (excitation), repellency, or toxicity, actions that can be evaluated using a laboratory-based assay system. In this study, the deterrent and toxic actions of three synthetic pyrethroids and DDT were characterized on six field strains of *Aedes aegypti* from Thailand under laboratory-controlled conditions using the high throughput screening system. All six strains showed significant contact irritant responses to the three synthetic pyrethroids, but significantly weaker irritant responses to DDT. Marked repellency responses were seen in all six *Ae. aegypti* test strains exposed to DDT, whereas the synthetic pyrethroids resulted in greater toxicity than DDT under similar test conditions. Although significantly different in actions, irritancy and repellency may reflect and be influenced by the background insecticide susceptibility status of a particular mosquito population. Results from this study can be used to guide decision making regarding more effective *Ae. aegypti* adult control in Thailand.

KEY WORDS *Aedes aegypti*, behavioral responses, HITSS, synthetic pyrethroids, DDT

Dengue fever and dengue hemorrhagic fever are considered the most important arthropod-borne viral disease manifestations in humans and globally a problem that continues to grow in scope and importance (Gibbons and Vaughn 2006). The four dengue virus serotypes are most commonly transmitted by *Aedes aegypti*, a common peridomestic and indoor day-biting mosquito with a widespread geographic distribution throughout most tropical and subtropical countries (Gubler 1998, Guzman and Kouri 2002). This mosquito preferentially feeds on human blood and is recognized as a highly efficient vector because of its proximate relationship with humans (Swaddiwudhipong et al. 1992, Edman et al. 1992, Chansaeng et al. 1993, Chareonviriyaphap et al. 2003). Currently, the primary methods for controlling dengue rely on chemical and nonchemical control applications against the mosquito to reduce human-vector contact (WHO 1999).

Theoretically, chemical compounds can protect humans from vector-borne diseases in two primary ways, as follows: either by killing or by nontoxic action modifying mosquito behavior to inhibit blood feeding (Davidson 1953, Roberts and Andre 1994, Roberts et al. 1997, Rutledge et al. 1999). Most previous works have been done on insecticide toxicity; little has been focused on nontoxic properties and actions (Roberts et al. 1997). Recently, three types of insecticidical actions were distinguished in the context of toxicants, contact irritants, and noncontact repellents effects (Grieco et al. 2007). For purposes of this report, we have defined contact irritancy as an excitatory effect resulting from insects making direct (tarsal) contact with chemical residues on a treated surface before eliciting a stimulus-mediated response, whereas repellency occurs as a result of spatial chemical detection from a distance, i.e., without making physical contact with an insecticide-treated surface (Roberts et al. 2000). The combined locomotor stimulant effects of both irritancy and repellency have long proven to inhibit or disrupt normal behavioral patterns and in particular drive sensitive insects away from treated surfaces (i.e., escape) and/or inhibit normal blood

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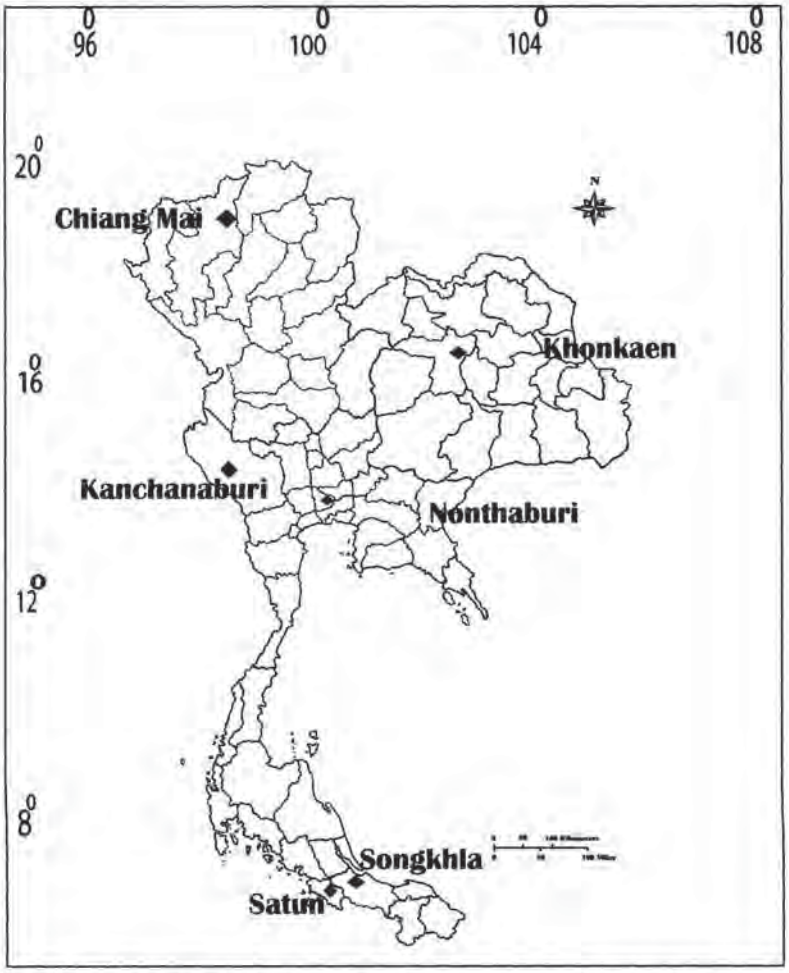

Fig. 1. Map of the six localities where *Ae. aegypti* were collected in Thailand.

feeding (Dethier et al. 1960; Kennedy 1947; Roberts et al. 1997, 2000; Chareonviriyaphap et al. 1997, 2004; Potikasikorn et al. 2005; Grieco et al. 2007).

There have been a few studies that have measured the three types of responses of mosquitoes to insecticides using both laboratory and field validation assay systems (Roberts et al. 1997, Chareonviriyaphap et al. 2002, Grieco et al. 2005). An improved excito-repellency test chamber system was developed to evaluate irritancy and repellency of mosquitoes (Chareonviriyaphap et al. 1997, Roberts et al. 1997). Subsequent modifications and improvements have been made to allow greater ease of setup and accuracy in evaluating the innate behavioral response of mosquitoes exposed to varying doses of residual insecticides (Chareonviriyaphap et al. 2002, Tanasinchayakul et al. 2006). Although found acceptable for field and laboratory uses, the excito-repellency chamber setup requires a relatively large amount of chemical and treated paper surfaces as a result of the size of the interior surfaces of each test system. The recent development of the high throughput screening system (HITSS) is a more compact test system requiring less chemical and treated material while providing repeatable, quantifiable measures of contact irritancy, spatial repellency, and toxicity using a relatively low number of test replications (Grieco et al. 2005). In this study, the HITSS was used to evaluate the behavioral responses of six field strains of *Ae. aegypti* against α-cypermethrin, deltamethrin, permethrin, and DDT in the laboratory. DDT served as the repellency standard for comparison with all other chemicals.

Materials and Methods

Mosquitoes. *Ae. aegypti* larvae and pupae were collected from natural and artificial larval habitats (containers) located within one village from six different locations in Thailand: Chiang Mai, Kanchanaburi, Khonkaen, Nonthaburi, Songkhla, and Satun (Fig. 1). All larvae and pupae were immediately transferred to the Department of Entomology insectary at Kasetsart University in Bangkok. Geographic coordinates and locations are provided in Table 1.

Table 1. Field sites and geographic coordinates where *Aedes aegypti* larval populations were collected

Province	Village	District	Geo-coordinates
Chiang Mai	Pang Mai Deang	Mae Teang	19° 14′ N 98° 82′ E
Kanchanaburi	Pu Teuy	Sai Yok	14° 20′ N 98° 59′ E
Khonkaen	Non Ton	Muang	16° 25′ N 102° 50′ E
Nonthaburi	Tha Sai	Muang	13° 53′ N 100° 29′ E
Songkhla	Bon Wua	Muang	7° 11′ N 100° 35′ E
Satun	Pi-marn	Muang	6° 37′ N 100° 03′ E

Mosquito larvae and pupae were reared to the adults under controlled insectary conditions (25 ± 5°C, 80 ± 10% RH, and 12:12 light:dark photoperiod). Female and male adults identified as *Ae. aegypti* were provided with cotton pads soaked with 10% sugar solution on first day of emergence. Adults from each strain were maintained in separate screen cages. Natural mated female mosquitoes were permitted to feed on blood from restrained live guinea pigs on day 4 postemergence. Two days after blood feeding, 10-cm-diameter oviposition dishes containing moist filter paper were placed in the cages for egg deposition. Eggs were dried at room temperature for 1–2 d before being immersed with water in individual hatching trays. At 2 d posthatch, ≈250 larvae of each strain were transferred to individual plastic rearing trays (20 × 30 × 5 cm) containing 1.5 liter of tap water and one teaspoon (≈2.5 g) of finely ground fish pellets. Pupae were transferred daily from larval trays to emergence cups and placed directly into screened 30-cm^3 cages. Adults were provided cotton soaked with 10% sucrose solution from time of eclosion to 24 h before use in test assays. Only F_1 and F_2 generations of each test strain were used in assay trials.

Chemical Compounds. Four insecticides were used in behavioral testing, including the following: 1) α-cypermethrin (1 a [S], 3 a-[±]-cyano-[3-phenoxyphenyl] methyl 3-[2, 2-dichloroethynyl]-2, 2 dimethylcyclopropanecarboxylate) (95% purity) obtained from BASF (Mt. Olive, NJ); 2) deltamethrin ([cyano-[3-phenoxyphenyl]-methyl] 3-[2,2-dibromoethenyl]-2,2-dimethyl-cyclopropane-1-carboxylate) (99% purity) provided by BASF; 3) permethrin (3 phenoxybenzyl [1 RS, 3 RS, 1 RS, 3 SR]-3-[2, 2-dichlorovinyl]-2, 2-dimethylcyclopropanecarboxylate) (92% purity) provided by Ladda (Bangkok, Thailand);, and 4) DDT (1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane, 1,1′-[2,2,2-trichloroethylidene] bis [4-chlorobenzene]) (98% purity) purchased from Sigma-Aldrich (St. Louis, MO; CAS 50-29-3, Product 386340).

World Health Organization Susceptibility Test. Each mosquito strain was tested for susceptibility to the four insecticides at operational field concentrations of 0.05% α-cypermethrin (WHO 1998a), 0.05% deltamethrin, 0.25% permethrin, and 4% DDT (WHO 1998b). Mosquitoes were assessed by exposing non-blood-fed, 3- to 4-d-old females to a single diagnostic dosage placed on insecticide-treated test papers following standard testing procedures and exposure times (WHO 1998b). After exposure, mosquitoes from paired test and control groups were transferred to separate holding containers, and mortality was recorded 24 h postexposure. Each test combination was replicated four times (25 female mosquitoes/test).

Insecticide-Treated Netting Strips. Nylon-organdy netting was cut into strips (11 × 25 cm) and treated either with insecticide active ingredient or chemical solvent (acetone) alone following HITSS protocols (Grieco et al. 2005, 2007). Three different concentrations at 10-fold serial dilutions of α-cypermethrin, deltamethrin, permethrin, and DDT were used in HITSS trials. Netting strips were treated with α-cypermethrin at 0.06, 0.6, and 6.0 nmol/cm^2; deltamethrin at 0.049, 0.49, and 4.9 nmol/cm^2; permethrin at 1.27, 12.7, and 127.8 nmol/cm^2; and DDT at 5.6, 56.4, and 564.2 nmol/cm^2. The highest concentrations for each chemical closely approximated the WHO-recommended field application rate (WHO 1998b). All netting strips were treated using a calibrated micropipette at the rate of 1.5 ml solution per 275 cm^2 and allowed to air dry for at least 15 min before use in the assay. Dry nets were placed inside corresponding treatment and control cylinders and remained in place during the entire test period. New treatment and control nets were prepared at the beginning of each test day.

Test Mosquito Preparation. Primed females were sorted into groups of 10 each for the contact irritancy assay (CIA) and 20 females each for the spatial repellency assay (SRA) and toxicity assay (TOX), as previously described (Grieco et al. 2005, 2007). Mosquitoes were sorted into groups of 10 or 20 and placed in individual plastic cups accordingly. On the day of testing, the plastic cups were transferred from the insectary to the testing laboratory, and specimens were removed by mechanical aspiration (attached to an air compressor) from each plastic cup into individual holding tubes. Holding tubes were subsequently placed into corresponding trays to separate control and treatment lots for use in CIA, SRA, and TOX assays.

Behavioral Tests. The HITSS has a modular design that allows for the quantitative observation of three test responses, contact irritancy, spatial repellency, and toxicity, using the same components in different configurations (Fig. 2). The standard order of assays used on a given day was CIA, SRA, and TOX. All trials were conducted during daytime and under controlled laboratory conditions for temperature 24 ± 2°C and relative humidity 70–80%.

Contact Irritancy Assay (CIA). Fig. 2A illustrates the components used in the CIA that employs a combination of clear cylinders and metal chambers that house the netting strips (control and treatment; Fig. 2A). A clear cylinder and a metal chamber are connected using a linking section containing a butterfly valve. Ten mosquitoes were introduced into each metal chamber using a mechanical aspirator. After a 30-s rest period, the butterfly valve was placed into the open position, 10 min after which the valve was closed and the number of mosquitoes having exited into the clear cylinders (i.e., number escaping) and the number of knockdown mosquitoes in both cylinders were recorded. For test trials, a control assay was simulta-

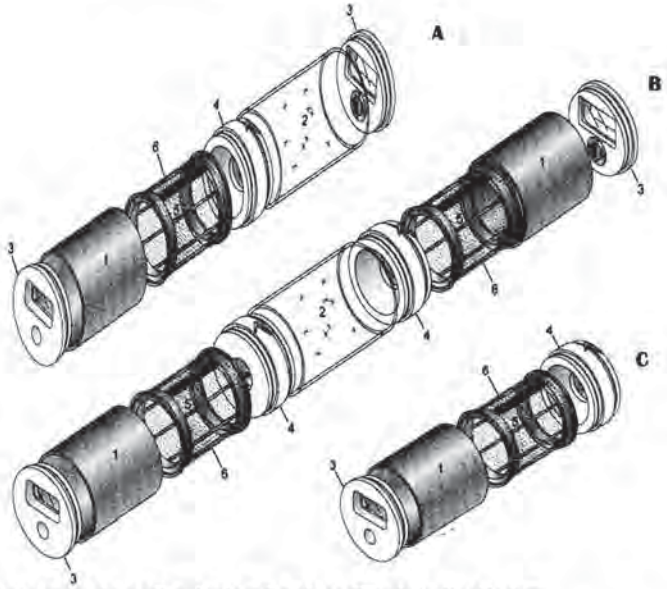

Fig. 2. Schematic drawing of the HITSS showing components parts of (A) the CIA, (B) SRA, and (C) TOX (from Grieco et al. 2005).

neously run using acetone-treated nets for comparison. The ratio of treatment to control assays was 2:1. To prepare for the next replicate, mosquitoes were transferred from the HITSS into individual control and treatment cages. Six replicates were performed for each chemical treatment concentration and mosquito strain.

Spatial Repellency Assay (SRA). The design of the SRA consists of one clear cylinder and two metal chambers (Fig. 2B). Each metal chamber contains either insecticide-treated netting (treatment) or netting treated with acetone solvent only (control). The clear cylinder is positioned between the two metal chambers and attached to each using linking sections with a butterfly valve. Twenty mosquitoes were introduced into the clear central cylinder using a mechanical aspirator. After a 30-s acclimation period, both butterfly valves were simultaneously opened. After 10 min, both valves were closed and the number of mosquitoes in each of the metal chambers counted along with the number knocked down in each metal chamber and clear cylinder. At the end of each replicate, mosquitoes were aspirated from all chambers and the assembly partially disassembled to allow ventilation of potential chemical saturation. Nine replicates were performed for each chemical treatment concentration and mosquito strain.

Toxicity Assay (TOX). The assembly configuration for this assay consists of individual metal chambers only (control and treatment) fitted with an end cap and funnel section (Fig. 2C). Each metal chamber holds a treated netting strip (insecticide or control) as used for the CIA and SRA. Twenty mosquitoes were introduced into each metal chamber, as previously described. After 1-h exposure, the number of knocked down mosquitoes was recorded from each metal

Table 2. Percentage of 24-h mortality of *Aedes aegypti*[a] strains after exposure to diagnostic concentrations of α-cypermethrin, deltamethrin, permethrin, and DDT

Chemical (concentration)[b]	Strain	No. mosquitoes[c]	% mortality[d] (mean ± SE)
α-Cypermethrin (0.05%)	Chiang Mai	75	100a
	Kanchanaburi	100	98.0 ± 1.16a
	Khonkaen	100	100a
	Nonthaburi	99	85.7 ± 4.05b
	Songkhla	100	91.0 ± 1.91b
	Satun	97	98.0 ± 2.17a
Deltamethrin (0.05%)	Chiang Mai	99	100a
	Kanchanaburi	98	100a
	Khonkaen	98	100a
	Nonthaburi	100	100a
	Songkhla	98	98.0 ± 1.54b
	Satun	97	100a
Permethrin (0.25%)	Chiang Mai	100	98.0 ± 1.15a
	Kanchanaburi	100	9.00 ± 3.42d
	Khonkaen	97	38.3 ± 9.66c
	Nonthaburi	100	5.00 ± 1.91d
	Songkhla	99	72.6 ± 3.20b
	Satun	96	65.4 ± 6.46b
DDT (4%)	Chiang Mai	99	37.2 ± 5.54a
	Kanchanaburi	100	2.00 ± 2.00b
	Khonkaen	100	3.00 ± 1.00b
	Nonthaburi	100	0b
	Songkhla	100	0b
	Satun	100	0b

[a] F_1-F_2 females, 3- to 5-d-old nonblood-fed, sugar starved 24 h before assay.

[b] WHO-recommended diagnostic concentration.

[c] Number of mosquitoes tested in four replicates of 25 each.

[d] Different letter designations (a, b, c, d) following percentage of mortality indicate a statistical difference ($P < 0.05$) between mosquito strains within each chemical tested.

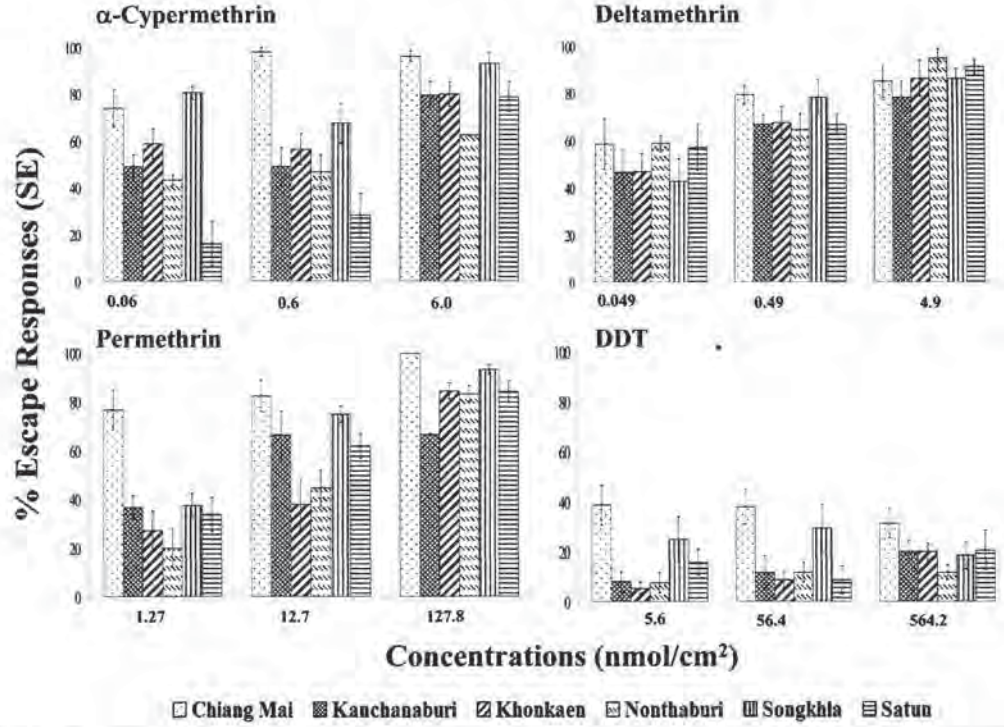

Fig. 3. Percentage of escape responses (with error bars) of six *Ae. aegypti* field strains in the CIA to three concentrations of α-cypermethrin, deltamethrin, permethrin, and DDT.

chamber, and then all specimens (knocked down and those still mobile) were transferred to individually labeled control and treatment holding cups and provided a cotton pad soaked with 10% sugar solution. Holding cups were maintained at 25°C and 80% RH, and mortality was recorded after 24 h. Six test replicates were performed for each chemical treatment concentration and mosquito strain.

Data Analysis. The analysis and interpretation of susceptibility test findings were determined according to World Health Organization criteria (WHO 1998b). The results were divided into three categories, as follows: 1) susceptible (percentage of mortality from 98 to 100%); 2) incipient insecticide resistance (percentage of mortality from 80 to 97%); and 3) resistant (percentage of mortality <80%). If mortality exceeded 20% in the control test, the test trial was rejected. If mortality in the control was above 5%, but below 20%, the results were corrected using Abbott's formula (Abbott 1925). Data from the HITSS assay were analyzed, as previously described (Grieco et al. 2005). CIA data were analyzed using the Wilcoxon two-sample test (Proc NPARI WAY, SAS Institute 1999) to examine the differences between the number escaping from treated and control chambers. For SRA data, a spatial activity index (SAI) was used to evaluate the repellent responses of mosquitoes. For each experimental replication, the SAI = $(N_c - N_t)/(N_c + N_t)$, where N_c is the number of females in the control chamber of the SRA device and N_t is the number of females in the treated chamber. The SAI values range from −1 to 1, with 0 indicating no response. SRA data were analyzed by a nonparametric Wilcoxon signed rank test (SAS Institute 1999) to determine whether the mean SAI for each test treatment was significantly different from zero. For toxicity data, percentage of knockdown and mortality was corrected using Abbott's formula and transformed to arcsine square root for analysis of variance. Mortality data derived from each treatment concentration were compared and separated using Tukey's honestly significant difference test at $P = 0.05$ (SAS Institute 1999).

Results

WHO Susceptibility Test. No mortality was recorded in the untreated controls over a 24-h holding period for all paired tests (Table 2). Four strains, Chiang Mai (100.0%), Khonkaen (100.0%), Kanchanaburi (98.0%), and Satun (98.0%), were found susceptible to α-cypermethrin, with incipient resistance/tolerance observed from Nonthaburi (85.8% mortality) and Songkhla (91.0% mortality) areas. Evidence of physiological resistance to permethrin was seen in all strains (5.0–72.6%), except Chiang Mai (98.0%). All six strains were highly susceptible to deltamethrin (98.0–100.0% mortality), whereas strong resistance to DDT was observed in all (0–37.2% mortality) (Table 2).

CIA. Escape responses of *Ae. aegypti* varied dramatically depending upon the test population (Fig. 3).

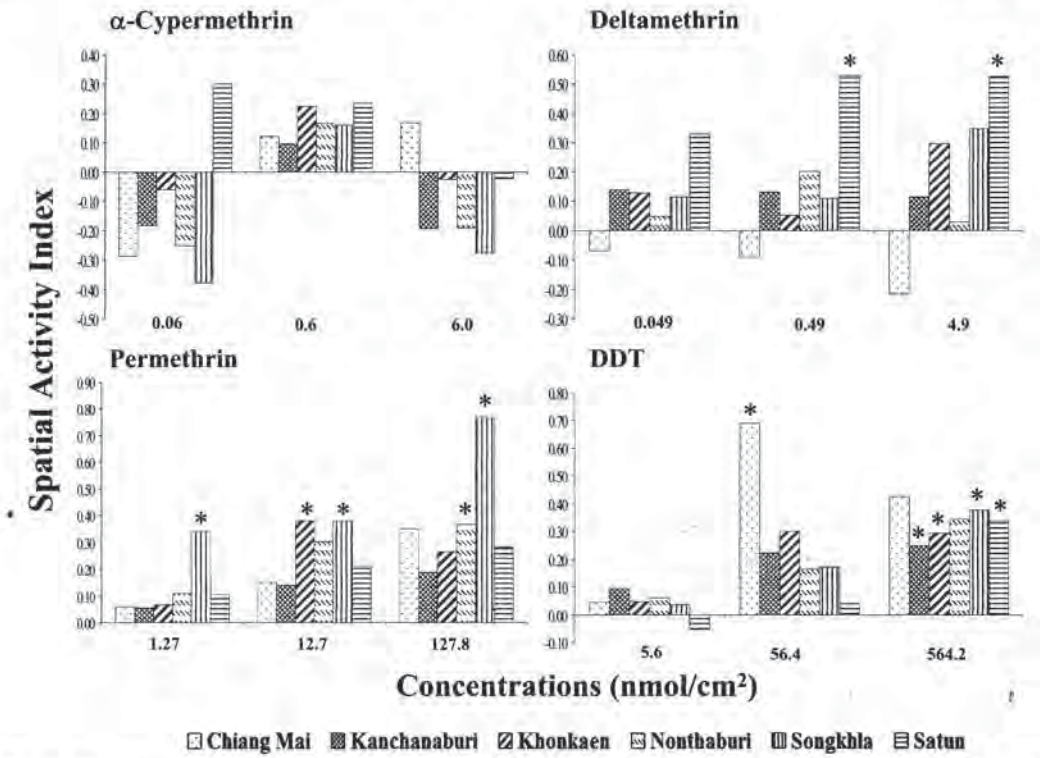

Fig. 4. Spatial repellent responses of six *Ae. aegypti* field strains to three concentrations of α-cypermethrin, deltamethrin, permethrin, and DDT. *, Denotes statistically significant (signed rank test, $P < 0.05$) repellent response compared with matched controls.

In general, there was a significant association between percentage of escaping and insecticide concentration in all strains against each of the three pyrethroids ($P < 0.05$). Statistical significance in percentage of escaping was also seen when each treatment concentration was compared with the matched control ($P < 0.05$). With DDT, no significant difference in percentage of escaping females was seen between the three different concentrations and regardless of test populations ($P > 0.05$).

SRA. Repellent responses of *Ae. aegypti* fluctuated among treatment concentrations and test compounds (Fig. 4). No statistical difference in spatial repellent response was observed in any test strains against all three concentrations of α-cypermethrin compared with controls ($P > 0.05$). At lowest and highest concentrations, this chemical actually resulted in negative SAI values in a high majority of test strains. In contrast, significant spatial repellent responses were observed with deltamethrin at 0.49 and 4.9 nmol/cm^2 in the Satun strain ($P < 0.05$). Permethrin elicited significant responses with all three test concentrations in the Songkhla strain and with 12.7 nmol/cm^2 in the Khonkaen strain, and at greatest concentration 127.8 nmol/cm^2 in the Nonthaburi strains ($P < 0.05$) (Fig. 4). Permethrin was the only test compound to produce a significant spatial repellent response at the lowest concentration (1.27 nmol/cm^2). A significant spatial repellent response was documented at the highest concentration of DDT (564.2 nmol/cm^2) in all test strains except Chiang Mai; however, this strain did show significant repellency at a 10-fold lower concentration (56.4 nmol/cm^2). Nevertheless, Chiang Mai mosquitoes showed movement away from DDT-treated netting with positive SAI values at all concentrations.

TOX. Percentage of mosquito mortality to all four chemicals was associated with increased chemical concentration. All three pyrethroids at all three concentrations were found highly toxic to most test mosquitoes, whereas DDT showed comparatively much lower toxicity in all strains (Fig. 5). The lowest concentration of deltamethrin showed higher rates of knockdown (59.6–92.4%) compared with α-cypermethrin (34.3–86.0%) and permethrin (5.53–98.2%) at the equivalent lowest test dose for each chemical. Although low knockdown occurred at the two lower concentrations of permethrin, high mortality rates resulted. The highest concentrations of α-cypermethrin, deltamethrin, and permethrin resulted in nearly 100% knockdown within the 60-min exposure time, ranging from 91.6 to 100%, 94.3 to 100%, and 96.8 to 100%, respectively. Overall, the lowest and highest rates of knockdown at all treatment concentrations of these three pyrethriods were observed in the Satun and Chiang Mai strains, respectively, with exception at the 6.0 nmol/cm^2 dose for α-cypermethrin, in which the

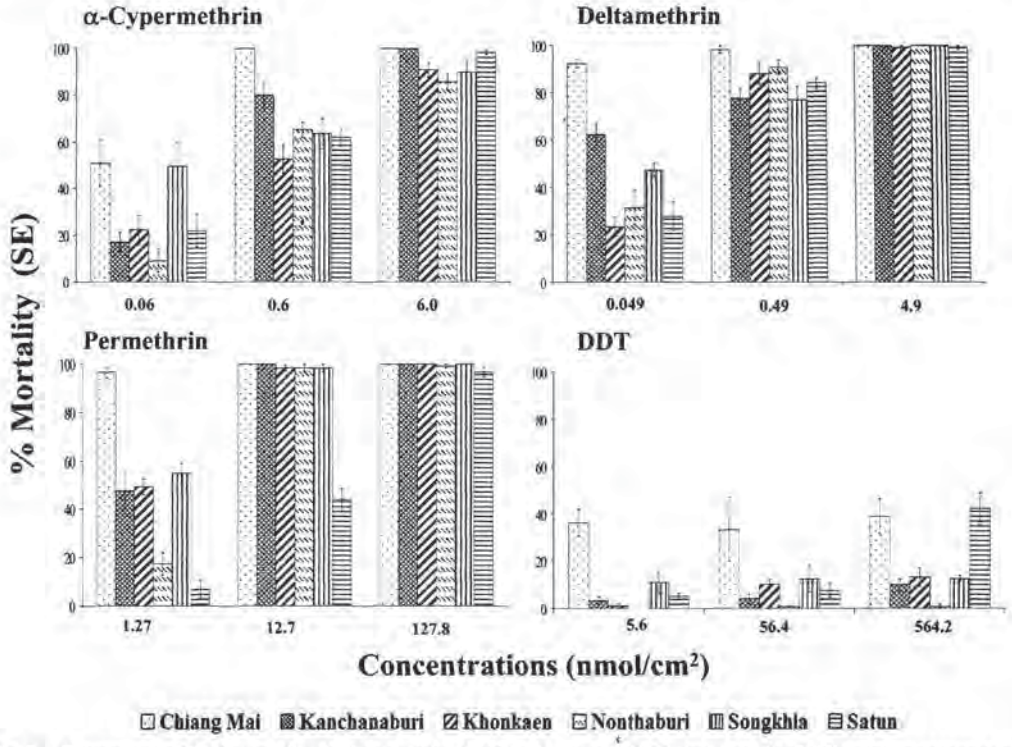

Fig. 5. Percentage of 24-h mortality (with error bars) of six *Ae. aegypti* field strains in the TOX to three concentrations of α-cypermethrin, deltamethrin, permethrin, and DDT.

highest knockdown was observed in the Kanchanaburi mosquitoes. DDT gave consistent, but modest levels of knockdown at all treatment concentrations for all test strains.

Discussion

This study clearly demonstrated that the majority of field-collected *Ae. aegypti* from northern, eastern, northwestern, and southern parts of Thailand exhibited comparatively high levels of resistance to permethrin (Table 2). Recently, several other strains of *Ae. aegypti* across Thailand have been found resistant to permethrin (Ponlawat et al. 2005, Jirakanjanakit et al. 2007). This apparent widespread resistance pattern could be related to the common use of commercial aerosol pest control products in many Thai households that contain permethrin or closely related synthetic pyrethroids and the wide distribution of pyrethroid-impregnated bed nets for mosquito control (Chareonviriyaphap et al. 1999, Ponlawat et al. 2005, Sathantriphop et al. 2006, Thanispong et al. 2008). Most strains were susceptible to α-cypermethrin with evidence of incipient resistance in the Nonthaburi strain and Songkhla in southern Thailand. In contrast, all *Ae. aegypti* test strains in the current study were found to be susceptible to deltamethrin, suggesting that this compound remains highly effective for control of adult *Ae. aegypti*. This result is in contrast with Yaicharoen el al. (2005), who reported physiological resistance to deltamethrin in several strains of *Ae. aegypti* from the Bangkok area. The reason for this is unclear, but it could be originated from the differences in the *Ae. aegypti* strains and locations. Moreover, ultra-low-volume applications of deltamethrin have been used commonly to attempt interruption of dengue virus transmission in urban areas. In all six strains, DDT resistance was markedly high.

The six *Ae. aegypti* field strains exposed to the four insecticides using the HITSS showed variable behavioral responses depending on type of exposure (CIA, SRA, and TOX), insecticide, and chemical concentration. In general, all *Ae. aegypti* strains demonstrated significant contact irritancy responses (Fig. 3). DDT elicited lower contact excitatory effects and similarly across all three concentrations, whereas significant spatial responses were mostly confined to the highest test concentration (i.e., near the field application rate of 2 g/m^2). All three pyrethroid compounds produced far higher toxicity than DDT in all strains with higher mortality associated with increasing concentration. These results conform closely to those of Grieco et al. (2007), showing that contact irritancy is the primary action of pyrethroid compounds and spatial repellency is the predominant endpoint effect of DDT on mosquitoes.

Unlike toxicity, certain insecticides possess the combined behavioral effects of irritancy and repellency, resulting in significant excitatory effects that stimulate mosquitoes to avoid insecticide-treated sur-

faces before receiving a lethal dose. Compared with irritancy, spatial repellency has been recognized as generally a more subtle, but nonetheless powerful action on those mosquitoes able to detect components of insecticides in a column of air without making direct contact-treated surface. The actual behavioral mechanisms behind these apparent undirected movements away from treated areas (e.g., orthokinesis, klinokinesis) as the result of exposure to strong locomotor stimulants remain unresolved. Irritant and repellent properties can operate in different ways to interfere with disease transmission. Repellents can disrupt normal behavioral patterns that prevent house entry, thereby reducing human-vector contact (i.e., blood feeding and pathogen transfer) and reducing the probability of developing resistance to the chemical (Lockwood et al. 1984). However, the benefit that contact irritancy contributes to disrupting disease transmission depends on the amount of time a mosquito vector spends inside a treated structure; the more profound the excitatory effect, the greater the likelihood blood feeding will be inhibited (Elliott 1972).

Our results show that *Ae. aegypti* strains found resistant to permethrin or α-cypermethrin continued to produce significant contact irritant responses at the lowest test concentrations. Therefore, lower doses than typical field application rates may be as operationally effective to reduce disease transmission risk. Likewise, high physiological resistance to DDT appears to have limited impact on suppressing spatial repellency in resistant *Ae. aegypti* strains. These results indicate the mechanisms of resistance in mosquitoes to counter the toxic actions of chemicals may differ significantly from those involved with irritancy and repellency.

Acknowledgments

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Human-Landing Patterns of *Anopheles dirus* sensu lato (Diptera: Culicidae) in Experimental Huts Treated with DDT or Deltamethrin

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Human-Landing Patterns of *Anopheles dirus* sensu lato (Diptera: Culicidae) in Experimental Huts Treated With DDT or Deltamethrin

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ABSTRACT *Anopheles dirus* females landing on humans inside experimental huts treated with residual applications of DDT or deltamethrin were observed during the wet season in Pu Teuy Village, Kanchanaburi Province, western Thailand. Two identical experimental huts were constructed in the fashion of typical local rural Thai homes. Pretreatment (baseline) human-landing collections (HLC) in both huts showed an early evening peak of activity between 1900 and 2000 h with no significant difference in numbers of mosquitoes captured between huts over a period of 30 collection nights. During posttreatment HLC, female mosquitoes continued to show greater landing activity inside huts fitted with insecticide-treated panels during the first half of the evening compared with the second half. A greater number (proportion) of *An. dirus* females landed on humans in the hut treated with deltamethrin compared with DDT. Comparing pre- and posttreatment HLC, the DDT-treated hut showed a 79.4% decline in attempted blood feeding, whereas exposure to deltamethrin resulted in a 56.3% human-landing reduction. An odds ratio was performed to demonstrate the relative probability (risk) of mosquitoes entering and attempting to blood feed in the two treated huts compared with untreated control huts. Mosquitoes were ≈0.47 times less likely to land on humans inside a DDT-treated hut compared with the deltamethrin-treated hut. Although both chemicals exerted strong excitatory responses, DDT appears to have a more pronounced and significant ($P = 0.002$) effect on behavior than deltamethrin, resulting in greater movement away from the insecticide source and thus potential reduction of blood-feeding activity.

KEY WORDS *Anopheles dirus*, behavioral response, experimental hut, deltamethrin, DDT

In Thailand, malaria remains a major and re-emerging health problem despite vector control programs that have been successful in reducing morbidity and mortality throughout much of the country (MPH 2006). Approximately 70% of the malaria cases are documented from the less developed border areas with eastern Myanmar, where efficient malaria vectors such as *Anopheles dirus* Peyton & Harrison are common (Scanlon and Sandhinand 1965, Kitthawee et al. 1990, MPH 2006). *An. dirus* s.l. is a well-known species complex of the *Anopheles leucosphyrus* group and closely associated with forest and forest-fringe habitats in Thailand (Baimai 1988). Unlike most species in the group that have infrequent contact with humans, a few members of *An. dirus* are regarded as excellent malaria vectors because of its highly endophagic and anthropophilic behavior. Natural high malarial infectivity rates (up to 13%) are not uncommon (Rosenburg and Maheswary 1982, Baimai et al. 1984). The most favored breeding habitats are shaded animal footprints, wheel-tracks, and temporary ground pools, making environmental control of this species particularly difficult. *An. dirus* larvae are occasionally found in water jars, cut tree stumps, and rot holes, adding to the problem of detection and control (Rattanarithikul et al. 2006).

An. dirus s.l. has shown varying behavioral responses to indoor use of insecticides (Ismail et al. 1974, 1975; Suwonkerd et al. 1990). In Thailand, indoor residual spraying (IRS) is routinely applied for interruption of human-vector contact and malaria transmission (Chareonviriyaphap et al. 1999, 2001). For many decades, DDT was the chemical of choice and

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was used extensively in most malaria-endemic areas in the country. Beginning in 1994, Thailand proceeded to replace DDT with synthetic pyrethroids for IRS to control malaria (Chareonviriyaphap et al. 1999).

Pyrethroids have been widely accepted for controlling disease vectors because of their relatively low mammalian toxicity and broad spectrum efficacy in controlling indoor mosquito populations (Elliott et al. 1978, Najera and Zaim 2002). Deltamethrin, a common synthetic pyrethroid, has been the mainstay for IRS to combat malaria transmission in Thailand (Pothikasikorn et al. 2005). However, like DDT, most pyrethroids exhibit strong excito-repellency in many mosquito species with avoidance of treated surfaces a common reaction (Roberts et al. 2000, Chareonviriyaphap et al. 2004, Pothikasikorn et al. 2005). "Excito-repellency," a composite term that has been in use for decades, is defined in this work in terms of an endpoint or multiple endpoints (i.e., avoidance and movement away from a treated surface or area because of excitatory effects caused by chemical exposure) involving a series of responses and one or a combination of behavioral mechanisms. Moreover, the use of the term "contact irritancy" is synonymous with excitation, whereas "avoidance" is more aptly applied to repellency. DDT and most pyrethroids act as strong locomotor stimulants in many mosquito species; however, the behavioral mechanism(s) for the physical displacement has not been determined and may be different for different species and compounds. As vector behavior is a critical component to malaria transmission (Elliott 1972, Pates and Curtis 2005), there have been numerous attempts to accurately measure avoidance responses and lethal effects of insecticides on mosquitoes using experimental huts (Smith 1965; Roberts et al. 1984, 1987; Roberts and Alecrim 1991; Chareonviriyaphap et al. 1997; Rozendaal et al. 1989; Bangs 1999; Grieco et al. 2000). Such information can help facilitate the choice of the most effective control methods.

In South Africa, Sharp et al. (1990) used experimental huts to compare responses of *Anopheles arabiensis* Paton after indoor spraying with either DDT or deltamethrin. They concluded that deltamethrin was preferable as it killed more mosquitoes, whereas DDT elicited a more profound escape response that caused more mosquitoes to exit the huts, thus escaping the toxic effects and surviving to potentially transmit malaria elsewhere. However, after the South African national malaria control program switched from DDT to deltamethrin, they witnessed malaria cases increase 4-fold in only 4 yr, a finding they attributed to pyrethroid-resistant *An. funestus* Giles captured exiting sprayed houses (Hargreaves et al. 2000). These same mosquito populations proved to be DDT susceptible, and a subsequent switch back to DDT helped to bring malaria quickly under control (Maharaj et al. 2005).

The South African experience illustrates that without a better understanding of the relationship between insecticide residues and mosquito behavior under natural settings, vector control strategies will continue to be hampered by poor knowledge of the actual chemical actions serving to prevent disease transmission. Response outcomes by malaria vectors to DDT and deltamethrin have previously been reported in Thailand (Ismail et al. 1974, 1975; Chareonviriyaphap et al. 2001, 2004; Sungvornyothin et al. 2001; Pothikasikorn et al. 2005; Polsomboon et al. 2008). Most work on the excitation and repellency of mosquitoes to insecticides has been conducted under laboratory-controlled conditions using an excito-repellency test system (Chareonviriyaphap et al. 2001, 2004; Sungvornyothin et al. 2001; Pothikasikorn et al. 2005), whereas fewer investigations have used field trials to evaluate the responses of *Anopheles* mosquitoes to insecticides using experimental huts (Ismail et al. 1975, Prasittisuk et al. 1996, Suwonkerd et al. 1997, Polsomboon et al. 2008). It remains difficult to extrapolate bioassay data on insecticidal effects (whether toxicity or excitation) and degree of protection against mosquito blood feeding inside a house or community-wide setting. Therefore, field studies are better suited to provide more direct evidence on the true biological and epidemiological impact of insecticides on disease transmission. The design of the experimental hut in carefully controlled field trials is a more objective way to obtain such information.

As very little has been documented on the indoor behavior of *An. dirus* females in response to either DDT or deltamethrin using experimental huts (Ismail et al. 1974, 1975), the results in this work are the first comparison of the behavioral responses of *An. dirus* to both DDT and deltamethrin using pre- and posttreatment observations.

Materials and Methods

Study Site. The study was conducted during the wet season at Pu Teuy Village, Sai Yok District, Kanchanaburi Province (14° 20′N, 98° 59′E, ≈300 m above sea level), western Thailand, ≈150 km northwest of Bangkok. The study site is located close to low hills and steep karst terrain, and is largely surrounded by either natural primary dense forest or small agricultural plots and commercial tree groves. The study site where the experiments are located is ≈800 m away from the nearest inhabited house. During the wet season in 2006 and 2008, there was a variety of preferred breeding sites for *An. dirus*, such as animal hoof prints and small, shaded fresh water pools. Indoor residual spraying using DDT had occurred routinely in the study area for many decades and was replaced with deltamethrin in 1994. IRS was discontinued in the area during the course of the investigation.

Insecticides. Two insecticides were compared, as follows: 1) deltamethrin ([S]-α-cyano-3-phenoxybenzyl [1R, 3R]-3[2,2-dibromovinyl]-2,2-dimethylcyclo-propanecarboxylate) (97% purity; this chemical was obtained from BASF [Mount Olive, NJ]); and 2) DDT (1,1′-[2,2,2-trichloroethylidene] bis [4-chlorobenzene]; 1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane) (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO), CAS 50-29-3, Product 386340.

Insecticide Susceptibility. The background physiological susceptibility of *An. dirus* to DDT (4%) and deltamethrin (0.05%) was assessed by exposing female mosquitoes to standard recommended discriminating concentrations applied on papers as recommended by WHO (1998, 2002). Impregnated papers (12 × 15 cm) were prepared in our laboratory using technical grade DDT and deltamethrin (Najera and Zaim 2002, WHO 2006). Additional filter paper was impregnated with 2 ml of acetone mixed with carrier (silicon oil) to serve as untreated controls. Batches of 25 nonblood-fed, sugar-starved, 3- to 5-d-old female mosquitoes were placed into respective holding tubes (control and treatment) and exposed to impregnated papers for 1 h. Immediately after exposure, the number of knockdown mosquitoes was recorded and all specimens were transferred into corresponding clean holding tubes provided with 10% sucrose cotton pads. Mortality was observed at 24 h postexposure, and results expressed as percentage mortality. Four replicates of paired control and treatment exposures were performed for each chemical.

Experimental Huts. Two identical experimental huts were used to study the entering and feeding behavior of *An. dirus.* Both huts had been previously used to evaluate the flight behavior of *Aedes aegypti* and *Anopheles minimus* (Chareonviriyaphap et al. 2005, Suwonkerd et al. 2006, Polsomboon et al. 2008). The huts were built using locally acquired materials following the design of Achee et al. (2005). Infrastructure consisted of sections of metal fence and custom-welded galvanized pipes for affixing the treated netting. Untreated wood planks, measuring 1 × 2.5 m, were joined together into panels measuring 1 × 3 m to serve as side walls. Floors were adjusted and aligned with cement blocks with an A frame-style zinc roof. The apex of the roof measured 3.5 m from the ground. The eaves on all four sides of the hut were sealed with 1/12-in aluminum wire mesh fastened across the eave opening. Huts were provided with three windows, one each per wall, and one door. To evaluate chemicals without having to apply compound directly to the wooden hut walls, a series of aluminum panels were designed for holding treated netting, which could be positioned on all interior wall surfaces of the hut (excluding floor and ceiling). The aluminum frame that secured the netting contained holes in each corner and were held in place to the hut walls using metal bolts. A 9-cm gap between the panel and the wood plank prevented the netting from touching the interior walls. The two huts were positioned 100 m apart on an open plot of land surrounded by forested terrain and agricultural fields.

Net Treatment. Polyester netting (a mesh size of 156 threads/square inch) was impregnated with either DDT at 2.0 g/m^2 or deltamethrin at 0.02 g/m^2 and prepared using acetone diluents following the method of Grieco et al. (2005, 2007). Three meter-square sections of netting were soaked with 18.6 ml of treatment solution in metal pans, and netting was kept completely submerged during the process. Additional net sections were treated with 18.6 ml of acetone only to serve as untreated controls. All nets were allowed to air dry for at least 60 min before use in the experimental huts.

Pretreatment Collection. Before commencing HLC, this study received formal human use approval from the Ethical Research Committee convened by the Research and Development Institute, Kasetsart University. Each study collector was offered antimalarial chemoprophylaxis to be taken voluntarily. Additionally, all collectors were afforded ready access to professional malaria diagnosis and immediate treatment if they contracted malaria during the course of the study.

Simultaneous indoor evening human-landing collections (HLC) were performed in two huts before treatment with insecticides to obtain baseline data on the temporal pattern of hut entry of *An. dirus.* The baseline collections also determined the comparability of the two huts with regard to *An. dirus* densities and hut entry patterns before treatment. Human mosquito collectors were divided into two teams of four persons each. Each hut had two collectors sitting inside with the first team collecting from 18.00 to 24.00 h, followed by the second team from 00.00 to 06.00 h. Treated netting (acetone only) was installed in both huts. All three windows and one door remained open during the period of collection to allow female mosquitoes to freely enter throughout the evening. HLC were conducted for 45 min with a 15-min break each hour. On each of the following nights of collection, collector teams were rotated by time period (first and second collection periods) and between huts to avoid collector bias. Each collector exposed both lower legs and captured all landing mosquitoes by mouth aspirator. Collected mosquitoes were retained in plastic holding cages labeled by hour and hut of collection and provided with cotton soaked with 10% sugar solution. Specimens were transferred to the field laboratory and morphologically identified (Rattanarithikul et al. 2006) the following morning. Specimens could only be identified to the level of *An. dirus* sensu lato. Hourly ambient indoor temperature and humidity were recorded during each hour of mosquito collection. Thirty consecutive collection nights were performed in each hut during June–July 2006.

Posttreatment Collection. For posttreatment collections, one hut served as the untreated control and the other hut served as the insecticide-treated structure. The interior of the treatment hut was lined with netting material treated with either 2 g/m^2 DDT or 0.02 g/m^2 deltamethrin. The control hut was lined with netting treated with acetone solvent only. The same procedures as used in the pretreatment collections were followed during posttreatment with one exception. To ensure no cross-contamination between chemical treatments (DDT or deltamethrin) used in the same hut, all aluminum panels and frames for holding treated netting were thoroughly cleaned by hand using a dilute blended detergent solution containing alkyl benzene sulfonate plus potassium salt (0.045%) and sodium lauryl ether sulfate (0.032%) (Teepol Pure, Sherwood Chemicals, Chachoengsao,

Thailand), followed by a careful wipe with 95% ethyl alcohol to remove any residual insecticide and cleaning solution from the surfaces. All three windows and one door remained open during the period of collection to allow female mosquitoes free entry into huts throughout the evening. Hourly ambient indoor temperature and humidity were recorded during each hour of collection. Thirty consecutive collection nights, per chemical, were performed in each hut. Collections in DDT-treated huts and controls occurred in August–September 2006, and for deltamethrin, collections occurred in June–July 2008.

Data Analysis. Pre- and posttreatment collection periods were grouped separately into four quarters by hour of collection: early evening (1800–2100 h), late night (2100–2400 h), predawn (2400–0300 h), and dawn (0300–0600 h). Comparison of control and treatment hut human-landing data was analyzed by nonparametric χ^2 statistic to determine whether captured number of mosquitoes was significantly different between huts. Univariate *t* tests (General Linear Model) were used for statistical multiple comparisons by collection period (four 3-h intervals: 1800–2100, 2100–2400, 2400–0300, 0300–0600) for estimation of differences in the percent reduction posttreatment HLC inside huts (control versus DDT and control versus deltamethrin) and HLC in each hut alone (DDT, deltamethrin, and untreated controls). All tests used SPSS software (SPSS version 15.0., Chicago, IL) with a discriminating level set at 0.05% ($P < 0.05$).

Correlation analysis (SPSS version 15.0., Chicago, IL) was used to investigate the association and estimated differences between number of human-landing *An. dirus* and the independent environmental variables ambient temperature, relative humidity, and rainfall recorded each hour. Mosquito and environmental data were aggregated into four 3-h time periods (1800–2100, 2100–2400, 2400–0300, and 0300–0600) during pre- and posttreatment collections in huts treated with DDT or deltamethrin with matched untreated control huts and environmental data. Pearson correlation coefficient (r) measurements were based on 48 collection aggregations during the period of observations in 2006 and 2008. The discriminating level for all tests was set at 0.05%.

A percent reduction statistic was used to compare the combined pretreatment HLC with posttreatment collections in the two huts and between chemicals as follows: $100 - (C_1 \times T_2 \div T_1 \times C_2)\ 100$, where C_1 = number of mosquitoes Hut-1 pretreatment; C_2 = number of mosquitoes control Hut-1 posttreatment; T_1 = number of mosquitoes Hut-2 pretreatment; and T_2 = number of mosquitoes Hut-2 posttreatment. A risk-odds ratio was performed to compare the probability (relative risk) of attempted feeding response by mosquitoes inside huts treated with DDT or deltamethrin compared with mosquitoes entering huts not exposed to a test chemical (Schlesselman 1982).

Results

Field-collected *An. dirus* s.l. females were exposed to the WHO-recommended discriminating concentrations of either DDT (4%)- or deltamethrin (0.05%)-treated papers to determine pretest susceptibility level to each compound. Adult female *An. dirus* (100 total) were found completely susceptible to both compounds after exposure to the respective diagnostic doses.

The temporal pattern of *An. dirus* human landing inside the experimental huts was observed during the rainy seasons in 2006 and 2008. From 30 pretreatment all-evening HLC, 768 and 836 *An. dirus* females were captured from huts 1 and 2, respectively. A prominent human-landing peak was seen between 1800 and 2100 h with a progressive decline in landing density thereafter. Statistically, the two experimental huts showed near equal probability of mosquitoes entering and landing on collectors in the two untreated huts ($\chi^2 = 4.139$, df = 3, $P = 0.247$).

DDT. In posttreatment trials, a combined 30 all-night HLC found *An. dirus* in the control hut was similar to that observed during pretreatment conditions (Table 1). However, an overall significant reduction in the number of *An. dirus* females was observed in the DDT-treated hut throughout the night (Table 2), with a more pronounced reduction in the number of mosquitoes collected during the first half (1800–2400 h) of the evening (Fig. 1). The lowest HLC density of female mosquitoes (23) occurred postmidnight (0000–0300 h). Landing mosquitoes during the early evening (1800–2100) were significantly greater ($P < 0.0001$) than all subsequent time intervals. A smaller, but insignificant peak was seen during the predawn collection (0300–0600) period (Table 2). A significantly higher overall number (766) of *An. dirus* females was captured in the untreated hut compared with DDT-treated structure (145) ($\chi^2 = 14.68$, df = 3, $P = 0.002$). Landing collections were also significantly different ($P < 0.001$) between DDT and the control hut across each 3-h time interval.

Deltamethrin. The temporal HLC pattern by *An. dirus* in the posttreatment control hut was found similar to that observed under prespray conditions (Table 1). Like DDT, a significant difference in number of overall mosquitoes landing on the collectors was observed between the control and deltamethrin-treated hut ($\chi^2 = 10.77$, df = 3, $P = 0.013$). Far fewer mosquitoes were collected from the deltamethrin-treated hut (360) than from the control hut (896), which was particularly noticeable in the early (first 3 h) evening HLC (Fig. 2). In both treated and control huts, there was a steady decrease in numbers collected as the evening progressed. The collection numbers in the treated hut during first half of the evening (1800–2400) were similar over the two time intervals, as were the numbers seen during the two second-half (2400–0600) collection intervals. All other time interval comparisons were statistically different from one another, including all quarterly collection periods compared with the control hut (Table 2). Landing collections

Table 1. Number of human-landing *Anopheles dirus* collected and aggregated into four time periods during 30 pre- and posttreatment collection nights each in huts treated with DDT or deltamethrin and matched (untreated) control huts

Chemicals	Hour of collection (% reduction in treated hut)				Sum (%)	% R[a]
	1800–2100	2100–2400	0000–0300	0300–0600		
Pretreat						
Hut 1	324	212	120	112	768 (47.9)	NA
Hut 2	350	233	154	99	836 (52.1)	NA
Posttreat						
DDT						
Hut 1	64	26	23	32	145 (15.9)	−79.39%
% R[a]	(−78.4)	(−85.1)	(−82.1)	(−68.2)		
Unsprayed						
Hut 2	320	192	165	89	766 (84.1)	
Deltamethrin						
Hut 1	120	121	65	54	360 (28.7)	
% R[a]	(−64.9)	(−45.7)	(−53.7)	(−41.1)		−56.26%
Unsprayed						
Hut 2	369	245	180	102	896 (71.3)	

[a] % reduction statistic = $100 - (C_1 \times T_2 \div T_1 \times C_2)$ 100, where C_1 = no. of mosquitoes Hut-1 pretreatment; C_2 = no. of mosquitoes control Hut-1 posttreatment; T_1 = no. of mosquitoes Hut-2 pretreatment; and T_2 = no. of mosquitoes Hut-2 posttreatment.

were significantly different ($P < 0.001$) between deltamethrin-treated and paired control hut across each 3-h time interval.

The impact of ambient temperature, relative humidity, and rainfall on landing collections throughout the night was analyzed for pre- and posttreatment collections and treated and control huts. Based on correlation analysis, no significant differences were seen for pre- and posttreatment collections and treated and control huts to account for differences in HLC collections. The number of *An. dirus* captured from the experimental huts in 2006 and 2008 was not significantly impacted by ambient temperature ($r = -0.013$, $P = 0.718$) and relative humidity ($r = 0.018$, $P = 0.636$). The total numbers of captured mosquitoes from wet season HLC were not significantly associated with total rainfall during each period (June–July 2006, $r = -0.065$, $P = 0.318$; August–September 2006, $r = 0.004$, $P = 0.955$; and June–July 2008, $r = -0.047$, $P = 0.472$).

An odds ratio statistic compared the relative probability of a mosquito to land on human collectors in the presence of either DDT or deltamethrin. Based on mosquitoes captured in the DDT-treated hut compared with deltamethrin, DDT appeared to reduce the likelihood of attempted blood feeding by a mosquito by more than half (odds ratio = 0.47), or conversely, *An. dirus* appeared >2 times less likely to land on a collector in a hut treated with DDT than deltamethrin. Overall, there was a 79.4% reduction in the number of *An. dirus* caught in the DDT-treated hut compared with the pre- and posttreatment control hut (Table 1 and Fig. 1) and a 56.3% reduction in the number of *An. dirus* collected from the deltamethrin-treated hut as compared with pre- and posttreatment HLC (Table 1 and Fig. 2). Comparing percent reduction during each of the four 3-h collection periods, DDT produced a consistently greater percent reduction each quarter compared with deltamethrin (Table 1) with a peak landing reduction for DDT in the second quarter (2100–2400 h) and for deltamethrin in the first quarter (1800–2100 h). When comparing the two insecticides, the DDT-treated hut produced a significantly greater reduction in overall mosquito-landing densities throughout the evening compared with its paired control than did deltamethrin ($P = 0.0023$).

Six paired time-interval comparisons of HLC percent reduction were made for DDT-treated hut versus control and comparisons in the treated hut only (Table 2). For treated versus control, only one time comparison was found not to be significantly different (2100–2400 versus 2400–0300, $P = 0.165$), whereas three paired time intervals in the treated hut alone were determined statistically similar. The same paired time interval comparisons were made for deltamethrin versus control and deltamethrin-treated hut only. The HLC percent reductions were significantly different for all six paired collection intervals for treated versus control. Within the treated hut, significant differences between time intervals were seen in four paired collection periods (Table 2). For combined DDT and deltamethrin collection intervals, only six of 24 comparisons were not found significantly different from one another; however, all but one was a pairing of sequential time intervals representing consecutive 6 h of collections.

Table 2. Statistical comparisons of posttreatment collection (3-h) intervals for determining differences in the HLC percent reduction inside huts compared with paired controls and treated huts alone

Time intervals	DDT vs control *P* value	DDT only *P* value	Delta vs control *P* value	Delta only *P* value
1800–2100 vs 2100–2400	0.000	0.000	0.000	0.924
1800–2100 vs 2400–0300	0.000	0.000	0.000	0.000
1800–2100 vs 0300–0600	0.000	0.000	0.000	0.000
2100–2400 vs 2400–0300	0.165	0.500	0.003	0.000
2100–2400 vs 0300–0600	0.000	0.590	0.000	0.000
2400–0300 vs 0300–0600	0.000	0.226	0.000	0.343

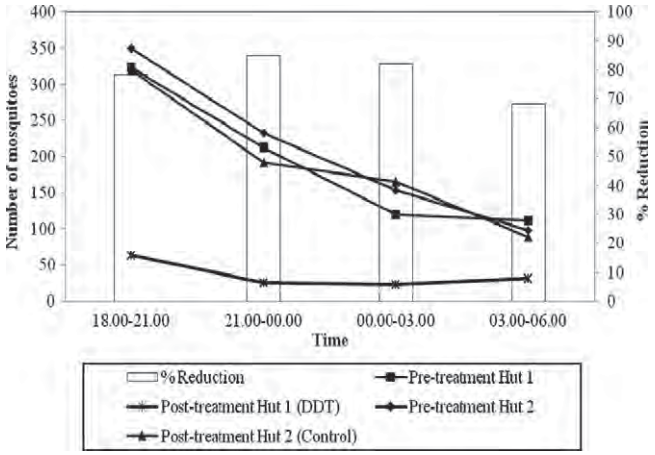

Fig. 1. Posttreatment number of *An. dirus* captured from human-landing collections during 30 collection nights at Pu Teuy Village inside a DDT-treated hut with matched (unsprayed) control hut.

Discussion

Our findings showed an unambiguous reduction in the number of female *An. dirus* attempting to blood feed in huts treated with either DDT or deltamethrin compared with pretreatment collections and matching posttreatment controls. *An. dirus* was less likely to land on humans inside a DDT-treated hut compared with the hut treated with deltamethrin, thus concluding that DDT appears to exhibit more pronounced excitatory-repellent effects and an overall greater reduction in human-landing activity, thereby translating to a lower risk of malaria transmission.

This study substantiates the strong excitation action of DDT reported for over 60 yr (Kennedy 1947, Tarzwell and Fisk 1947, Roberts et al. 2000). There was nearly an 80% reduction of *An. dirus* females collected in the hut treated with DDT compared with the matched untreated control. Hut studies with anopheline vectors in Belize C.A. drew similar conclusions showing DDT elicited strong excitatory and repellency responses (Bangs 1999, Grieco et al. 2000). Earlier work in Thailand found *An. dirus* females showed strong avoidance (repellency) of entering experimental huts treated with DDT (Suwonkerd et al. 1990). Similar work of Roberts and Alecrim (1991) in Brazil observed that *Anopheles darlingi* females completely avoided or disappeared after experimental huts were sprayed with DDT.

Deltamethrin also dramatically decreased landing *An. dirus* populations from inside the experimental hut, albeit the temporal human-landing pattern was different from that of DDT. Although there was a gradual decrease in number of *An. dirus* throughout the night, the overall percent reduction was significantly less than that seen for DDT. Corroborative findings were reported with house entering and exit behavior of *Anopheles vestitipennis* Dyer & Knab after huts were sprayed with deltamethrin or DDT, respectively (Grieco et al. 2000). They found that a significantly greater proportion of female mosquitoes en-

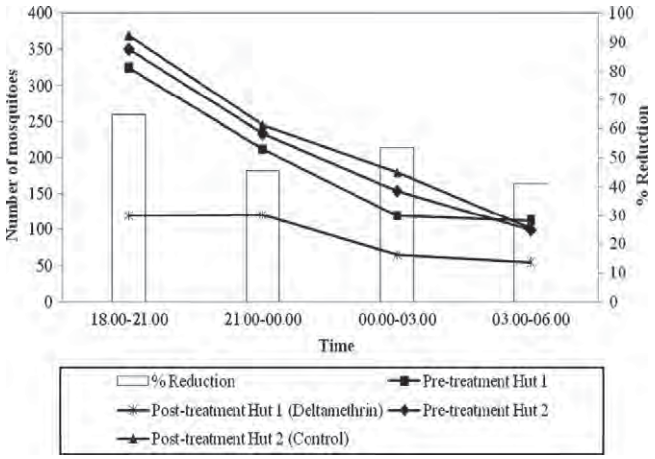

Fig. 2. Posttreatment number of *An. dirus* captured from human-landing collections during 30 collection nights at Pu Teuy Village inside a deltamethrin-treated hut with matched (unsprayed) control hut.

tered the hut treated with deltamethrin compared with DDT, indicating a more powerful spatial repellency function of the latter. There was a 97% reduction of mosquitoes entering a DDT-treated hut compared with only a 66% reduction with deltamethrin.

This study was not designed to determine the time of mosquito entry, but rather a measure of densities and time of landing on a host inside an experimental structure treated with either DDT or deltamethrin compared with a paired untreated control hut. Observations on indoor resting mosquitoes were not assessed; therefore, it is possible that host-seeking mosquitoes entering a hut during the early part of the evening might have rested on untreated surfaces (e.g., ceiling) for many hours before attempting to land on human collectors. The overall reduction in human-landing mosquitoes produced by both compounds could be a result of the combined effects of spatial repellency, contact excitation, and toxicity; however, use of HLC as outcome data alone cannot distinguish the importance of each effect.

The findings in this study are subject to several limitations. Using only morphological criteria for the identification of *An. dirus*, we were not able to distinguish between closely related sibling species in the Dirus Complex that occur in sympatry, particularly *An. dirus* and *Anopheles baimaii* Sallum and Peyton. Both species are regarded as efficient malaria vectors in Thailand and are common in Kanchanaburi Province (Rattanarithikul et al. 2006). Another weakness in the study design was not holding captured mosquitoes for a 24-h period to observe survival frequency between *An. dirus* captured in treated and untreated huts. Although we conclude that the combination of excitation and repellency is the primary reason for the significant reduction of *An. dirus* in HLC in the insecticide-treated huts, it is unclear what impact DDT and deltamethrin may have had in reducing mosquito longevity after contact with the toxicants.

Lastly, more accurate comparison between DDT- and deltamethrin-treated huts would have been facilitated had collections occurred at closer time intervals from one another. Although both measurement periods occurred during very similar periods of the year and observed climatic conditions were not significantly different, the collections were separated by a period of ≈2 yr. Our inability to properly correct for any subtle fluctuations in weather and differences in mosquito population densities between the two periods may have possibly influenced the comparisons. Therefore, we still caution any strict interpretation of our findings and actual differences between DDT and deltamethrin affecting indoor landing collections. However, we remain confident the percent reduction statistic for treated versus matched control and use of the odds ratio remain valid methods for comparison of different chemicals in measuring differences in landing mosquitoes inside treated huts. Whereas more accurate statistical comparisons between treatments and collections conducted during the same time period are available, nevertheless, contrasting odds ratios appears a valid alternative comparison measure between respective chemicals and temporal disparity in collections.

Environmental conditions during the observation periods can potentially confound the results and comparisons in hut studies. Ambient temperature, relative humidity, wind speed, and precipitation are among several external factors that can influence mosquito behavioral patterns and activity periodicities (Clements 1999). In this study, ambient temperature, relative humidity, and precipitation were not significantly different between study periods and appear not to have influenced differences seen in total number of mosquitoes captured during the three collection periods. This study was not designed to specifically investigate the impact of environmental variables on landing patterns of *An. dirus* in both treated and control huts during the two wet seasons. Many studies have shown clear associations between environmental conditions in the peridomestic environment playing a critical role by influencing mosquito flight activity, indoor and outdoor resting, and presumably host feeding. Better information on how climatic factors influence anopheline behavior would be useful when designing future experimental hut studies and serve as a baseline for the natural movement patterns of species when evaluating the response to test compounds.

To better understand the potential effects of an insecticide on mosquitoes, a probability model examining the repellent, excitatory, and toxic actions of chemicals and their function in the control of malaria by interrupting or preventing human-vector contact was developed (Roberts et al. 2000). From a control standpoint, excito-repellency was defined as the combined biological response of deterring mosquitoes from entering a sprayed house or causing mosquitoes to prematurely exit a house before blood feeding; this as a result of either direct contact and/or indirect detection (noncontact repellency) with an insecticide. This model has proven useful for guiding the testing of insecticides and interpretation of findings for preventing disease transmission.

Excito-repellency, although not technically a behavior as its usage may commonly indicate, involves a series of responses and behaviors that may vary from one insect/compound combination to another, but have similar outcomes, i.e., excitation leading to movement away from the source (Dethier et al. 1960). For example, the endpoint of movement away from the source is what is commonly measured using the excito-repellency escape chamber (Chareonviriyaphap et al. 1997, Pothikasikorn et al. 2005). Although these studies have surmised that the compound is detected through tarsal contact or by chemical molecules suspended in air, the actual behavioral mechanism for the diversionary movement remains unknown. We observed strong evidence of excitation caused by locomotor stimulants, DDT and deltamethrin; however, an ability to quantify the mechanism by which the physical movement away from a source occurs would be equally valuable (Fraenkel and Gunn 1940). Movement away from the DDT or deltamethrin might be the result of mechanisms such as orthokinesis (undi-

rected movement with increased speed), klinokinesis (increased turning rate with greater stimulus detected, and a straighter flight path when the stimulus decreases), or taxis (directed movement; for example, with respect to wind direction or visual cues). Behavioral analysis that specifically quantifies the type of movements made by *An. dirus* when in contact with these compounds and the precise cues involved would help to shed light on the behavioral mechanisms involved.

In this study, we introduced a new statistic, and to our knowledge, the first time the odds ratio has been used in the analysis and interpretation of experimental hut studies. We concluded that DDT reduced the likelihood (risk) of attempted mosquito blood feeding by more than half compared with mosquitoes in the presence of deltamethrin. In disease transmission terms, this would theoretically translate into an equivalent reduction in risk of infection. The rationale for its novel use is as follows. Our study was prospective in design, examining a select population cohort in time and space. Although experimental hut studies conducted under natural conditions cannot accurately define the population size under observation, we can nevertheless hypothesize and observe the influence of a single factor (i.e., insecticide exposure) and the probability of occurrence of a given outcome or response (i.e., attempted blood feeding). The assumption is that mosquitoes entering the untreated control huts were representative of the general population and comparable with respect to the frequency by which they would have been influenced by the presence of insecticide in treated huts. For analysis, we designated DDT as exposed and deltamethrin as the nonexposed comparison group. In the conventional epidemiological setting, prospective (or longitudinal) studies normally imply a large population under observation over a prolonged period of time. In our biological study, the population size in question was unknown, but was large enough to satisfactorily generate consistent and analyzable data. The 60 pre- and posttreatment evening collections were considered sufficiently robust to make confident conclusions.

In conclusion, we attribute the reduced number of mosquitoes captured in human landings to the excitatory effects of DDT and deltamethrin vice effects from other potential external factors such as environmental conditions at the time of study. Although not quantified in this study, other studies have found that this excitation commonly results in movement away from the treated area, yet the behavioral mechanisms for this movement are unknown. Ultimately, determining the behavioral mechanism or combination of mechanisms is fundamental to understanding how these chemicals work at sublethal concentrations for reducing human landings and movement away from treated huts. Whereas the data presented cannot define the nature or relative importance of each response, it does clearly demonstrate the effectiveness of DDT to deter normal indoor human-landing behavior by *An. dirus.* Whereas deltamethrin did not appear to cause as dramatic a reduction in the landing population as DDT, it still produced a significant decrease of *An. dirus* attempting to land on humans inside the huts compared with untreated controls. The variation in the landing patterns of *An. dirus* suggests that the two compounds may be operating differently regarding their respective chemical actions contributing to deliberate avoidance of treated surfaces-DDT primarily through spatial repellency and deltamethrin by contact excitation (irritancy).

This study demonstrates the importance of assessing the outcomes of excitatory-repellency effects under field conditions for a better understanding of how insecticidal chemicals function to control mosquitoes and reduce disease transmission risk. Further studies will be conducted using behaviorally active insecticides with use of entrance and exit traps involving collections of knockdown/killed mosquitoes indoors to determine the relative importance of each effect on the vector population, and whether it is primarily a single or combination of lethal and sublethal responses that results in the dramatic reduction of indoor landing activity.

Acknowledgments

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Feeding response of *Aedes aegypti* and *Anopheles dirus* (Diptera: Culicidae) using out-of-date human blood in a membrane feeding apparatus

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ABSTRACT: The colonization of *Aedes aegypti* and *Anopheles dirus* was performed using out-of-date human blood from a blood bank as a nutritional supply dispensed from a common artificial feeder. Preserved human blood was collected and used for feeding on days 5, 15, and 25 after date of expiration and dispensed from a common artificial feeder to rear the mosquitoes. *Ae. aegypti* had a feeding rate of 78.7, 62, and 18% at the respective intervals while *An. dirus* had a rate of 80, 56.8, and 7.3% on the same respective days. Direct feeding on live hamsters resulted in a rate of 96 and 90% for *Ae. aegypti* and *An. dirus*, respectively. Although egg production rates decreased from the day 5 feeding to the day 25 feeding, all of the developmental stages resulting from *An. dirus* fed at day 5 and 15 showed insignificant differences when compared with direct feeding on the blood of a hamster. ***Journal of Vector Ecology* 35 (1): 149-155. 2010.**

Keyword Index: *Aedes aegypti*, *Anopheles dirus*, colonization, out-of-date blood, artificial feeding, Thailand.

INTRODUCTION

The laboratory colonization of *Aedes aegypti* and *Anopheles dirus*, the main human vectors for dengue and malaria in Thailand, respectively, is of major importance for purposes of research, control, and experimentation. For most mosquito species, blood-feeding is essential for the production of viable eggs and blood-feeding on laboratory animals has some definite advantages. It has been reported that females of *Aedes* and *Anopheles* mosquitoes maintained on blood from live animals laid a higher number of eggs during their lifetimes and had greater longevity than those fed only sugar (Day et al. 1994, Straif and Beier 1996).

Various species of live animals, including mice, rats, gerbils, guinea pigs, hamsters, rabbits, chickens, non-human primates, and humans, have been used successfully to blood-feed mosquitoes (Nayar and Sauerman 1977, Klein et al. 1986, Canyon et al. 1999, Harrington et al. 2001, Braks et al. 2006, Xu et al. 2006, Xue et al. 2008). However, the use of live animals has many drawbacks, including pain and distress caused by blood feeding and additional/unsustainable expense for the maintenance of animals in the laboratory (Kasap et al. 2003). Moreover, there are evidences of accidental disease transmission and hypersensitivity to mosquito bites (Bailey et al. 1978). Evidently, it is somewhat effective, in terms of cost, safety and welfare of animals, to employ an artificial membrane feeding technique.

An artificial membrane feeding technique has been utilized for at least 40 years. The technique has been proven to be not only an effective method for delivering a blood meal to mosquitoes but has also been used to determine infection and transmission thresholds of viruses, *Plasmodium* spp., and filarial worms in the mosquitoes (Collins et al. 1964, Rutledge et al. 1964, Ponnudurai et al. 1971, Sattabongkot et al. 2003, Pothikasikorn et al. 2007). Various kinds of membrane have been applied in this technique, for instance, the skin of bat's wings, latex condoms, and paraffin films (Bailey et al. 1978, Wirtz and Rutledge 1980, Hagen and Grunewald 1990, Novak et al. 1991, Kasap et al. 2003, Rampersad and Ammons 2007). As for the blood meal, blood from various hosts, such as avian blood, rodent blood, mammalian blood, and stored human blood from blood banks have been used (Bunner et al. 1989, Nasirian and Ladonni 2006).

An effective storage system for human blood has been sought for the past 90 years (Hess 2006). At the present time, the common RBC storage solutions (anticoagulants) work well for blood banks, allowing three to seven weeks storage. There are many blood storage solutions or anticoagulant formulae in use from the blood banks of Thailand, for instance, acid citrate dextrose (ACD), citrate phosphate dextrose (CPD), (citrate phosphate dextrose plus adenine (CPDA-1), and citrate phosphate dextrose with additive solution (CPD-AS). However, the storage solution ACD is the one commonly used in many blood banks in hospitals throughout Thailand. This solution provides for 21 days of effective preservation of RBC. After day 21, RBC will lose some components and hemolysis may commence

(Sawant et al. 2007). Hence, the standard of 21 days is used to determine the expiration date of preserved human blood from blood banks. Since expired preserved human blood is discarded every day, it would be interesting to determine whether the two important vectors, *Ae. aegypti* and *An. dirus,* can be colonized using out-of-date preserved human blood supplied by a blood bank through an artificial feeding technique. This technique will provide an alternative blood source from the common practice of feeding the mosquitoes on live hamsters for purposes of colonization.

MATERIALS AND METHODS

Mosquitoes

This investigation used material from colonies of *Aedes aegypti* and *Anopheles dirus* that have been maintained under laboratory conditions at Kasetsart University, Bangkok, Thailand. Newly emerged adults were provided cotton pads soaked with 10% sucrose solution. Seven-day-old non-blood-fed *Ae. aegypti* females were used in this study. Due to the same difficulties in handling test specimens of *An. dirus,* only 12-day post-emergence females were used in the experiment. All females were sugar starved for at least 12 h before using (Rutledge et al. 1964).

Feeding apparatus and technique

Out-of-date preserved human blood was obtained from the blood bank of Ramathibodi Hospital, Bangkok, Thailand. The blood was stored in an ACD solution and refrigerated at 4° C until use. The blood was collected on days 5, 15, and 25 after the expiration date from the same blood bag and used as a feeding substrate. Ordinarily, a hamster from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Bangkok, Thailand, was used for blood feeding, strictly following the NLAC-MU guidelines.

Mouse skin was chosen as a membrane for the artificial feeding set-up due to its effectiveness and availability as previously described by Pothikasikorn et al. (2007). Briefly, the carcasses of mature mice obtained from the laboratory of the Microbiology and Biology Departments at the Faculty of Science, Mahidol University were skinned, the fur clipped, and all fat and connective tissues scraped or pulled off. The mouse skin was then frozen until use (Pothikasikorn et al. 2007).

The feeder apparatus was made of heat-resistant glass to which was attached a cylindrical water-jacket to contain the blood meal. The feeding component was set up and kept warm through the entry of tepid water from a lower inlet and its exit from an upper outlet connected to the water bath (Rutledge et al. 1964). The bottom of the cylinder had a circular lip over which the membrane could be securely tied with a rubber band to close the base of the feeder. Five ml of blood meal was placed inside the feeder. Plasticine was used to close the top of the tube of the chamber. Prior to feeding, the blood was warmed and maintained between 32-37° C in the cylinder of the feeder apparatus.

Feeding method

For each set of outdated preserved human blood, 150 females of each mosquito species were divided into three groups of 50 each. Each group was placed in a plastic bowl cage (6 cm high and 12 cm in diameter) covered with nylon mesh. Three feeders, each fitted with a mouse-skin membrane, were set up in tandem so that each one could be placed on top of a separate mosquito holding cage. Feeding tests were conducted on the mosquitoes from each cage with each session lasting for 30 min for *Ae. aegypti* and 60 min for *An. dirus* (Pothikasikorn et al. 2007). While mosquitoes fed, the artificial blood meal was periodically shaken to prevent precipitation of blood cells. The feeder temperature ranged from 32° to 37° C (mean 34.5° C) while the ambient temperature range was 23°-27° C (mean 25° C) and the relative humidity was 80%. Direct feeding on the hamster was done by placing the hamster in the 30 cm^3 stainless steel cage with batches of 50 female mosquitoes fed for 30-60 min. Due to ethical reasons and complicated processes, fresh human blood could not be obtained. Instead, the mosquitoes were fed on a hamster as controls.

Determination of developmental rates

After being fed, the mosquitoes were removed and examined to determine the feeding rate. The surviving female mosquitoes were transferred to the 30 cm^3 stainless steel cages and provided with 10% sucrose solution.

Three to four days after females were fully engorged, *An. dirus* deposited eggs on a 3 x 15 cm piece of filter paper lining a labeled 150 ml beaker containing 75 ml of clean water. Three to four days after feeding, *Ae aegypti* deposited their eggs on a 2.5 cm diameter piece of absorbent paper placed on cotton soaked with clean water inside a small plastic bowl (2.0 cm diameter and 1.5 cm high), and the number of eggs of both mosquito species were recorded to determine the oviposition rate.

One to two days after oviposition, the papers with eggs were removed from the cages and placed in a 22x35x7 cm pan half-filled with clean water and the number of larvae was recorded to determine the hatching rate.

Larvae were reared to the pupal stage in a 22x35x7 cm pan filled half-way with clean water and fed daily with ground rabbit chow for nutrition. The rate of pupation and adult formation were recorded respectively. Adults were provided a 10% sucrose solution on soaked cotton pads.

We subsequently compared the direct feeding on a hamster method and the expired human blood feeding method with each of the developmental rates using a one-way ANOVA – LSD throughout the study. Analysis was performed using the Statistix 9.0 Analytical Software (Tallahassee, FL, U.S.A.).

RESULTS

Ae. aegypti and *An. dirus* were reared using out-of-date human blood from a blood bank in an artificial feeding apparatus using a mouse skin as the membrane, collected on days 5, 15, and 25 after its expiration date. The feeding

Table 1. Feeding rate of *Ae. aegypti* and *An. dirus,* comparing direct feeding on hamsters to feeding on out-of-date preserved human blood. Percentages in each row followed by the same letter are not significantly different ($P \leq 0.05$) by one-way ANOVA – LSD.

Mosquito species	No. engorged females / total (%)			
	Direct feeding on live hamster	Human blood 5 d post-expiration	Human blood 15 d post-expiration	Human blood 25 d post-expiration
Aedes aegypti	144/150 (96.0)[a]	118/150 (78.7)[b]	93/150 (62.0)[c]	27/150 (18.0)[d]
Anopheles dirus	135/150 (90.0)[a]	120/150 (80.0)[b]	85/150 (56.7)[c]	11/150 (7.3)[d]

Table 2. Effect of feeding responses for outdated preserved human blood on the oviposition rate, hatching rate, and pupation rate to adult formation rates of *Ae. aegypti* and *An. dirus* in comparison to direct feeding on a hamster. Percentages in each column followed by the same letter are not significantly different ($P \leq 0.05$) by one-way ANOVA – LSD.

Control / outdated blood	No. eggs/engorged females (mean ± SD)		Sum of larvae (mean % hatching rate ± SD)		Sum of pupae (mean % pupation rate ± SD)		No. of adults (mean % adult formation rate ± SD)	
	Ae. aegypti	*An. dirus*	*Ae. aegypti*	*An. dirus*	*Ae. aegypti*	*An. dirus*	*Ae. aegypti*	*An. dirus*
Live hamster	2,211/144 (15.3 ± 1.1)[a]	2,400/135 (17.9 ± 1.1)[a]	1,419 (64.6 ± 6.2)[a]	1,172 (48.8 ± 0.1)[a]	654 (46.1 ± 2.6)[a]	630 (53.7 ± 3.2)[a]	417 (63.8 ± 0.6)[a]	488 (77.5 ± 0.7)[a]
human blood 5 d post-expiration	1,504/118 (12.7 ± 0.2)[b]	1,870/120 (15.6 ± 0.1)[a]	793 (52.7 ± 0.8)[b]	877 (46.9 ± 2.1)[a]	463 (58.3 ± 2.3)[b]	460 (52.5 ± 1.2)[a]	272 (58.6 ± 2.6)[a]	387 (84.1 ± 1.0)[a]
human blood 15 d post-expiration	932/93 (10.0 ± 0.0)[c]	745/85 (8.8 ± 1.5)[b]	484 (52.0 ± 1.0)[b]	345 (46.6 ± 2.6)[a]	266 (55.3 ± 6.0)[b]	180 (52.1 ± 1.1)[a]	167 (62.9 ± 7.9)[a]	150 (83.3 ± 0.0)[a]
human blood 25 d post-expiration	240/27 (8.8 ± 0.9)[c]	51/11 (2.8 ± 4.9)[c]	113 (47.9 ± 3.7)[b]	22 (43.1 ± 24.9)[b]	62 (54.3 ± 3.5)[b]	8 (12.1 ± 21.0)[b]	28 (43.1 ± 9.3)[b]	6 (25.0 ± 43.3)[b]

rates of *Ae. aegypti* and *An. dirus* significantly ($P \leq 0.05$) decreased as the out-of-date blood became older (Table 1 and Figure 1). For example, the feeding rate of *Ae. aegypti* on blood collected on day 5 after its expiration date was approximately four times greater than on blood collected on day 25 after its expiration date. Similarly, the feeding rate of *An. dirus* on blood collected on day 5 after its expiration date was approximately eleven times greater than on blood collected on day 25 after its expiration date (Table 1). Additionally, the rates of feeding on various ages of out-of-date blood were significantly lower than the rates of feeding on live animal controls for both mosquito species ($P \leq 0.05$).

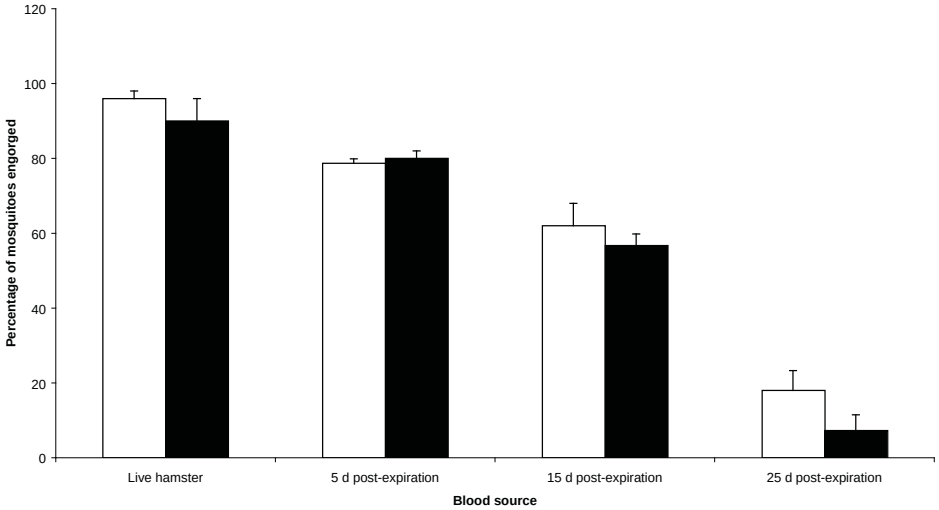

Figure 1. Effect of blood source on feeding rate of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

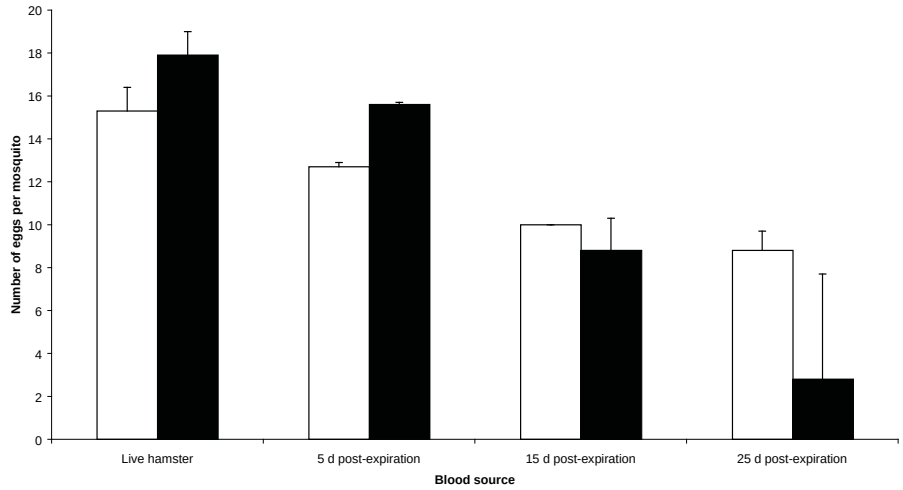

Figure 2. Effects of blood source on egg production of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

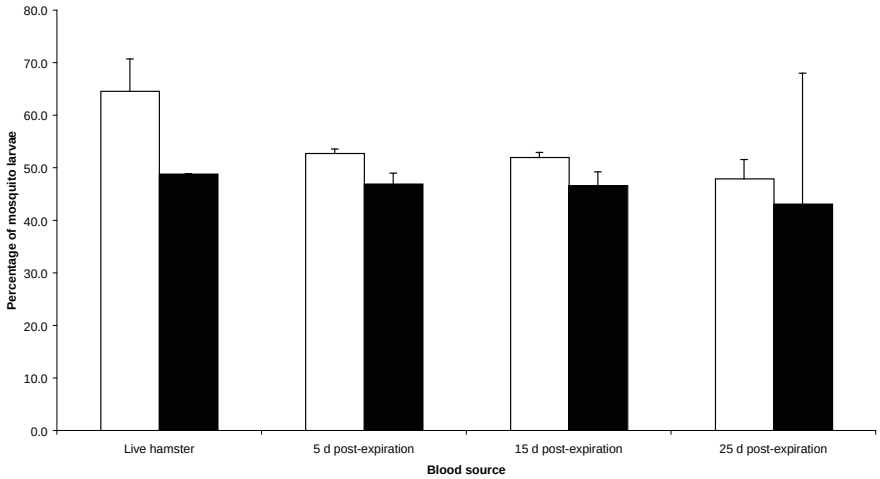

Figure 3. Effects of blood source on egg hatching for *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

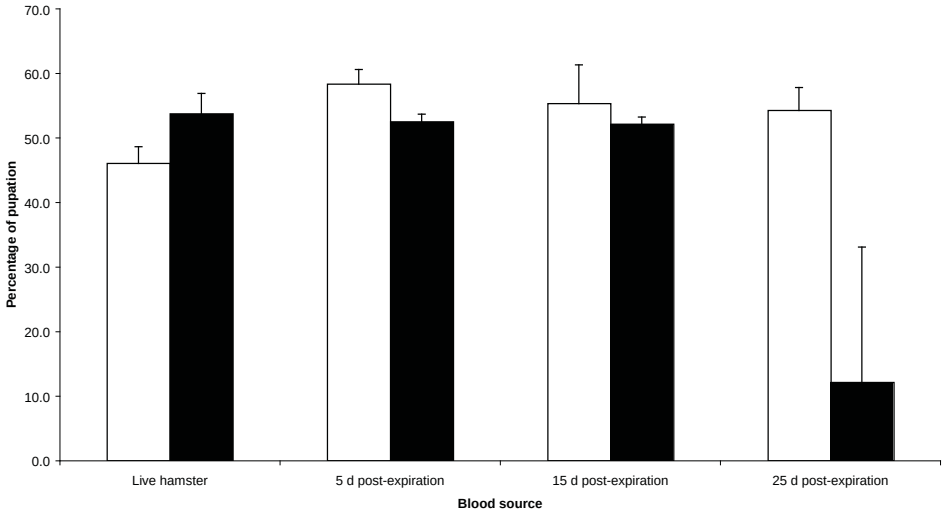

Figure 4. Effects of blood source on the pupation of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

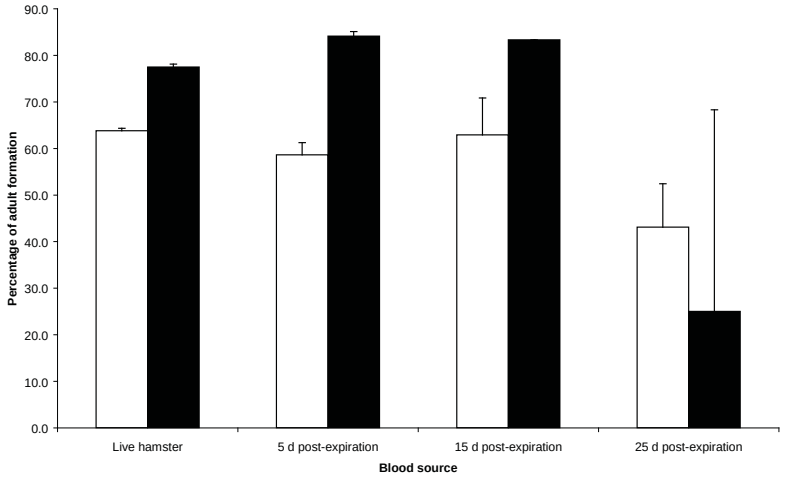

Figure 5. Effects of blood source on adult development of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

Feeding rates on live hamsters were 0.96 for *Ae. aegypti* and 0.90 for *An. dirus*, whereas rates were comparatively low on blood collected on day 25 after its expiration date for both species (0.18 for *Ae. aegypti* and 0.07 for *An. dirus*) (Table 1). No mortality was observed in test populations of both species reared under laboratory conditions.

Age of out-of-date blood appears to affect the fecundity rates of both species of mosquitoes as shown in Table 2 and Figure 2-5. The egg production rate of *Ae. aegypti* mosquitoes steadily decreased when fed on out-of-date human blood collected on day 5, 15, and 25 after expiration date, ranging from 12.7, 10.0, to 8.8 eggs per female, respectively, compared to 15.3 eggs per female in the control group (Table 2). The rates of hatching from egg to 1st instar larva of *Ae. aegypti*, when fed on days 5, 15, and 25 after blood expiration, still showed significant decreases when compared with the control ($P \leq 0.05$). In contrast, pupation rates (1st instar larva developing to pupa) were significantly higher in out-of-date blood fed groups than the control group ($P \leq 0.05$). While the group that fed on the most aged out-of-date blood (25 d after expiration date) showed a significant reduction in the adult development rate, there was no significant difference in such rate among mosquitoes fed on the more recently out-of-date blood compared to the control group ($P \leq 0.05$).

DISCUSSION

Although blood meals from live animals such as hamsters and guinea pigs have been used routinely in maintaining mosquito colonies in laboratories, their drawbacks include ethical issues and often unsustainable costs. This study has shown that recently expired stored human blood, normally discarded by blood banks, is an

alternative to live animal feeding.

The decrease in the feeding success on out-of-date blood of both mosquitoes shown in Figure 1 may be because the expired blood is less attractive as a food source to mosquitoes. It has been reported that stored RBCs become fractionated as they age, thus depleting essential substances such as adenine nucleotides, potassium, diphosphoglycerate (DPG), ATP stores, amino acids, and lipid (Hosoi 1959, Galun et al. 1984, Tinmouth and Chin-Yee 2001, Greenwalt et al. 1991, Hess 2006, Muller-Steinhardt et al. 1997, Gammon et al. 2000, Sawant et al. 2007, Hurd et al. 1995**,** Dimond et al. 1956, Briegel 1990, Scott et al. 1993, Harrington et al. 2001). Although the more recent out-of-date blood did not affect the fecundity of *An. dirus*, the decreases in nutritional components in aged out-of-date blood seemed to significantly decrease the fecundity rate of both species compared to the control group. This is probably due to the qualitative differences in host blood (hamster vs human blood) which would affect mosquito egg production (Nayar and Sauerman 1977). Spielman and Wong (1974) reported that the amino acid isoleucine was found in lower titers in human blood compared with other vertebrate species such as chicken and mice, and suggested that this might be a limiting factor in mosquito egg production. As confirmed by Harrington et al. (2001), *Ae. aegypti* may thus benefit from an accumulation of more energy reserves and a fitness advantage when they ingest human blood (low isoleucine) rather than rodent or chicken blood (high isoleucine).

Although the recent out-of-date bloods (5 and 15 d after expiration dates) seemed to reduce the hatching rates of *Ae. aegypti* mosquitoes (Figure 3), the rate of adult development was not affected by the out-of-date blood (Figure 5). More interestingly, the rate of pupation of this mosquito species fed on the recent out-of-date blood was significantly increased compared to the control group (Figure 4). As for *An. dirus* mosquitoes, the recent out-of-date bloods (5 and 15 d after expiration dates) did not appear to significantly affect rates of hatching, pupation, or adult development (Figures 3, 4, 5). A possible explanation may be related to the nutrition of female mosquitoes. Blood and sugar are essential for survival and reproduction in female mosquitoes (Briegel and Horler 1993, Foster 1995), and the recent out-of-date human blood could provide essential components required for reproduction and development.

In summary, it was found that all of the surviving female mosquitoes fed preserved human blood outdated by between 5 and 15 d displayed only slight differences in fecundity when compared to the controls. We therefore suggest that preserved human blood which is out-of-date by up to 10 d could be used as a source of nutrition to rear *Ae. aegypti* and *An. dirus* mosquitoes as an alternative to directly feeding on a hamster. These experiments show that the freshness of the blood showed no advantage over outdated preserved human blood samples provided that cell lysis had not occurred in the RBC during collection and storage.

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Population Structure of *Stomoxys calcitrans* (Diptera: Muscidae) From Nine Regions of Thailand

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Population Structure of *Stomoxys calcitrans* (Diptera: Muscidae) From Nine Regions of Thailand

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ABSTRACT Starch gel electrophoresis of isozymes was used to estimate gene flow among nine populations of *Stomoxys calcitrans* (L.) (Diptera: Muscidae) from Thailand. Of the 13 putative loci, nine polymorphic loci were detected. Limited genetic differentiation among populations was observed ($F_{ST} = 0.060$). The highest level of polymorphism was observed in flies from eastern Trat and northern Chiang Mai provinces (69.2%), whereas the lowest level of polymorphism was seen in flies from central Saraburi Province (23.1%). Gene flow between populations varied from 3.27 to 27.53 reproductive migrants per generation. Among the nine populations sampled, no correlation was seen between genetic and geographical distances showing that sampled *S. calcitrans* fit closely in the same cluster taxa. The electrophoresis of ten isozymes shows a genetic homogeneity of *S. calcitrans* populations at the scale of Thailand.

KEY WORDS *Stomoxys calcitrans*, genetics, isozyme, gene flow, Thailand

The genus *Stomoxys* (Muscidae: Stomoxyinae) contains at least 18 described species (Zumpt 1973). Both sexes of all species are blood-feeding insects and considered significant economic pests and disease vectors of livestock and other warm-blooded animals in many parts of the world (Bruce and Decker 1958, Zumpt 1973, Masmeatathip et al. 2006, Mullens et al. 2006). Among these flies, the cosmopolitan *Stomoxys calcitrans* (L.) is an aggressive, vicious biter, and will readily attack humans (Harwood and James 1979, Wall and Shearer 1997). This species can serve as mechanical or biological vectors of several important veterinary pathogens such as *Trypanosoma evansi* (Steel) or *T. vivax* (Ziemann) in horses, cattle, camels and dogs, several species of gastrointestinal *Habronema* nematodes of equines, and the filarial parasite of cattle, *Setaria cervi* (Rudolphi). Stable flies also have been implicated in the transmission of polio virus, equine infectious anemia, anthrax, and fowl pox (Greenberg 1971, Harwood and James 1979, Lehane 1991, Wall and Shearer 1997). Although most active near livestock, *S. calcitrans* can be a significant nuisance to humans on beaches and in residential areas near agricultural areas, with a typical flight range of ≈1–2 km from their origins (Jones et al. 1991).

In Thailand, five species have been identified in the genus *Stomoxys* with the most prevalent being *S. calcitrans* (Masmeatathip et al. 2006). *S. calcitrans* is found in many areas of Thailand, mainly in the central and northeastern regions (Sucharit and Tumrasvin 1981, Echeverria et al. 1983). Recent investigations have indicated that *S. calcitrans* is widespread throughout Thailand where domestic and wild animals are common (Masmeatathip et al. 2006; V.M., unpublished data). Wind-assisted active migration (appetitive flight) or passive wind-borne dispersion play a role in the movement patterns of *S. calcitrans* (Williams and Rogers 1976, Hogsette and Ruff 1985). Other findings suggest that greater movement over 1–3 km seems to be a normal characteristic of *S. calcitrans* (Eddy et al. 1962, Bailey et al. 1973). In addition, Hogsette and Ruff (1985) acknowledged a wind-assisted flight range of 225 km in Florida.

A better understanding of the biology of *S. calcitrans*, especially population genetics, is needed before initiating fly control activities. Knowledge of population structure can help estimate migration between/among different stable fly populations, provide insight into the epidemiology and transmission of pathogens, and support more responsive and effective fly control. In this study, we measured the genetic relationships among populations of *S. calcitrans* collected from nine locations of Thailand by using allele variation frequencies of isozymes.

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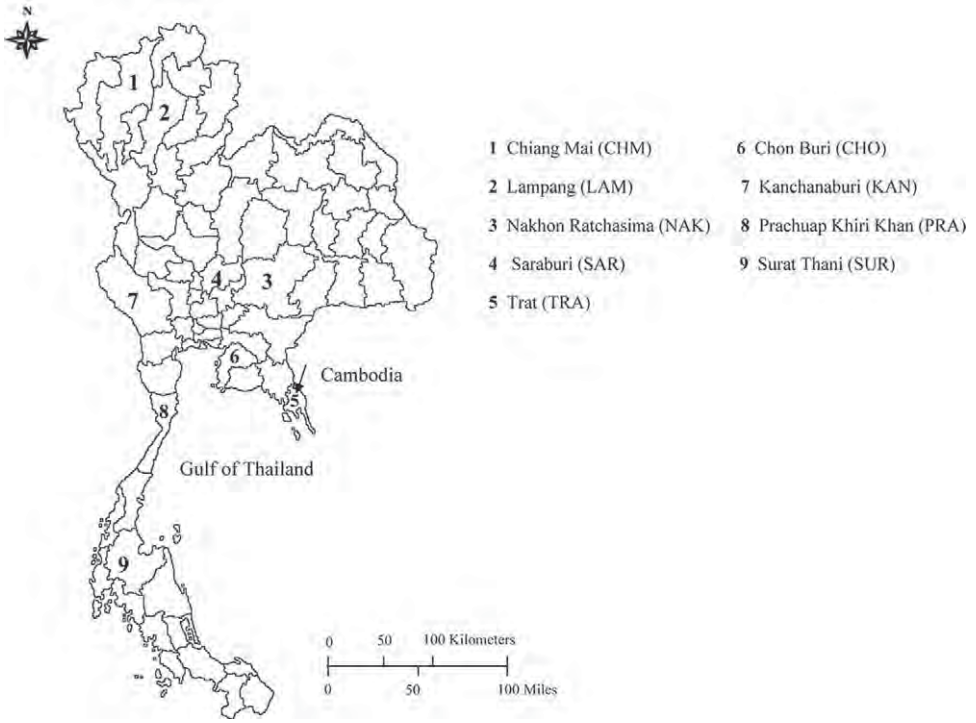

Fig. 1. Map of Thailand with the different provinces and sample sites.

Materials and Methods

Study Sites. *S. calcitrans* were collected from nine provincial regions of Thailand [North: Chiang Mai (CHM) and Lampang (LAM), Northeast: Nakhon Ratchasima (NAK), Central: Saraburi (SAR), East: Chon Buri (CHO) and Trat (TRA), West: Kanchanaburi (KAN) and South: Prachuap Khiri Khun (PRA) and Surat Thani (SUR)] (Fig. 1). To evaluate the genetic significance of the observed genetic difference among *S. calcitrans* populations, *Stomoxys uruma* (Shinonaga & Kano) also was included as an outgroup. *S. uruma* was collected from Nakhon Ratchasima (OUT), northeastern Thailand. GPS coordinates and a brief description of the locations are given in Table 1.

Collection Method. At each collection site, nine "Vavoua" traps (Laveissiere and Grebaut 1990) were placed on the ground near livestock, ≈10 m apart (0600–1800 hours). Collections of *S. calcitrans* occurred for at least two consecutive days at each site. Sample sizes are indicated for each population in Table 3. Flies were identified to species according to Zumpt (1973), and the abdomen was removed to avoid blood contamination. All specimens were brought back to the laboratory at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, and kept frozen in liquid nitrogen <1–3 d before further analysis. Strict segregation of specimens was maintained to prevent sample contamination between localities.

Starch Gel Electrophoresis. Horizontal starch gel electrophoresis was conducted following methods of Harris and Hopkinson (1976) and Manguin et al. (1995). Each fly was ground in 25 μl of grinding buffer

Table 1. Descriptions of stable fly collection sites

Pop	Reference point	Characteristics of collection sites
CHM	18° 48′ N, 98° 58′ E	Industrial dairy, Maejo University, San Sai District, Chiang Mai Province. Approximately 80 cows.
LAM	18° 17′ N, 99° 28′ E	The Thai Elephant Conservation Center, Thung Kwian forest park, Hang Chat District, Lampang Province. Approximately 100 elephants.
NAK	15° 0′ N, 102° 6′ E	Dairy, Wang Nam Khiao District, Nakhon Ratchasima Province. Approximately 40 cows.
SAR	14° 31′ N, 100° 52′ E	Industrial dairy, Dairy Farming Promotion Organization of Thailand, Muak Lek District, Saraburi Province. Approximately 200 cows.
CHO	13° 24′ N, 101° 0′ E	Khao Kheow Open Zoo established a Zoological Park (Khao Kheow Open Zoo) is located in Si Racha District, Chon Buri Province, eastern Thailand. There are ≈300 species, 8,000 animals in this zoo.
TRA	12° 13′ N, 102° 30′ E	Dairy, Bo Rai District, Trat Province. Approximately 20 cows.
KAN	14° 1′ N, 99° 31′ E	Dairy, Pu Teuy Village, Sai Yok District, Kanchanaburi Province. Approximately 25 cows.
PRA	11° 49′ N, 99° 47′ E	Dairy, Huai Sat Yai sub-district, Hua Hin District, Prachuap Khiri Khan Province. Aappro ximately 30 cows.
SUR	9° 8′ N, 99° 19′ E	Dairy, Mueang Surat Thani District, Surat Thani Province. Approximately 20 cows.

Table 2. Enzymes and loci used in starch gel electrophoresis on adult *S. calcitrans*

Enzyme system	E.C.[a]	No. loci[b]	Buffer
Acp (acid phosphatase)	3.1.3.2	1	Morpholine
Aox (aldehyde oxidase)	1.2.3.1	1	Morpholine
Mez (malic enzyme)	1.1.1.40	1	Morpholine
Mdh (malate dehydrogenase)	1.1.1.37	2	Morpholine
Pgm (Phosphoglucomutase)	2.7.5.1	1	Morpholine
Gpd (α-glycerophosphate dehydrogenase)	1.1.1.8	1	TMEDTA
Got (Glutamate-oxaloacetate-transaminase)	2.6.1.1	2	TMEDTA
Had (β-hydroxyacid dehydrogenase)	1.1.1.30	1	TMEDTA
Idh (isocritrate dehydrogenase)	1.1.1.42	2	TMEDTA
6-Pgd(6-phosphogluconate dehydrogenase)	1.1.1.44	1	TMEDTA
		13	

[a] Enzyme Commission number.
[b] Number of scored bands per phenotype.

(0.605 g of Trizma base, 0.02 g of EDTA, 0.005 g of NADP, and 50 ml of H_2O), and the homogenate absorbed into two 0.4- by 1.4-cm cellulose polyacetate wicks (Gelman Sciences Inc., Ann Arbor, MI). Ten enzyme systems were analyzed with two different buffer systems: morpholine (Morph) and Tris-malate-EDTA (TMEDTA) (Pasteur et al. 1988), run for 6 h at a constant 16 V/cm, stained and incubated at 37°C for 15–60 min (Table 2; Harris and Hopkinson 1976, Manguin et al. 1995). Loci were considered polymorphic when the frequency of the most common allele was <0.95. The migration distances of protein bands which are most close to the origin (cathode) were identified as the first locus, with the higher loci indicated by increasingly higher number. The most common allele was designated as 100 (Pasteur et al. 1988).

Data Analysis. Chi-square tests were performed to test for significant differences between observed and expected allelic frequencies between and among sampled populations. Analysis of allele frequencies, heterozygosity per locus, conformity to Hardy–Weinberg (HW) expectations and genetic distances were calculated using BIOSYS-1 (Swofford and Selander 1989). F_{IS} was used as a mean index of fixation of individuals relative to the total of subpopulations (Wright 1978). Differentiation among populations was evaluated with F-statistics (F_{ST}), a measure of the amount of differentiation among populations (Wright 1978). Effective migration rate (N_em) was estimated from the F_{ST} values as *Nem* (Wright 1978).

$$N_em = (1 - F_{ST})/4\,F_{ST} \qquad [1]$$

GENEPOP-3.1 (http://genepop.curtin.edu.au/) was used to estimate the degree of isolation by distance between localities (Raymond and Rousset 1995, Rousset 2008). This was computed by the relationship between pairwise estimates of F_{ST} and logarithms of geographical distance to determine whether geographical distance between populations serves as an effective barrier to gene flow by using SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

From our results, two different types of peptide chains were produced. The first was made from two peptide chains and known as dimer, a heterozygote with three bands. The second peptide chain was a monomer, the enzyme made of one polypeptide chain, whereas a heterozygote produces only two bands. From 10 enzyme systems, 13 putative loci were detected (Table 2). The number of polymorphic loci by populations was seven (CHM), eight (LAM), seven (NAK), three (SAR), nine (TRA), five (CHO), seven (KAN), seven (PRA), and six (SUR). Allele frequencies are presented in Table 3.

From 117 locus comparisons, 31 loci exhibited deviation from Hardy–Weinberg equilibrium ($P < 0.05$). The numbers of loci showing deviation from HW were six, five, four, four, four, three, two, and one in TRA, KAN, LAM, CHM, CHO, NAK, PRA, SUR, and SAR populations, respectively (Table 3). Higher percentages of polymorphic loci were observed in the CHM and TRA fly populations (69.2%) compared with the other seven populations, ranging from 23.1 to 61.5%. The greatest number of alleles per locus (2.5 ± 0.2) was observed in the TRA population and the greatest heterozygosity (Hobs = 0.161 ± 0.066) was observed in the KAN population (Table 4). The lowest variability was observed in the SAR population (23.1%), which had both low alleles per locus (1.9) and levels of heterozygosity (Hobs = 0.072 ± 0.032). Observed heterozygosities from all locations were not significantly different from the Hardy–Weinberg expected heterozygosities ($t_{0.025} = 2.120$, df = 16, $P = 0.09$) (Table 4).

FST showed an average value of 0.060. F_{IS} was 0.138 when all populations were analyzed (Table 5). Five loci, *Got-1, Got-2, Idh-1, Mez-1,* and *Pgd-1,* among a total of 13, demonstrated low heterozygosity, with F_{IS} values of 0.668, 0.673, 0.706, 0.696, and 0.585, respectively.

Gene flow among populations was calculated by estimating N_em where N_e is the effective population size and m is the migration rate between populations. Because m is the proportion of migrants (number of migrants/N_e), N_em is actually an estimate of the number of migrants independent of population size, still maintaining the present levels of genetic differentiation between sampled populations. Among all test populations, N_em estimated from F_{ST} (0.060) was 3.92, with a wide range of 3.27–27.53 (Table 6). The lowest gene flow was found between CHM and KAN, SUR, and KAN (3.27 migrants per generation), lower gene flow was found between LAM and KAN, CHO and KAN, SAR and KAN (3.59, 3.84, 3.85 migrants per generations, respectively), whereas the highest was observed between LAM and SUR (27.53 migrants per generation) (Table 6). An analysis among all populations indicated that there was no correlation between genetic and geographic distance among the nine populations of *S. calcitrans* ($r^2 = 0.014$, df = 35, $P = 0.493$) (Table 6). The phenogram (Fig. 2) shows that the nine sampled populations of *S. calcitrans* in Thailand occur

Table 3. Allele frequency and sample size (*n*) of nine collections of *S. calcitrans*

Locus		Allele	*S. calcitrans* pop								
			CHM	LAM	NAK	SAR	TRA	CHO	KAN	PRA	SUR
Acp-1	*n*		25	30	27[a]	43	30	33	19[a]	30	28
		75	0.000	0.000	0.130	0.174	0.017	0.106	0.105	0.050	0.000
		100	0.880	0.933	0.796	0.802	0.917	0.848	0.763	0.900	0.982
		195	0.120	0.067	0.074	0.023	0.067	0.045	0.132	0.050	0.018
	χ^2		0.381	0.113	8.627	2.435	0.195	0.935	27.065	0.304	0.000
Aox-1	*n*		30[a]	30	21	21	29	18	25	27	28
		76	0.150	0.083	0.024	0.024	0.017	0.278	0.000	0.037	0.000
		100	0.750	0.833	0.905	0.905	0.810	0.722	1.000	0.833	0.857
		128	0.100	0.083	0.071	0.071	0.172	0.000	0.000	0.130	0.143
	χ^2		15.005	5.164	0.171	0.171	2.737	0.111	—	1.412	0.670
Gpd-1	*n*		30	29[a]	27	43[a]	28	32	25	26	27
		64	0.000	0.034	0.019	0.000	0.018	0.000	0.040	0.000	0.000
		100	0.950	0.897	0.852	0.977	0.875	1.000	0.920	0.769	0.898
		142	0.050.05	0.069	0.130	0.023	0.107	0.000	0.040	0.231	0.111
	χ^2		0.055	57.145	1.274	85.012	0.482	—	0.139	2.115	0.346
Got-1	*n*		28[a]	30[a]	28	43	30[a]	32	25	30	28
		100	0.929	0.950	1.000	0.988	0.967	1.000	1.000	1.000	0.964
		148	0.071	0.033	0.000	0.012	0.000	0.000	0.000	0.000	0.036
		240	0.000	0.017	0.000	0.000	0.033	0.000	0.000	0.000	0.000
	χ^2		36.706	59.018	—	0.000	59.018	—	—	—	0.019
Got-2	*n*		30[a]	30[a]	28	43	30[a]	32[a]	25	30	28
		−100	0.900	0.967	1.000	0.977	0.967	0.938	0.900	0.983	0.982
		−273	0.100	0.033	0.000	0.023	0.033	0.063	0.100	0.017	0.018
	χ^2		35.457	59.018	—	0.000	59.018	42.034	3.687	0.000	0.000
Had-1	*n*		30	30	28	43	29[a]	33[a]	25[a]	27	28
		92	0.000	0.033	0.000	0.000	0.034	0.000	0.000	0.019	0.000
		100	0.933	0.950	1.000	1.000	0.931	0.970	0.960	0.889	0.875
		110	0.067	0.017	0.000	0.000	0.034	0.030	0.040	0.093	0.125
	χ^2		0.113	0.055	—	—	57.038	65.016	49.021	4.133	1.264
Idh-1	*n*		30	30	26[a]	43	29[a]	33[a]	25	28[a]	26
		86	0.000	0.000	0.019	0.000	0.034	0.000	0.000	0.000	0.000
		100	0.950	0.983	0.904	1.000	0.793	0.970	1.000	0.964	0.962
		108	0.050	0.017	0.077	0.000	0.172	0.030	0.000	0.036	0.038
	χ^2		0.550	0.000	34.043	—	88.800	65.016	—	55.019	0.020
Idh-2	*n*		26	27	28	43	29	33	25	28	28
		54	0.000	0.111	0.036	0.000	0.067	0.000	0.100	0.018	0.068
		100	1.000	0.889	0.964	1.000	0.900	1.000	0.900	0.982	0.932
		130	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000
	χ^2		—	0.346	0.019	—	0.117	—	0.242	0.000	0.899
Mdh-1	*n*		28	29[a]	27	43	30	32	25	24	22
		57	0.000	0.017	0.093	0.035	0.000	0.000	0.180	0.104	0.045
		100	0.964	0.931	0.907	0.965	1.000	0.969	0.820	0.896	0.995
		200	0.036	0.052	0.000	0.000	0.000	0.031	0.000	0.000	0.000
	χ^2		0.019	18.334	0.221	0.037	—	0.016	1.054	0.255	0.024
Mdh-2	*n*		7	11	9	43	16	17	25[a]	9[a]	10
		−100	1.000	1.000	0.889	0.942	0.906	0.882	0.580	0.944	1.000
	χ^2	−157	0.000	0.000	0.111	0.058	0.094	0.118	0.420	0.056	0.000
	n		—	—	0.067	0.130	0.111	0.221	12.414	0.000	—
Mez-1			30	29	27	43	30	30	25[a]	24	27
	χ^2	100	1.000	1.000	1.000	1.000	0.983	1.000	0.960	1.000	1.000
		111	0.000	0.000	0.000	0.000	0.017	0.000	0.040	0.000	0.000
			—	—	—	—	0.000	—	49.021	—	—
Pgd-1	*n*		30[a]	30	17	30	30[a]	20	25[a]	26[a]	30[a]
		54	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000
		100	0.883	0.983	0.972	1.000	0.900	1.000	0.960	0.885	0.933
		108	0.117	0.017	0.000	0.000	0.067	0.000	0.000	0.115	0.067
		155	0.000	0.000	0.000	0.000	0.033	0.000	0.040	0.000	0.000
	χ^2		9.429	0.000	—	—	67.797	—	49.021	12.188	8.816
Pgm-1	*n*		29	28	26[a]	43	30[a]	33[a]	23	19	27[a]
		43	0.086	0.000	0.000	0.000	0.017	0.030	0.022	0.000	0.000
		68	0.138	0.107	0.058	0.012	0.033	0.030	0.022	0.000	0.056
		87	0.000	0.000	0.038	0.012	0.000	0.000	0.087	0.000	0.000
		100	0.759	0.804	0.808	0.953	0.867	0.909	0.848	0.974	0.870
		135	0.017	0.089	0.096	0.023	0.083	0.030	0.022	0.026	0.074
	χ^2		6.920	3.037	17.023	0.076	29.278	65.124	10.704	0.000	17.175

[a] Deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

n, number of samples; CHM, Chiang Mai; LAM, Lampang; NAK, Nakhon Ratchasima; SAR, Saraburi; CHO, Chon Buri; TRA, Trat; KAN, Kanchanaburi; PRA, Prachuap Khiri Khun; SUR, Surat Thani.

Table 4. Genetic variability and mean heterozygosity at 13 loci of pooled samples of *S. calcitrans*

Collection site	Avg. alleles per locus (± SD)	% polymorphic loci[a]	Mean heterozygosity	
			H_{obs}	H_{exp}[b]
CHM	2.0 ± 0.2	69.2	0.102 ± 0.031	0.150 ± 0.038
LAM	2.3 ± 0.2	61.5	0.104 ± 0.030	0.125 ± 0.030
NAK	2.3 ± 0.3	53.8	0.115 ± 0.033	0.136 ± 0.037
SAR	1.9 ± 0.3	23.1	0.072 ± 0.032	0.069 ± 0.026
TRA	2.5 ± 0.2	69.2	0.107 ± 0.028	0.160 ± 0.029
CHO	1.8 ± 0.2	38.5	0.090 ± 0.041	0.105 ± 0.036
KAN	2.2 ± 0.3	53.8	0.161 ± 0.066	0.172 ± 0.044
PRA	2.1 ± 0.2	53.8	0.122 ± 0.037	0.135 ± 0.032
SUR	1.9 ± 0.1	46.2	0.121 ± 0.030	0.125 ± 0.028
Total			Avg.	0.131 ± 0.033
			$t_{0.025} = 1.310^{ns}$	

[a] Locus considered polymorphic when frequency of the most common allele < 0.95.
[b] Unbiased estimate and standard error (Nei 1978).

in a common genetic cluster as indicated by a low genetic distance (<0.017).

Linkage disequilibrium analysis was tested and showed significant differences in overall pairs of loci in which were departure from the Hardy–Weinberg equilibrium. In TRA population, linkage disequilibrium was significantly different in pairs of loci which departure from the Hardy–Weinberg equilibrium. For example *Aox-1*/*Idh-1* ($P = 0.0092$), *Aox-1*/*Pgd*-1 ($P = 0.0405$), *Gpd-1*/*Got-1* ($P = 0.0353$), *Gpd-1*/*Idh*-2 ($P = 0.0241$), *Gpd-1*/*Mez-1*($P = 0.0362$), *Got-1*/*Got-2* ($P = 0.0345$), *Got-1*/*Had-1*($P = 0.0342$), *Got-1*/*Mez-1*($P = 0.0326$), *Got-1*/*Had-1*($P = 0.0338$), *Got-2*/*Mez-1*($P = 0.0335$), *Had-1*/*Mez-1*($P = 0.0338$) and *Idh-1*/*Pgd-1*($P = 0.0013$).

Discussion

Insect population dispersal via passive and active movements is considered an important means of natural gene flow. This dispersal would influence the genetic structure and gene flow between *S. calcitrans* populations. Defining the population structure of stable flies in association with capacity to transmit diseases is of epidemiological importance and can assist predictive modeling and timely planning for allocation of insect monitoring and application of control. The temporal and spatial differences with respect to expression of enzymes that are associated with variability of vectorial capacity for disease pathogens may be influenced by the patterns of gene flow between and within populations. For example, a correlation exists between genetic distance and variation in the ability of *Aedes aegypti* (L.) to competently replicate and transmit dengue viruses (Bosio et al. 2000, Ocampo and Wesson 2004).

Table 5. F-statistics analysis of polymorphic loci in nine populations of *S. calcitrans*

Locus	F_{IS}[a]	F_{ST}
Acp-1	0.071	0.042
Aox-1	0.080	0.062
Gpd-1	0.027	0.050
Got-1	0.668	0.030
Got-2	0.673	0.029
Had-1	0.306	0.034
Idh-1	0.706	0.063
Idh-2	−0.124	0.059
Mdh-1	−0.050	0.051
Mdh-2	−0.341	0.176
Mez-1	0.696	0.027
Pgd-1	0.585	0.048
Pgm-1	0.096	0.036
Mean	0.138	0.060

[a] Inbreeding coefficient.
$F_{IS} = 1$, *total absence heterozygote*; $F_{IS} = -1$, total absence homozygote.

The genetic structure of *S. calcitrans* populations in Thailand has not previously been reported. The genetic variation and gene flow between and among nine different geographical populations of *S. calcitrans* were compared using isozyme electrophoresis. The percentage of polymorphic loci (69.2%) and mean heterozygosities (2.5) observed in this study were higher than those observed for populations in the United States (Jones et al. 1987, Szalanski et al. 1996). Several polymorphic loci (*Aox-1*, *Had-1*, *Got-1*, *Got-2*, and *Gpd-1*), in this study were not observed by those authors. However, our findings do correspond more closely to percentage of polymorphic loci and mean heterozygosity reported in stable fly populations by Krafsur (1993).

The low genetic variability in the SAR population (23.1%) may be due to several contributing factors; as example the frequent use of insecticides. The SAR population was collected from a large, industrial-size dairy farm with one to two applications per week of synthetic pyrethroid, for controlling flies. In addition, progressive urbanization and other human activities near the SAR site may have greatly reduced preferred ecological habitats and other animal hosts to sustain large fly populations with a potential significant impact on the genetic structure of *S. calcitrans* in the area. This finding differs with other studies on genetic differentiation of *Ae. aegypti* mosquitoes (Failloux et al. 1995, Lerdthusnee and Chareonviriyaphap 1999). In these cases, higher genetic variability was seen in populations associated with relatively open, more rural farming systems; and in which fly control activity is limited and preferred breeding habitats and hosts are more abundant.

Departure from the Hardy–Weinberg equilibrium occurred in six loci of the TRA population, indicating that mating is not random, and there is a reproductive isolation between individuals. Disequilibrium was due to lack of heterozygote in TRA population. This population was collected near the Cambodian border and this area is surrounded by steep mountains on the eastern side and by the Gulf of Thailand on the western side. *S. calcitrans* from this site exhibited significant deficiency of heterozygotes that could have arisen from a sampling bias. The Trat Province normally experiences prolonged rainy period (8–10 mo/yr). The relative geographical isolation, less conducive weather conditions and steep mountain may have reduced the reproductive success and may have resulted in a smaller, less diverse population.

Table 6. Above diagonal, geographic distance (kilometers); below diagonal, pairwise F-statistics and effective migration rate (N_em) of all (*S. calcitrans*) loci at nine collection sites

	CHM	LAM	NAK	SAR	TRA	CHO	KAN	PRA	SUR
CHM	—	92	777	625	1,011	777	824	977	1,340
LAM	0.014 (17.61)	—	687	535	914	680	727	880	1,243
NAK	0.029 (8.37)	0.018 (13.64)	—	152	399	280	387	540	903
SAR	0.038 (6.33)	0.029 (8.37)	0.018 (13.64)	—	380	201	235	388	751
TRA	0.020 (12.25)	0.017 (14.46)	0.017 (14.46)	0.033 (7.32)	—	234	443	596	959
CHO	0.024 (10.17)	0.029 (8.37)	0.031 (7.81)	0.029 (8.37)	0.034 (7.10)	—	209	362	725
KAN	0.071 (3.27)	0.065 (3.59)	0.037 (6.51)	0.061 (3.85)	0.055 (4.29)	0.061 (3.84)	—	295	658
PRA	0.029 (8.37)	0.024 (10.17)	0.020 (12.25)	0.034 (7.10)	0.018 (13.64)	0.043 (5.56)	0.060 (3.92)	—	364
SUR	0.025 (9.75)	0.009 (27.53)	0.026 (9.36)	0.039 (6.16)	0.016 (15.38)	0.047 (5.07)	0.071 (3.27)	0.018 (13.64)	—

Conversely, the significant deviation from Hardy–Weinberg equilibrium and heterozygosity deficiency seen in the KAN population could be the result of dispersal and migratory behavior as both larval habitats and animal hosts are readily available and no geographical barriers exist in this area. Passive transportation of immature stages in manure for agricultural use may play a significant role in population structure for the KAN fly population. This event is quite common in the agricultural area, especially Kanchanaburi Province, where manure from cattle is brought in and out once every 2 mo. Therefore, both active and passive movements offer possible dispersal pathways for *S. calcitrans* into and out of the KAN site.

Higher genetic diversity and average allele per locus were observed in TRA, NAK, and LAM fly populations compared with the other six population samples. The NAK population was collected from a small rural village in the Tub Lan National Park; similarly, the LAM population was collected from The Thai Elephant Conservation Center. These populations would be expected to be exposed to few, if any, population control activities (Sukonthabhirom et al. 2005).

The high value of F_{IS} (0.138) indicates high inbreeding within the subpopulation of *S. calcitrans.* The N_em value, derived from the F_{ST}, among all nine populations was 3.92 reproductive migrants/generation, which was lower than the N_em value obtained from stable fly studies in Nebraska (5.85) (Szalanski 1995), in Reunion Island (12.25) (Gilles et al. 2004), and in Gabon and France (2.3) (Dsouli et al. 2009). However, Szalanski (1995) reported that very few (<10) migrant stable flies are needed each generation to maintain genetic homogeneity among populations.

The unbiased genetic distances obtained indicate that there is no significant genetic difference between the nine populations examined. The resulting phenogram produced four closely related clusters. The first cluster included the LAM, SUR, TRA, and PRA populations, the second cluster the NAK and SRA populations, the third cluster the CHM and CHO populations, and the fourth cluster the KAN population, slightly apart from the others. KAN had the greatest deviation from HW equilibrium but with no significant difference in genetic background with the others. The better understanding of rate of gene flow and genetic structure of different vector populations over time may help identify more efficient control methods.

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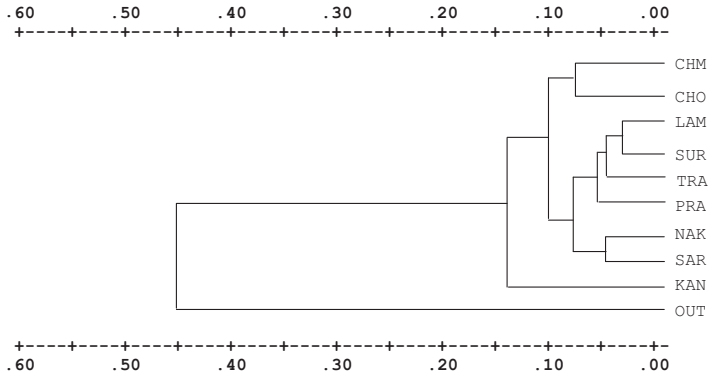

Fig. 2. Unweighted pair group method averaging phenogram from modified Roger's distance (Wright 1978) matrix among all nine populations of *S. calcitrans* and one population of *S. uruma* (cophenetic correlation = 0.926).

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Geographic Distribution of Stomoxyine Flies (Diptera: Muscidae) and Diurnal Activity of *Stomoxys calcitrans* in Thailand

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ABSTRACT Stomoxyine flies (*Stomoxys* spp.) were collected in 10 localities of Thailand using the Vavoua traps. These localities represented four major ecological settings, as follows: small local dairy farms, large industrial dairy farms, a national park, and one elephant conservation area. Three species of stable flies were identified in the following proportions: *Stomoxys calcitrans* (91.5%), *Stomoxys indicus* (7.9%), and *Stomoxys sitiens* (0.6%). The number of flies collected differed significantly among collection sites ($\chi^2 = 360.15$, df = 3, $P < 0.05$). The greatest number of stomoxyine flies was captured in dairy farms. Seasonal and daily activity of *S. calcitrans* was observed during a 1-yr period at two selected locations (Dairy Farming Promotion Organization of Thailand and Khao Kheow Open Zoo). *S. calcitrans* was more abundant during the rainy season (March–September), but was not associated with the total rainfall ($r^2 = 0.0002$, $P > 0.05$). Peak of daily flight activity of males *S. calcitrans* was at 1000 and 1600 h, whereas females showed an increase of activity all along the day until 1600 h. A better understanding of stomoxyine fly behavior related to patterns of daily activity will facilitate and improve the efficiency of fly control measures in private and government sectors.

KEY WORDS *Stomoxys* spp., distribution, seasonal and diurnal activity, Vavoua traps, Thailand

The genus *Stomoxys* (Muscidae: Stomoxyinae) contains at least 18 described species (Zumpt 1973). They are obligate blood-sucking insects with some species considered significant economic pests of livestock and other warm-blooded animals in many parts of the world (Zumpt 1973, Mullens et al. 1988, Masmeathathip et al. 2006). Among these, *Stomoxys calcitrans* (L., 1758), known as stable fly, is the most important and cosmopolitan species. Both male and female stomoxyine flies feed on blood, generally once each day. Adult flies have a typical flight range of 1 mile (≈1.6 km). The biology of stable flies is described elsewhere (LaBrecque et al. 1975, Berry et al. 1976, Smith et al. 1985).

Stomoxyine flies can be a severe problem in dairies and feedlots, where they breed in moist soil and similar substrates (Meyer and Petersen 1983). Severe biting activity can result in a reduction in animal weight and milk production. Significant economic losses as a result of reduction of anticipated gross weight gain and 30–40% decreases in milk yields have been observed (Hall et al. 1982, Mullens et al. 1988). In the United States, the estimated economic loss to the beef and dairy industry is nearly 400 million dollars annually (Smith et al. 1987). The high number of flies biting cattle and other affected animals may have a direct influence on the epidemiology of communicable diseases. Several stomoxyine fly species have been implicated as mechanical vectors of anaplasmosis (*Anaplasma marginale*), trypanosomosis (*Trypanosoma* spp.), and different viruses (e.g., bovine leucosis virus, bovine herpesvirus-2, and lumpy skin disease virus) (Buxton et al. 1985, Mihok et al. 1995, Torr et al. 2006, Carn 1996).

Stomoxys species have been found to have a wide host range (Warnes and Finlayson 1987). In Egypt, domestic donkeys and horses are the preferred hosts (Hafez and Gamal-Eddin 1959). Warnes (1984) found *S. calcitrans* preferred to feed on cattle and horses in the United Kingdom. Numerous host factors appear to influence the long-range olfactory responses of stable

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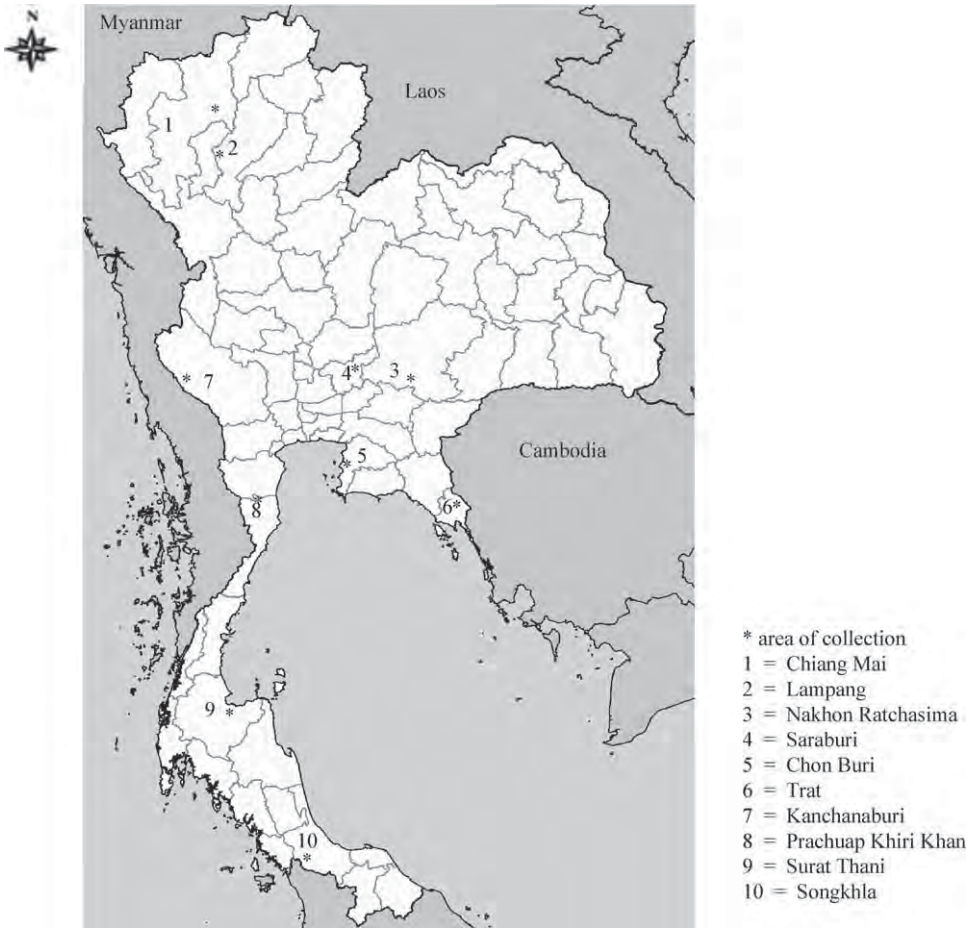

Fig. 1. Collection sites of stomoxyine flies in Thailand.

flies that include age, size, sex, and nutritional state. Pheromones produced by cattle also play a role attracting some stable flies (Torr et al. 2006).

Surveys of adult stable fly populations can be assessed using several different techniques. Many studies have employed the use of direct counts or collections from host animals, especially leg counts to access fly densities (McNeal and Campbell 1981, Berry and Campbell 1985). Various trapping devices and techniques have been developed to collect flies (Gersabeck and Merritt 1983, Foil and Hogsette 1994). These include sticky traps (Williams 1973, Broce 1988) and also the Vavoua trap (Laveissiere and Grebaut 1990), originally designed for tsetse fly collection. This trap has proved very efficient at capturing various *Stomoxys* spp. in many regions of Africa (Holloway and Phelps 1991, Mihok et al. 1995) and in La Reunion Island (Gilles et al. 2007).

Whereas most studies on stomoxyine flies, particularly *S. calcitrans*, have been documented in Africa and the United States, relatively little is known in Thailand about the presence of different stomoxyine fly species, their distribution, and biology. Recently, Masmeatathip et al. (2006) described the seasonal abundance of *Stomoxys* species in Thailand, but the study was limited to one location and did not compare the species diversity among different ecological settings. In this current study, stomoxyine fly species were surveyed and identified from 10 provinces, representing four different habitats throughout Thailand. In addition, a monthly stable fly collection was performed during a 1-yr period at Dairy Farming Promotion Organization of Thailand, Saraburi Province (SB), and Khao Kheow Open Zoo, Chon Buri Province (CB), to study the seasonal and daily activity. The results of these studies might support more effective fly control programs in private and government sectors.

Materials and Methods

Collection Sites. Stable fly collections were made in 10 geographical locations in Thailand (Fig. 1). Geographic coordinates and a brief description of collection sites are provided in Table 1.

Stable Fly Collection. At each collection site, nine Vavoua traps (Laveissiere and Grebaut 1990) were randomly placed around sample sites at ≈10 m apart.

Table 1. Stomoxyine fly collection sites in Thailand

Collection site provinces	Coordinates	Characteristics	Dates of collection
Nong Han, San Sai, Chiang Mai	18° 48′ N, 98° 58′ E	Industrial dairy farm, Maejo University; ~80 cows	April 2007
Wiang Tan, Hang Chat, Lampang	18° 17′ N, 99° 28′ E	Thai Elephant Conservation Center, Thung Kwian Forest Park; ~40 elephants	April 2007
Thai Samakkhi, Wang Nam Khiao, Nakhon Ratchasima	15° 0′ N, 102° 6′ E	Local dairy farm, Wang Nam Khiao District; ~40 cows	Mar. 2007
Mit Taphap, Muak Lek, Saraburi	14° 31′ N, 100° 52′ E	Industrial dairy farm: Dairy Farming Promotion Organization of Thailand; ~200 cows	Mar. 2007
Bang Phra, Si Racha, Chon Buri	13° 24′ N, 101° 0′ E	National park, Khao Kheow Open Zoo; a variety of natural and resident wild life	May 2007
Pong Kanang, Mueang, Trat	12° 13′ N, 102° 30′ E	Local dairy farm: Bo Rai District; ~20 cows	May 2007
Tha Sao, Sai Yok, Kanchanaburi	14° 1′ N, 99° 31′ E	Local dairy farm: Military Development Office; ~30 cows	April 2007
Huoy Sat Yai, Hua Hin, Prachuap Khiri Khan	11° 49′ N, 99° 47′ E	Local dairy farm: Dairy Farming Cooperatives; ~20 cows	April 2007
Makham Tia Mueang, Surat Thani	9° 8′ N, 99° 19′ E	Local dairy farm: Mueang District; ~20 cows	Sept. 2007
Nam Noy, Hat Yai, Songkhla	7° 0′ N, 100° 28′ E	Local dairy farm: Hat Yai District; ~20 cows	Sept. 2007

For the distribution study, stomoxyine flies were sampled from 0600 to 1800 h for a 2-d period in each site during the period March–September 2007 (Table 1). A more intensive monthly stable fly collection was performed from June 2007 to May 2008 for 2 d per month in two locations, Dairy Farming Promotion Organization of Thailand, SB, and Khao Kheow Open Zoo, CB. For this study, four Vavoua traps were placed from 0600 to 1800 h and stable flies were captured at 2-h intervals. In each study, the flies were preserved in 95% ethanol and recorded by date, hour of capture, and location. The specimens were brought back to the Department of Entomology, Faculty of Agriculture, Kasetsart University (Bangkok, Thailand) for the identification according to Zumpt (1973). Air temperature and relative humidity were also recorded at 2-h periods.

Data Analysis. Spatial and temporal data (time, date, place, species, number of specimens, and environmental conditions) were used in the analysis as appropriate. χ^2 test was used to evaluate the differences in number of stable flies among categories at different collection sites. The interaction between number of *S. calcitrans* captured and the yearly total rainfall at individual site was analyzed using variables with bivariate normal distribution (Kleinbaum et al. 1987). The GLM procedure in SPSS program package (version 13, SPSS, Chicago, IL) was used to evaluate the differences between numbers of stable flies collected at each location. All statistical significance was set at $P < 0.05$.

Results

A survey of stomoxyine fly species was made in 10 locations within six geographical regions of Thailand, including the following: 1) Chiang Mai and Lampang (north), 2) Nakhon Ratchasima (northeast), 3) Saraburi (central), 4) Chon Buri and Trat (east), 5) Kanchanaburi and Prachuap Khiri Khan (west), and 6) Surat Thani and Songkhla (south) (Fig. 1). Four potential *Stomoxys* habitats were sought, including industrial farms (two sites), local dairy farms (six), Thai Elephant Conservation Center (one), and a national park (one) (Table 1).

Stomoxyine fly captures made from March to September 2007 for the distribution study are summarized in Table 2. During this period, a total of 811 specimens, representing three different species, was captured. *S. calcitrans* (91.5% of captures) was found the most prevalent and present in all sites. *Stomoxys indicus* (7.9%) was present in nine sites, and *Stomxys sitiens* (0.6%) was found only in three sites (Table 2).

The greatest proportion of *S. calcitrans* (30.3%) has been recorded from the western provinces (Kanchanaburi and Prachuap Khiri Khan); 22.9% have been captured from northern provinces (Chiang Mai and Lumpang); 20.1% from northeast-central (Nakhon Ratchasima and Saraburi); 22.5% from the east (Chon Buri and Trat); and only 4.2% from southern provinces (Surat Thani and Songkhla) (Table 2).

Collectively, 81.5% (661) of stomoxyine flies were captured from dairy farms, 12.9% (105) from the National Park in Chon Buri Province, and 5.5% (45) from the Thai Elephant Conservation Center in Lampang Province. Among the eight dairy farms, *S. calcitrans* was found the most abundant (Table 3), representing 92.4% of the captured flies, whereas *S. indicus* represented 7% and *S. sitiens* was relatively rare (0.8%). All three species were recorded from local dairy farms

Table 2. Total numbers of stomoxyine flies collected in 10 collection sites

Collection sites (province)	Stomoxyine flies			
	S. calcitrans	*S. indicus*	*S. sitiens*	Total
Chiang Mai	129	1	0	130
Lumpang	41	4	0	45
Nakhon Ratchasima	9	11	0	20
Saraburi	140	6	0	146
Chon Buri	90	15	0	105
Trat	77	17	0	94
Kanchanaburi	111	2	1	114
Prachuap Khiri Khan	114	2	1	117
Surat Thani	28	6	3	37
Songkhla	3	0	0	3
Total	742	64	5	811

Table 3. Total number of stomoxyine flies among four different habitats

Habitats	*S. calcitrans*	*S. indicus*	*S. sitiens*	Total
Industrial dairy farm (2)	269	7	0	276
Local dairy farm (6)	342	38	5	385
National park (1)	90	15	0	105
Elephant conservation Center (1)	41	4	0	45
Total	742	64	5	811

(six), whereas only two species (*S. calcitrans* and *S. indicus*) were captured from the two industrial dairy farms. *S. calcitrans* was also the predominant species seen at the National Park (85.7%), compared with *S. indicus* (14.3%). The prevailing species in the elephant center was *S. calcitrans* (91.1%), followed by very small numbers of *S. indicus* (Table 3). The differences in numbers of stomoxyine flies among the different habitats were found highly significant by χ^2 test ($\chi^2 = 360.15$, df = 3, $P < 0.05$).

A monthly stable fly collection was performed at Dairy Farming Promotion Organization of Thailand, SB, and Khao Kheow Open Zoo, CB, from June 2007 to May 2008. A total of 3,374 and 1,696 *S. calcitrans* was captured at SB and CB, respectively (Table 4). The sex ratios (5.6 and 2.1 for SB and CB, respectively) were always in favor of males (Table 4). Significant differences in number of stable flies collected during the 12 mo at both locations were obtained ($P < 0.05$). In SB, stable fly abundance increased when the collection began, reached a peak in July 2007, and declined from August 2007 to January 2008. In CB, the seasonal trend was very similar. The major peak of *S. calcitrans* abundance was observed in May 2008 in SB and in March 2008 in CB, and the secondary peak was in July 2007 (Fig. 2). No association between the density of *S. calcitrans* and the yearly total rainfall was observed ($r^2 = 0.0002$, $P > 0.05$).

The diurnal activity of males and females of *S. calcitrans* at SB and CB is shown in Fig. 2. The numbers of stable flies collected from different periods of the day (0600–0800, 0800–1000, 1000–1200, 1200–1400, 1400–1600, and 1600–1800 h) are statistically different ($P < 0.05$). Males show clearly two peaks of diurnal activity (1000 and 1600 h). The diurnal activity of females was less clear, with a continuous increase until 1600 h (Table 5 and Fig. 2).

Discussion

There are very few publications on stomoxyine fly species in Thailand (Sucharit and Tumrasvin 1981, Masmeatathip et al. 2006). The data obtained from the current study provide better insight on species distribution, bionomics, and diurnal activity of this group of biting flies in Thailand. These data should also prove useful to help to identify the respective roles of these species as nuisance and potential vectors of pathogens. We used the Vavoua trap as a proven method to attract and capture these diurnally active flies. If some stomoxyine flies do have a more nocturnal activity, they were not captured with this trap. This is perhaps the case with *S. indicus* (Zumpt 1973). In the current study, *S. calcitrans* was found to be the most widely distributed species and strongly associated with both small and large dairy farms in Thailand.

The comparatively high numbers of stomoxyine flies, and *S. calcitrans* in particular, collected in dairy farms are most likely the consequence of relative high host density for blood-feeding adults and suitable soil and environmental conditions for stable fly larvae to complete their life cycle. This combination appears to play a significant role in stable fly abundance. In addition, the mixture of manure with silage and spilled feed in dairy farms appears a highly favorable medium for developing stable fly larvae (Masmeatathip et al. 2006). Romero et al. (2006) found that female stable flies are attracted to oviposition sites by stimuli caused by bacteria present in manure nearby.

Stable fly abundance and peak densities are the consequence of appropriate environmental conditions, i.e., moisture, light intensity, rainfall, and temperature to maintain acceptable breeding habitats. Mullens and Meyer (1987) observed a single seasonal peak of *S. calcitrans*, being the most prevalent during the summer season from May to June, whereas marked bimodal and trimodal peaks have been documented in other locations in the United States, presumably influenced by ambient temperatures in more temperate

Table 4. Numbers of male and female *S. calcitrans* collected at Dairy Farming Promotion Organization of Thailand, Saraburi Province (SB), and Khao Kheow Open Zoo, Chon Buri Province (CB)

Month	No. *S. calcitrans* at SB			No. *S. calcitrans* at CB		
	Males (%)	Females (%)	Total	Males (%)	Females (%)	Total
June 07	235 (84)	46 (16)	281	91 (74)	32 (26)	123
July 07	496 (88)	67 (12)	563	183 (65)	97 (35)	280
Aug. 07	290 (86)	46 (14)	336	129 (69)	58 (31)	187
Sept. 07	96 (65)	52 (35)	148	52 (50)	52 (50)	104
Oct. 07	132 (85)	24 (15)	156	18 (42)	25 (58)	43
Nov. 07	38 (90.5)	4 (9.5)	42	26 (48)	28 (52)	54
Dec. 07	41 (87)	6 (13)	47	18 (49)	19 (51)	37
Jan. 08	17 (61)	11 (39)	28	15 (50)	15 (50)	30
Feb. 08	146 (91)	15 (9)	161	43 (75)	14 (25)	57
Mar. 08	202 (86)	34 (14)	236	420 (75)	143 (25)	563
April 08	516 (83)	109 (17)	625	70 (74)	25 (26)	95
May 08	657 (87.5)	94 (12.5)	751	80 (65)	43 (35)	123

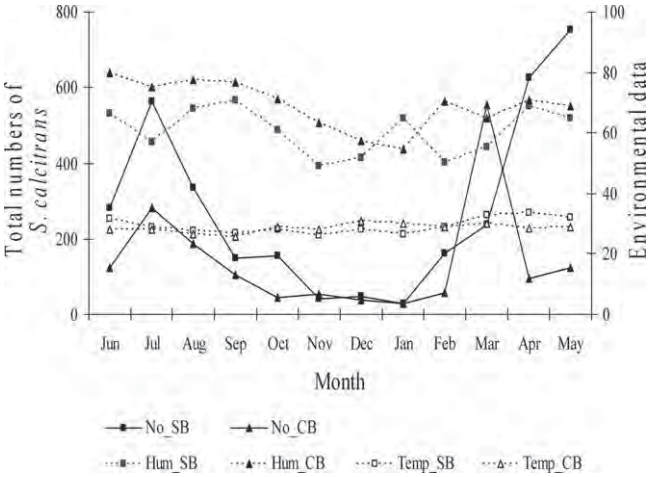

Fig. 2. Total numbers of *S. calcitrans* collected at Dairy Farming Promotion Organization of Thailand, SB, and Khao Kheow Open Zoo, CB.

climates (Lysyk 1998). In Thailand, Masmeatathip et al. (2006) reported a wet season peak density of *S. calcitrans* associated with rainfall. In our study, the majority of adult stable flies were captured during the dry period from March to May.

The results from the National Park were conspicuously different from the dairy farm settings. The park is a large and open area, and most warm-blooded animals may either rest or otherwise be absent during the daytime, resulting in poor availability for adult stable flies to blood feed. Furthermore, the park's environment may have lacked sufficient and suitable medium for stable fly development. In a similar situation, Mihok and Clausen (1996) monitored stomoxyine flies with Vavoua traps on a single day in a forested area of the Nairobi National Park, Kenya, finding fairly robust numbers of species (six), but all in very low densities.

Similarly, the Thai Elephant Conservation Center appears not to be an ideal ecological setting for stomoxyine flies, considering the low numbers of flies captured. Only *S. calcitrans* was present in any meaningful number. We also suspect that a feedlot and livestock area that was located near the elephant center (≈3 km distance) was the primary breeding habitat for *S. calcitrans* in this area. Foil and Hogsette (1994) reported that the stable flies can disperse up to 5 km or more in search of blood meals.

In Thailand, investigations on the daily activity of *Stomoxys* species have been limited. Masmeatathip et al. (2006) reported a bimodal activity of *S. calcitrans*, with the first peak period being between 0800 and 1000 h and another less marked period between 1600 and 1800 h. Besides, there were a number of studies reporting a bimodal activity of *S. calcitrans*, in Florida (Simmonds 1944); Mauritius (Kunz and Monty 1976); Manaus, Brazil (Charlwood and Lopes 1980); and Kansas (Semakula et al. 1989). In contrast, unimodal activity pattern on the daily feeding of *S. calcitrans* was observed in Uganda (Coaker and Passmore 1958, Harley 1965). In our study, there were two distinct daily peaks of male *S. calcitrans*, occurring at 1000 and 1600 h, and one unclear peak of female at 1600 h. In Brazil, a fairly constant peak of female activity was reported throughout the day (Charlwood and Lopes 1980). Marked number of *S. calcitrans* was captured from the dairy farms compared with the forest setting, indicating that *S. calcitrans* is more associated with the anthropic ecological setting.

Our study has several limitations. First, fly collections between the different sites were not conducted contemporaneously; therefore, seasonal and temper-

Table 5. Numbers of male and female *S. calcitrans* collected at Dairy Farming Promotion Organization of Thailand, Saraburi Province (SB), and Khao Kheow Open Zoo, Chon Buri Province (CB), from 6 am to 6 pm

Time	No. *S. calcitrans* at SB			No. *S. calcitrans* at CB		
	Males (%)	Females (%)	Total	Males (%)	Females (%)	Total
0600–0800	272 (9.5)	53 (10.4)	325	98 (8.6)	37 (6.7)	135
0800–1000	708 (24.7)	70 (13.8)	778	287 (25.1)	57 (10.3)	344
1000–1200	448 (15.6)	83 (16.3)	531	180 (15.7)	109 (19.8)	289
1200–1400	421 (14.7)	76 (15)	497	153 (13.4)	100 (18.2)	253
1400–1600	659 (23)	122 (24)	781	244 (21.3)	144 (26.1)	388
1600–1800	358 (12.5)	104 (20.5)	4621	83 (16)	104 (18.9)	287

Percentages are expressed as per day per sex.

ature differences between sites may have greatly influenced collection numbers. Second, because we used only one trapping method, it is unclear whether this single method favored one *Stomoxys* species over another. Third, most of wild mammals are nocturnal and not accessible to diurnal flies. Perhaps other species of *Stomoxys* do exist in such areas, but should be captured using light traps during the night or traps in the canopy (Mavoungou et al. 2007). Use of multiple trapping methods and mark-release-recapture studies of *Stomoxys* species, particularly *S. calcitrans*, will be subjects for further investigation.

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Host feeding responses of *Aedes aegypti* (L.) exposed to deltamethrin

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ABSTRACT: Escape responses of mated and unmated nulliparous *Aedes aegypti* mosquitoes were compared using three different concentrations of deltamethrin in the presence or absence of a live animal host using an excito-repellency (ER) test system. Both insecticide contact (excitation) and non-contact (repellency) test configurations were compared. For contact trials, mated mosquitoes showed similar escape movements among the three concentrations when host stimuli were absent. Significant differences in responses were seen between the lower concentrations of (LC_{50} and LC_{75}) deltamethrin with and without hosts present ($P<0.05$). Presence or absence of host stimuli produced no significant differences in escape response for unmated females when exposed to the highest concentration (LC_{90}) of deltamethrin. Our findings indicate that as deltamethrin concentrations decrease to sublethal levels, mating status and host cues play a more significant role in escape behavior. Therefore, insemination can influence the outcome of feeding success and flight movement of nulliparous female *Ae. aegypti* in contact with deltamethrin and in the presence of live host stimuli. The ER assay system serves as a useful tool for observing excitation and repellency responses of *Ae. aegypti* to insecticides in the presence or absence of other environmental and biological cues that can affect mosquito behavior. ***Journal of Vector Ecology* 36 (2): 361-372. 2011.**

Keyword Index: *Aedes aegypti*, deltamethrin, host stimuli, feeding responses, excitation, repellency.

INTRODUCTION

Despite decades of organized malaria and dengue control activities, both diseases remain major health concerns in Thailand (MOPH 2009). Because no effective and acceptable vaccine or medical treatment is currently available for combating dengue (and Chikungunya virus), the control of these diseases in Thailand has focused primarily on the monitoring and reduction of the mosquito vectors. *Aedes aegypti* is a highly anthropophilic species and often resides in and near human dwellings preferentially feeding on humans (Gubler 1997, Thavara et al. 2001, Suwonkerd et al. 2006). This species can utilize many types of artificial and natural container habitats for oviposition and larval development, making source reduction as a method of control difficult. Other methods to prevent dengue transmission are to reduce human-vector contact using insecticides that attack the adult mosquito. Synthetic insecticides, particularly pyrethroids, have been used extensively (especially during high transmission periods or outbreaks) to control dengue vector mosquitoes in Thailand (Chareonviriyaphap et al. 1999, Somboon et al. 2003, Jirakanjanakit et al. 2007, Thanispong et al. 2008). In general, pyrethroids have been the insecticides of choice for many decades because of their high insecticidal activity, relatively low mammalian toxicity, rapid degradation in the environment, and relative lower cost compared to alternative class compounds. Deltamethrin is currently one of the most commonly used insecticides in public health programs and has been the mainstay for the emergency control of *Ae. aegypti* adults in Thailand since 1994 (Kongmee et al. 2004).

Pyrethroids have been widely used for controlling disease vectors due to their relatively low mammalian toxicity and broad spectrum efficacy in controlling indoor mosquito populations (Elliott et al. 1978, Najera and Zaim 2002). However, like DDT, most pyrethroids exhibit strong excito-repellency action in many mosquito species with physical displacement (avoidance) of treated surfaces a common reaction (Roberts et al. 2000, Chareonviriyaphap et al. 2004). DDT and most pyrethroids act as strong locomotor stimulants in many mosquito species, however the physiological and behavioral mechanism(s) for deterrence has not been determined and may vary for different species and compounds. Excito-repellency (or avoidance), a composite term that has been in common use for decades, is more appropriately defined in terms of an endpoint or multiple endpoints (i.e., movement away from a treated surface or area because of excitatory effects caused by chemical exposure) involving a series of responses to a stimulus and one or more combinations of behavioral mechanisms. It should be noted that terminologies with unintentional anthropomorphic (irritancy) or teleological (avoidance) connotations are synonymous with excitation and repellency, respectively.

When mosquitoes are exposed to an insecticide, two different behavioral responses are recognized, commonly referred to as irritancy and repellency (Rutledge et al 1999,

Roberts et al. 2000). Irritability occurs when insects actually make physical contact with chemical residues before eliciting a stimulus-mediated (excitation) escape response, whereas repellency is defined as a stimulus (or stimuli) acting from a distance from the insecticide-treated surface that deters insects from entering treated areas or otherwise disrupts normal patterns of behavior (e.g., blood-feeding).

A better understanding of the responses and mechanisms of *Ae. aegypti* exposed to insecticides are operationally relevant for maximizing effective control. The design of a flexible laboratory test system to accurately measure excitatory and spatial repellent responses is extremely useful as a chemical product screening device and a means to better utilize chemicals in control operations. In this study, an excito-repellency (ER) test system designed by Chareonviriyaphap et al. (2002) with minor modifications was used to evaluate the exit movement patterns of *Ae. aegypti* exposed to residual insecticides in the presence of a host attractant. A variety of test designs using the ER system exist, but this represents the first attempt to examine the possible influence of host cues on escape and blood feeding responses during concurrent exposure to an insecticide, thereby further expanding the utility of the system. The general excito-repellency assay system is designed to observe escape movement away from a surface due to contact excitation or non-contact spatial repellency that measures the effects of an insecticide acting from a distance (Roberts et al. 1997).

Although chemical control methods remain among the most effective means of reducing transmission by preventing mosquito bites (WHO 1999), the movement patterns of *Ae. aegypti* in response to insecticide exposure and competing stimuli remains poorly understood. Movement patterns of *Ae. aegypti* exposed to chemicals alone or in the presence of various host types has been performed using experimental huts in Thailand (Suwonkerd et al. 2006, Grieco et al. 2007); however, no complementary tests have been conducted under laboratory-controlled conditions to determine if suitable experimental designs are compatible with findings from the field. A few studies have examined the effects of different nutritional and physiological conditioning of adult female *Ae. aegypti* mosquitoes on escape responses (Chareonviriyaphap et al. 2006, Polsomboon et al. 2008). We decided to introduce a live host as a possible competing factor that might significantly influence a mosquito's response to active ingredients normally performing as strong deterrents to normal behavioral patterns. Additionally, examining the possible effects mating status might have on a mosquito's ability to respond to insecticide while in the presence of a host was deemed relevant, as post-insemination is a presumed driver of programmed behavioral sequences (e.g., flight-activity rhythm, responsiveness to host cues) differing from that of non-mated females and depending on blood-fed status and state of ovarian development (Clements 1999). The aim was to determine if additional stimuli (live host chemical/olfactory, physical and visual cues) can influence (e.g., activate, enhance, suppress) excitation and repellency responses and whether mating status in nulliparous/non-gravid females might influence those responses.

MATERIALS AND METHODS

Aedes aegypti was originally collected as immature stages from one locality in Pu Teuy Village (Sai Yok District, Kanchanaburi Province, Thailand). Mosquitoes were reared in the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, under environmentally controlled conditions (25±5°C, 80±10% RH, and 12:12 L:D photoperiod). Immature stages were reared in plastic pans under identical population density and physical and nutritional conditions throughout the study. Pupae were transferred into the cups containing tap water and placed in screened (30 cm^3) cages. Teneral adult males and females were identified to species and provided cotton pads soaked with 10% sugar solution. Following free mating, two- to five-day-old female mosquitoes were allowed to feed on a live, restrained guinea pig (*Cavia cobaya*). An oviposition site (Petri dish) containing tap water and filter paper (Whatman® No. 1) was placed in each cage for egg deposition following ovarian development. The resultant progeny and adults from F_1 to F_3 generations were utilized for testing.

Two different test populations, representing either mated or unmated nulliparous six-day-old female *Ae. aegypti* were used for the tests. Population 1 consisted of mated, nulliparous mosquitoes derived from females held together with male mosquitoes to allow free mating up to the day of test. Random samples of females were dissected to examine the spermathecae to confirm the percentage of successful mating. Population 2 consisted of unmated, nulliparous mosquitoes obtained by segregating females from male mosquitoes before emergence to prevent mating. This was accomplished by placing individual pupae into a small vial containing 10 ml of clean water until emergence, then combining all females. All mosquitoes were denied access to any blood source and only provided with 10% sugar solution soaked on cotton pads. All sugar was removed approximately 12 h before each test.

Deltamethrin [IUPAC: (S)-alpha-cyano-3-phenoxy-benzyl (1R,3R)-3-(2,2-dibromovinyl)-2, 2-dimethyl cyclo-propanecarboxylate] (98% active ingredient) was provided by BASF. All treated test papers were produced at the Department of Entomology, Kasetsart University. Technical grade insecticide was dissolved in a mixture of silicone oil (Dow Corning 556) and analytical grade acetone in the ratio of 0.34:1.66. Whatman® No. 1,12 x 15 cm^2 filter papers were prepared and used for dose response assays and 27.5 × 35.5 cm^2 papers were used for excito-repellency tests. All treated papers were prepared according to World Health Organization specifications (WHO 1998, 2006) and treated with 2 ml prepared insecticide solution per 180 cm^2 paper. Control papers were treated with carrier diluents only (silicone oil + acetone). All papers were allowed to air dry for 24 h and used in tests within 48 h of preparation.

Two test populations of *Ae. aegypti*, either mated or unmated, were exposed to various concentrations of

deltamethrin in order to determine baseline percent lethal concentrations (LC) sufficient to kill 50%, 75%, and 90% of exposed female mosquitoes. For each test, five cylinders (two controls and three containing chemical treatment) were used (WHO 1981a). Twenty-five mosquitoes were introduced into each cylinder and held for 1 h. Mosquitoes were then transferred to holding containers and provided a 10% sucrose solution. Six different concentrations of deltamethrin were used in all test combinations and each replicated three times.

Six-day-old, either mated or unmated, nulliparous mosquitoes, were selected for determining susceptibility to deltamethrin. Three concentrations of deltamethrin were used: 0.002%, 0.01%, and 0.05%. The highest concentration, 0.05%, served as the discriminating (diagnostic) dose based on recommendations for anopheline mosquitoes (WHO 1998). WHO test kits and procedures were used (WHO 1981b). Twenty-five, non-blood-fed female mosquitoes were placed into each holding tube lined with clean (untreated) paper for 1 h to observe the health of mosquitoes before insecticide exposure. Dead and moribund mosquitoes were removed before beginning the test exposure. Live healthy mosquitoes from each holding tube were transferred to either an insecticide-treated or control tube for 1 h. At the end of 1 h, the number of knockdown mosquitoes were recorded. All mosquitoes were transferred to separate clean holding tubes and provided with 10% sugar solution. Final mortality was recorded at 24 h post-exposure. All test combinations were replicated three times. The susceptibility status was categorized based on World Health Organization criteria (WHO 1981b, 1998).

A series of excito-repellency response assays was designed to observe the exit movement of female *Ae. aegypti* (Chareonviriyaphap et al. 2002). The test designs allowed recording of both contact excitation (irritancy) and non-contact (repellency) to deltamethrin with and without a host present and comparing responses between mated and unmated mosquitoes. Live adult guinea pigs (*Cavia cobaya*) served as host cues. The basic test design included two test chambers (deltamethrin-treated) and two paired control chambers (untreated). Each test trial had two chambers (test and control) with and two chambers without a host placed inside. Four-chamber trials were either set up as a contact format (allowing the mosquito direct physical tarsal exposure to the treated paper) or non-contact (treated papers protected by a fine-mesh screen barrier denying the mosquito physical contact with the chemical) format. Chambers receiving a host had only one restrained animal each. Twelve h before testing, all mosquitoes were deprived of sugar and provided with water only. Following chamber preparation, 25 female *Ae. aegypti*, either mated or unmated, were introduced into each of the four test chambers using a mouth aspirator. Mosquitoes were allowed a 3-min adjustment period to chamber conditions followed by opening the escape funnel to begin the observation period. The number of mosquitoes escaping from the chamber into the receiving cage was recorded at 1-min intervals for 60 min. Mosquitoes that successfully blood-fed were recorded regardless of amount of blood imbibed. Immediately after 1 h exposure**,** the numbers of dead or knockdown mosquitoes, with and without blood that either remained inside the chamber or escaped, were recorded separately. All live specimens (knockdown and those capable of flight) that either escaped or remained inside the chamber were collected and held separately by category in small holding containers and provided with 10% sugar solution. Mortality was recorded following 24 h post-exposure. All tests were performed between 08:00-16:00 and otherwise kept under identical laboratory conditions (temperature, humidity, illumination).

Each test trial was required a minimum of 100 six-day-old female mosquitoes (25 mosquitoes/chamber). The minimum number of female mosquitoes needed for this study was 4,800 based on use of three chemical concentrations x two physiological states (mated and unmated) x four chambers per trial (paired treated + control, with host and no host) x two exposure conditions (contact and non-contact formats) x four replicates per trial set design. For tests involving LC_{90} concentrations, a total of eight trials were run for each chemical dose increasing the minimum number of female mosquitoes required to 6,400. In all, eight different test combinations with matched controls were conducted for each of three concentrations of deltamethrin and replicated a minimum of four times.

For preparation of host cues, each guinea pig had a patch of skin exposed by carefully clipping the hairs to allow mosquitoes an easier access to the skin surface. Each animal was placed in a restraint device without sedation for a period of 1 h while allowing the 25 mosquitoes the opportunity to blood feed on the host for those 60 min. Guinea pigs were allowed 20 min free of the restraint device before replicating the test. The same set of animals was used throughout the study. All procedures and rules governing the use of live animals in this research met all stipulations by the Thailand Research Fund Organization and were approved by the Committee on Animal Use, Kasetsart University, under study protocol number RSA 5180020.

A log-probit (dose-response) analysis (PROC PROBIT, SAS ver. 9, SAS Institute, Cary, NC) was used to estimate percent lethal concentrations based on dose-mortality response assays (Finney 1971). Insecticide susceptibility test data were analyzed using methods outlined by W.H.O. (WHO 1981a). The Kaplan-Meier survival analysis (Kleinbaum 1995) method was used to estimate probability of mosquito escape in response to differences in mosquito behaviors and mating status between test conditions (Survivorship procedures: PROC LIFETEST, SAS ver. 9, SAS Institute, Cary, NC). For analysis, mosquitoes that escaped were regarded as "deaths" and those remaining in the test chamber labeled as "survivals" (Chareonviriyaphap et al. 1997). Survival analysis was also used to estimate escape time (ET) from data collected over the 60-min period. Time in minutes for 25%, 50%, and 75% (ET_{25}-ET_{75}) of the test population to escape respective chamber test designs was estimated. A log-rank method (Mantel and Haenzel 1959) was used to compare the patterns of escape

behavior between different treatment designs (contact and non-contact, host and no host, and insemination status). Statistical significance for these tests was set at $P<0.05$.

The proportion of both mated and unmated nulliparous *Ae. aegypti* that successfully blood-fed and did not escape during contact and non-contact trials among the three different concentrations of deltamethrin with a live host present were recorded for each treatment and analyzed using PROC GENMOD, link function: logit (SAS ver. 9.2, SAS Institute, Cary, NC).

RESULTS

Susceptibility tests using three different serial dilutions (0.002, 0.01, and 0.05%) of deltamethrin performed on mated and unmated, nulliparous *Ae. aegypti* found high susceptibility (mortality > 97%) to the recommended operational 0.05% concentration (Table 1). The probit estimates and confidence intervals (lower and upper fiducial limits) of deltamethrin LC_{50}, LC_{75}, and LC_{90} for mated, nulliparous, non-blood-fed, six-day-old female *Ae. aegypti* were 0.0071% (0.00563-0.00886), 0.0143% (0.01123-0.01935), and 0.0269% (0.01976-0.04134), respectively, with slope ± SE 2.21±0.239; and for unmated females 0.0065% (0.00517-0.00855), 0.0134% (0.01050-0.01812), and 0.0255% (0.01872-0.03916), respectively, with slope ± SE 2.17±0.235 (data not shown). There was no statistical difference between lethal concentration estimates and insemination status. The ability of a small percentage of the test population to survive the diagnostic dose after 24 h suggests the occurrence of low-grade (incipient) physiological resistance but there was no statistical difference in insecticide susceptibility response between mated and unmated females ($P > 0.05$).

Aedes aegypti escape responses during 60 min exposures and subsequent percent mortality of escaped and non-escaped mosquitoes following a 24 h holding period in contact and non-contact ER chamber configurations with or without live host stimuli are presented in Tables 2 and 3. *Ae. aegypti* showed similar degrees of contact escape responses to all three concentrations without host cues, ranging from 31.3-41.0% for mated and 30.9-39.2% for unmated mosquitoes. In all cases, percent escape in non-contact assays was significantly lower ($P<0.05$) than contact trials under the same conditions and in only a few instances were statistical differences seen between paired control and non-contact tests, different doses, and host presence or absence (see below). Percent mortality of escaped mosquitoes was very low in contact trials (0-4.1%) and none seen in non-contact tests. The highest mortality recorded was in non-escaped mated (24.35%) and unmated (20.97%) females exposed to LC_{90} without hosts. In non-contact trials, the highest percent mortality was observed with non-escaped, unmated females exposed to LC_{75} with host cues present (4.21%). As expected, percent mortality for escaped and non-escaped mosquitoes in all control tests was extremely low (range: 0-2.08%) (Tables 2 and 3).

For all three doses, there were no significant differences in escape response between mated and unmated females in non-contact trials. Figures 1A-C compare the dose-response patterns of escape and percentage of mosquitoes remaining in the test chambers under different contact and non-contact test conditions and three deltamethrin concentrations (control survival analysis not shown). The escape patterns show the probability of exiting the treated and control chambers at one min intervals during the 60 min exposure. In contact trials, escape patterns of mated females at LC_{50} and $LC_{75,}$ without host, were greater than other test combinations (Figures 1A and 1B). In contrast, a higher escape response was observed in contact trials for unmated females with host at the highest dose compared to other test combinations (Figure 1C).

Analysis of escape responses, exclusive of blood feeding outcome, is presented in Tables 4 and 5. Within trial log-rank comparisons of escape probability between paired control and contact, control and non-contact, contact and non-contact trials, and contact and non-contact controls are presented in Table 4. Significant differences in escape patterns were observed among all contact tests compared to paired controls and matched non-contact tests; in most cases the values were highly significant ($P<0.0001$). With two exceptions (mated with and without hosts), there were no significant differences in escape patterns between non-contact and paired controls ($P>0.05$). Comparisons of contact and non-contact controls showed varying response of escape with significant differences seen in all but one set with host (unmated) and only one set with host (unmated).

Paired-dose comparisons of escape responses of *Ae. aegypti* among different concentrations between mated and unmated mosquitoes, in contact and non-contact trials, with and without host stimuli are presented in Table 5. There were significant differences between all pairs in contact trials with hosts ($P<0.05$), except between LC_{50} vs. LC_{75} among unmated females. In contact trials without hosts, no significant differences were seen between concentrations regardless of mating status. Likewise, no significant differences were seen between dose comparisons in non-contact trials with or without host ($P>0.05$), except a slight difference ($P=0.047$) recorded between LC_{50} and LC_{90} among unmated mosquitoes.

Escape time (ET) for 25%, 50%, and 75% of mosquitoes to depart chambers was estimated as data would allow. In contact trials, at LC_{90} and ET_{25}, with and without hosts for mated and unmated females was 7 and 3 min, and 8 and 10 min, respectively. With one exception (contact trial with host, at LC_{90} and ET_{50}, for unmated females: 13 min), all other estimates could not be made based on insufficient numbers of mosquitoes successfully escaping. All non-contact trials had fewer than 25% of mosquitoes escaping within 1 h exposure (data not shown).

Logistic regression was applied to the data at each treatment level. Both contact and non-contact control results had a significant effect on the overall model ($P<0.0001$ and $P<0.0340$, respectively) (Table 6). Mating status (insemination) influenced blood feeding response in the contact controls ($P<0.0093$, 95% C.I. -1.7204, -0.2426).

Table 1. Baseline mean percent mortality of mated and unmated nulliparous six-day-old *Ae. aegypti* exposed to three concentrations of deltamethrin using standard WHO contact susceptibility test procedures.

Conditions	Dosage (%)	Treatment		Control	
		No. tested	% Mortality±SE	No. tested	% Mortality±SE
Mated	0.002	75	13.33 ± 1.33	50	0
	0.01	74	60.78 ± 3.67	50	0
	0.05	75	97.33 ± 2.67	50	0
Unmated	0.002	75	13.33 ± 3.53	50	0
	0.01	73	65.41 ± 5.49	50	0
	0.05	75	98.67 ± 1.33	50	0

Table 2. Contact percentage escape and mortality of mated and unmated nulliparous *Ae. aegypti* with 1 h exposure to three concentrations of deltamethrin, with and without host stimuli.

Insecticide	Conditions	Dose	Host	No. Tested	% Escaped	% Mortality	
						Escaped	Not Escaped
Del	Mated	LC_{50}	Y	97	15.46	0	2.44
Control			Y	99	4.04	0	0
Del			N	99	31.31	0	1.47
Control			N	98	12.24	0	0
Del		LC_{75}	Y	99	27.27	0	1.39
Control			Y	97	4.12	0	0
Del			N	97	39.21	0	3.45
Control			N	98	13.27	0	0
Del		LC_{90}	Y	194	44.85	2.30	4.81
Control			Y	198	4.55	0	0.53
Del			N	195	41.03	3.75	24.35
Control			N	198	5.05	0	0
Del	Unmated	LC_{50}	Y	97	20.62	0	3.90
Control			Y	98	6.12	0	0
Del			N	97	30.93	0	5.97
Control			N	98	10.20	0	0
Del		LC_{75}	Y	99	28.28	3.57	1.41
Control			Y	98	2.04	0	0
Del			N	98	34.69	0	6.25
Control			N	98	10.20	0	0
Del		LC_{90}	Y	196	62.24	4.10	12.16
Control			Y	196	11.22	0	0.57
Del			N	204	39.22	3.75	20.97
Control			N	200	11.00	0	0.56

Table 3. Non-contact percentage escape and mortality of mated and unmated nulliparous *Ae. aegypti* with 1 h exposure to three concentrations of deltamethrin, with and without host stimuli.

Insecticide	Conditions	Dose	Host	No. Tested	% Escaped	% Mortality	
						Escaped	Not Escaped
Del	Mated	LC_{50}	Y	99	1.01	0	2.04
Control			Y	100	7.00	0	0
Del			N	100	1.00	0	1.01
Control			N	99	3.03	0	0
Del		LC_{75}	Y	99	3.03	0	0
Control			Y	98	5.10	0	0
Del			N	99	0	0	2.02
Control			N	99	2.02	0	0
Del		LC_{90}	Y	196	4.08	0	0
Control			Y	196	3.06	0	0
Del			N	196	0	0	1.02
Control			N	198	4.04	0	0
Del	Unmated	LC_{50}	Y	99	8.08	0	0
Control			Y	98	11.22	0	0
Del			N	98	6.12	0	0
Control			N	100	7.00	0	0
Del		LC_{75}	Y	98	3.06	0	4.21
Control			Y	98	5.10	0	0
Del			N	97	1.03	0	0
Control			N	100	1.00	0	2.08
Del		LC_{90}	Y	202	1.98	0	0.51
Control			Y	196	2.04	0	0
Del			N	200	1.00	0	0.51
Control			N	198	1.01	0	0

At the LC_{50} and LC_{75} there were significant differences of percent blood-fed for mated mosquitoes ($P < 0.0064$, C.I. -1.9930, -0.3266 and $P < 0.0016$, C.I. -2.0303, -0.4777, respectively).

In the LC_{50} contact trials, the percentage of mated and unmated mosquitoes that did not escape (84.5% and 79.4%, respectively) from treated chambers containing a live host showed 67.1% (55/82) of mated females successfully blood-fed, whereas 55.8% (43/77) of unmated females contained host blood. At the lowest concentration (0.002%), there was only a slightly greater likelihood that non-escaped mated females would successfully blood-feed compared to unmated females. At the highest dose (0.05%), 55.2% of mated mosquitoes remained inside insecticide-treated chambers containing host stimuli, of which 71.0% (76/107) successfully blood-fed, whereas only 37.8% of unmated mosquitoes remained in the chambers after 60 min with a lower percent blood-feeding (59.5%, 44/74) compared to mated mosquitoes. Our findings indicate that as deltamethrin concentrations decrease to sublethal levels, mating status and host cues play a more significant role in escape behavior. Therefore, insemination can influence the outcome of feeding success and flight movement of nulliparous female *Ae. aegypti* in contact with deltamethrin and in the presence of live host stimuli.

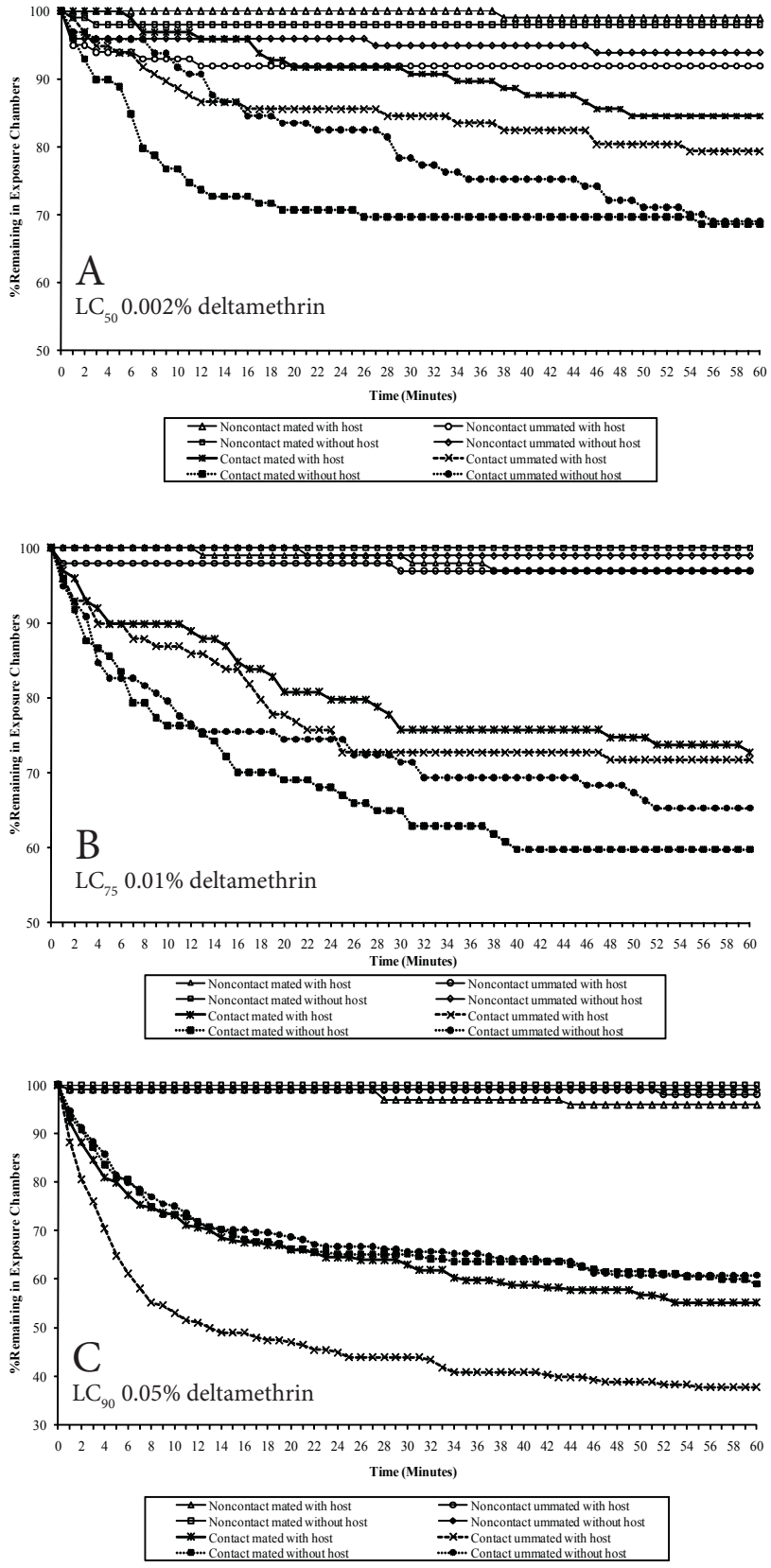

Figure 1. (A-C). 60-min probability escape patterns using survival analysis comparing mated and unmated nulliparous *Aedes aegypti* in contact and non-contact trials with or without presence of live host stimuli when exposed to (A) 0.002% LC_{50}, (B) 0.01% LC_{75}, and (C) 0.05% LC_{90} concentrations of deltamethrin.

Table 4. Comparison of the escape responses between contact trials (CT) and paired control (CC), non-contact trials (NT) and paired control (NC), paired contact (CT) and non-contact trials (NT), and paired contact (CC) and non-contact controls (NC) for mated and unmated *Ae. aegypti* exposed to three different concentrations of deltamethrin with or without live host cues present.

Host	Conditions	Dosage	CC vs CT	NC vs NT	CT vs NT	CC vs NC
With Host	Mated	LC_{50}	0.0056*	0.0313*	0.0002*	0.3376
		LC_{75}	<0.0001*	0.4600	<0.0001*	0.7319
		LC_{90}	<0.0001*	0.7086	<0.0001*	0.0814
	Unmated	LC_{50}	0.0027*	0.4691	0.0162*	0.1980
		LC_{75}	<0.0001*	0.4720	<0.0001*	0.2624
		LC_{90}	<0.0001*	0.9869	<0.0001*	<0.0001*
Without Host	Mated	LC_{50}	0.0009*	0.3076	<0.0001*	0.0148*
		LC_{75}	<0.0001*	0.1562	<0.0001*	0.0031*
		LC_{90}	<0.0001*	0.0452*	<0.0001*	0.0101*
	Unmated	LC_{50}	0.0004*	0.8052	<0.0001*	0.4325
		LC_{75}	<0.0001*	0.9857	<0.0001*	0.0051*
		LC_{90}	<0.0001*	0.9972	<0.0001*	<0.0001*

*Statistically significant ($P < 0.05$) differences within test conditions and pairings.

Table 5. Multiple paired comparisons of escape responses between three different concentrations of deltamethrin in either mated and unmated nulliparous *Aedes aegypti* exposed to contact and non-contact trials either with or without live host cues.

Host	Conditions	Dosage	Contact trial (P)	Non-contact trial (P)
With Host	Mated	LC_{50} vs LC_{75}	0.0333*	0.3105
		LC_{50} vs LC_{90}	<0.0001*	0.1705
		LC_{75} vs LC_{90}	0.0027*	0.6894
	Unmated	LC_{50} vs LC_{75}	0.2034	0.1249
		LC_{50} vs LC_{90}	< 0.0001*	0.0476*
		LC_{75} vs LC_{90}	< 0.0001*	0.6244
Without Host	Mated	LC_{50} vs LC_{75}	0.2081	0.3197
		LC_{50} vs LC_{90}	0.0991	0.1594
		LC_{75} vs LC_{90}	0.8344	1.000
	Unmated	LC_{50} vs LC_{75}	0.4111	0.0566
		LC_{50} vs LC_{90}	0.0750	0.0026*
		LC_{75} vs LC_{90}	0.4220	0.6024

*Statistically significant ($P < 0.05$) differences within test conditions and pairings.

Table 6. Logistic regression (Generalized linear model) of proportion of mated and unmated nulliparous *Ae. aegypti* that successfully blood-fed and did not escape during contact and non-contact trials between the three different concentrations of deltamethrin with a live host present.

Parameter	df	Estimate	S.E.	Likelihood Ratio 95% C.I.		Wald Chi-Square	*P* value
Intercept	1	0.4308	0.1454	0.1481	0.7192	8.77	0.0031
CC	1	0.9845	0.2402	0.5207	1.4644	16.80	<.0001*
NC	1	0.4565	0.0363	0.2153	0.8814	4.50	0.0340*
CT	1	-0.1829	0.2771	-0.7251	0.3640	0.44	0.5091
NT	0	0.0000	0.0000	0.0000	0.0000	0.0	0.0 [1]
Mated	1	1.3122	0.2512	0.8301	1.8176	27.28	<.0001*
Unmated	0	0.0000	0.0000	0.0000	0.0000	0.0	0.0 [1]
LC_{50}	1	0.6532	0.2817	0.1121	1.2200	5.38	0.0204*
LC_{75}	1	0.1998	0.2600	-0.3054	0.7159	0.59	0.4421
LC_{90}	0	0.0000	0.0000	0.0000	0.0000	0.0	0.0 [1]
CC*Mated	1	-0.9783	0.3763	-1.7204	-0.2426	6.76	0.0093*
LC_{50}*Mated	1	-1.1565	0.4242	-1.9930	-0.3266	7.43	0.0064*
LC_{75}*Mated	1	-1.2497	0.3955	-2.0303	-0.4777	9.98	0.0016*

*Statistically significant ($P < 0.05$).

[1]If a column of the model matrix corresponding to a parameter is found to be linearly dependent, or *aliased*, with columns corresponding to parameters preceding it in the model, PROC GENMOD assigns it zero degrees of freedom and displays a value of zero for both the parameter estimate and its standard error.

DISCUSSION

This study focused on how the insemination of nulliparous (non-blood-fed) mosquitoes might influence behavioral responses (blood-feeding and escape) to residual deltamethrin depending on the presence or absence of live host cues. In other words, when presented with concurrent strong stimuli (chemical and host), whether insemination might have an effect on the escape response and blood-feeding success and suppress or delay the excitatory-deterrent effects of the insecticide. Potential confounders such as age and nutritional and physiological states were carefully controlled and environmental parameters (temperature, humidity, and light) were maintained within a defined range to limit potential non-study factors as confounders.

The *Ae. aegypti* used in this study were susceptible (mortality >97%) to deltamethrin at the recommended operational concentration (0.05%), thus removing physiological resistance as a potential confounder in analysis and interpretation. There was no significant difference in response between mated and unmated females for all three concentrations (0.002%, 0.01%, and 0.05%) indicating that whether insemination occurred had no effect on mortality. However, most published data indicate that behavioral responses to spatial repellent and contact irritant actions are independent of the toxic action of a compound (Achee et al. 2009). In Thailand, synthetic pyrethroids, including deltamethrin, have been the primary adulticides used to control *Aedes* mosquitoes in both the private and public domains (Chareonviriyaphap et al. 1999, Paeporn et al. 2005). Although *Ae. aegypti* appeared to show a slight tolerance to deltamethrin, these findings indicate that when applied to walls or other surfaces such as bednets, at a concentration of 0.05% or greater, this chemical would be effective as a control agent against this species in Pu Teuy Village and possibly elsewhere in Thailand.

A discussion of the merits of each analytical test used for comparisons of data sets for statistical inference is beyond the scope of this paper. In addition to sample size limitations, we could not control for all sources of potential variation, including inherent mosquito behavioral heterogeneity in response to external cues and chemicals, therefore some inference testing (distribution-free nonparametric methods, e.g., log-rank) used in this study was less powerful statistically compared to parametric approaches (e.g., GLM). Nevertheless, we assume samples were randomly drawn from a binomial distribution, that individual responses (means) were within normal approximations, and that all trials were mutually independent, which may not have been the case in all instances (Kramer et al. 2010). Although potential test-to-test variability (outside of sampling error) is of concern (e.g., day-to-day variation in response), the statistical options used to compare mixed

factorial data sets remains provisional until we have a much better understanding of the degree of extra-binomial dispersion and factors influencing mosquito biting/feeding behavior and avoidance (escape) response to sub-lethal concentrations of chemicals.

A variety of factors such as test designs and environmental and biological conditions at the time of testing have been shown to affect behavioral responses in mosquitoes (Sungvornyothin et al. 2001, Polsomboon et al. 2008). For example, intrinsic factors that can influence susceptibility and behavioral responses of female mosquitoes include carbohydrate (energy) reserves, age, blood, engorgement, time from last blood feeding, and gonotrophic status (Busvine 1964, Xue and Barnard 1999). All of these factors were controlled for as best as possible. The blood feeding response in *Ae. aegypti* can be inhibited for a period of time after sugar feeding (Klowden and Briegel 1994). The physiological state of mosquitoes has also been shown to be an important factor influencing escape movement from chemical-treated surfaces (Roberts et al. 1984, Sungvornyothin et al. 2001, Chareonviriyaphap et al. 2006). In particular, chronological age can influence both physiological susceptibility (toxicity) (Raffaele et al. 1958, Lines and Nassor 1991) and degree of excitation caused by insecticides (Busvine 1964). Few investigations have been performed on the effect of age on behavioral response to chemicals, but generally, older mosquitoes have been found to be less prone to react than younger females, possibly associated with lower or depleted energy reserves in later life (Kaschef 1970). In our study, all tests used only six-day-old females.

In contact tests with a host present, a clear dose response was seen showing female mosquitoes (mated and unmated) with significantly greater escape response as chemical concentrations increased. This dose effect was not as evident when tests were conducted without host presence (Table 6). At the highest concentration (0.05%), the greatest escape response was seen among unmated females with host present, 62.2% successfully exiting chambers within 60 min. Escape activity was generally lower in both mated and unmated mosquitoes with decreasing dose, thereby increasing the time mosquitoes were present in the chamber to potentially acquire a blood meal. Excitation responses are greatly reduced after a recent blood meal (Busvine 1964, Qutubuddin 1967, Roberts et al. 1984, Sungvornyothin et al. 2001, Kongmee et al. 2004, Chareonviriyaphap et al. 2006, Polsomboon et al. 2008), partly a consequence of the dramatic increase in body weight and less inclination to take flight. Under similar test conditions, unfed mosquitoes (mated and unmated) often demonstrated stronger excitation/repellent behavior than blood-fed females exposed to DDT (Busvine 1964). Reduced flight activity would hypothetically increase the chance of acquiring a lethal concentration while resting on a treated surface.

Without host stimuli present, mated and unmated mosquitoes showed very similar escape responses, ranging from 31% to 41% of mosquitoes exiting during a 60 min exposure to deltamethrin. Higher percent mortality (24.35%) was observed in unmated females that remained in the chamber exposed to the highest dose. The results on escape response to deltamethrin between unmated and mated mosquitoes are comparable to that reported by Kongmee et al. (2004) and Polsomboon et al. (2008) demonstrating a more pronounced contact excitation effect compared to spatial repellency.

For deltamethrin, repellency did not appear to play a strong role in escape compared to contact irritancy. Both mated and unmated *Ae. aegypti* demonstrated very weak escape responses, with or without host, in nearly all non-contact trials compared to matched controls. Pyrethroids, in general, perform primarily as contact irritants and have relatively poor spatial repellent qualities (Grieco et al. 2007). *Ae. aegypti* mosquitoes may also have a greater tolerance (i.e., less sensitive) to exogenous chemicals detected from a distance compared to other mosquito species. *Anopheles* mosquitoes have also demonstrated strong irritancy to certain insecticides compared to generally much weaker repellency; nevertheless, in many cases repellency was still shown to be a more significant action compared to paired non-contact controls (Chareonviriyaphap et al. 1997, 2001, 2004, Sungvornyothin et al. 2001).

In contact escape, *Ae. aegypti* showed no significant differences between mated and unmated females at all concentrations without a host and at LC_{50} and LC_{75} with a host. Only at the highest dose were differences seen that might be attributed to mating condition. Despite potential knockdown and strong contact excitatory action of deltamethrin, mosquitoes that acquired a lethal dose may have been influenced by attraction to the host animal to remain longer inside the chamber. We surmised that mated mosquitoes, in particular, may be more inclined to remain longer inside treated chambers because of the attractive emanations produced by the guinea pig. Host availability in the presence of a strong excito-repellent insecticide may be potentially more hazardous to attracted mosquitoes. On the other hand, unmated mosquitoes in the presence of a host demonstrated significantly higher escape response than mated females exposed to the highest concentration. This may be attributed to unmated females' greater preference for insemination before initiating a search for blood meals. Movement patterns of mated *Ae. aegypti* that enter pyrethroid-sprayed structures with human hosts present demonstrate that insecticides showing relatively poor repellency may not prevent mosquitoes from entering, but that strong contact irritant action can cause them to prematurely exit the treated house before taking a blood meal (Grieco et al. 2007).

The difference in patterns of escape between mated and unmated females in the presence of a host was statistically significant for deltamethrin only at the highest contact concentration and not significant when the host was absent. This would indicate host stimuli become less effective attractants to unmated females when exposed to increased chemical concentrations. We speculate that the different patterns of escape behavior between mated and unmated females in the presence of a host may reflect differences in

response to host cues and the effects of post-insemination (i.e., virgin mosquitoes being less responsive than mated females). Degree of host attractiveness to blood-seeking *Ae. aegypti* has been shown to influence movement patterns in and out of experimental huts depending on the type of animal host used in the experiment (Suwonkerd et al. 2006). *Ae. aegypti* is a highly anthropophilic species and is far more likely to feed on humans than other animal hosts (Christophers 1960, Harrington et al. 2001, Ponlawat and Harrington 2005). Using only guinea pigs in this study may not reflect as accurately the true response of female *Ae. aegypti* if presented with alternative or more preferred hosts; therefore, extrapolation of these results to natural conditions should be used with caution. Moreover, even generalized behavioral responses of *Ae. aegypti* to sub-lethal doses of deltamethrin may not reflect those in other mosquito species, particularly *Anopheles*. The current test design using exogenous chemicals that may disrupt normal behavioral patterns combined with host cues to study feeding response needs to be investigated further with alternative chemicals and mosquito species.

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Locomotor Behavioral Responses of *Anopheles minimus* and *Anopheles harrisoni* to Alpha-Cypermethrin in Thailand

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LOCOMOTOR BEHAVIORAL RESPONSES OF *ANOPHELES MINIMUS* AND *ANOPHELES HARRISONI* TO ALPHA-CYPERMETHRIN IN THAILAND

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ABSTRACT. Excito-repellency responses of 3 test populations, representing 2 sibling species within the Minimus Complex, *Anopheles minimus* and *An. harrisoni*, were characterized for contact irritant and noncontact repellent actions of chemicals during and after exposure to α-cypermethrin at half the recommended field (0.010 g/m^2), the recommended field (0.020 g/m^2), and double the recommended field concentration (0.040 g/m^2), using an excito-repellency escape chamber system. Two field populations of *An. minimus* and *An. harrisoni* collected from the malaria-endemic areas in Tak and Kanchanuburi provinces in western Thailand, respectively, were tested along with a laboratory population of *An. minimus* maintained since 1993. Females of all 3 test populations rapidly escaped after direct contact with treated surfaces for each concentration. In general, increased escape responses in the *An. minimus* test populations were proportionate to increased insecticide dosages. The greatest escape response for *An. harrisoni* was observed at the operational field concentration of α-cypermethrin. The noncontact repellency response to α-cypermethrin was comparatively weak for all 3 test populations, but significantly different from each paired contact test and respective noncontact controls. We conclude that strong contact irritancy is a major action of α-cypermethrin, whereas noncontact repellency plays no role in the escape responses of 2 species in the Minimus Complex in Thailand.

KEY WORDS *Anopheles minimus*, *Anopheles harrisoni*, behavior, alpha-cypermethrin, excito-repellency

INTRODUCTION

In spite of significant achievements in malaria control in the past decades, approximately 30,000–40,000 confirmed malaria cases occur in Thailand annually. Malaria remains prevalent along the relatively undeveloped borders between Thailand and eastern Myanmar, northern Malaysia, and western Cambodia (Chareonviriyaphap et al. 2000; DDC 2006, 2010). Approximately 70% of all malaria cases are reported along the Myanmar border where the *Anopheles minimus* complex is abundant and plays an important role in disease transmission (DDC 2010, Manguin et al. 2010).

Two sibling species, *An. minimus* Theobald and *An. harrisoni* Harbach and Manguin, within the Minimus Complex are found in sympatry in Pu Teuy Village, Kanchanaburi Province, western Thailand (Sungvornyothin et al. 2006b, Manguin et al. 2010). *Anopheles minimus* has a much wider geographic distribution and is a primary vector of malaria throughout much of its range, whereas *An. harrisoni* is more restricted and focal in occurrence and appears to play a more minor role in malaria transmission (Manguin, personal communication). In Thailand, vector control has been a primary method for malaria abatement. For decades, DDT was used for indoor residual spraying (IRS) but was withdrawn in 2000 based on perceived adverse impact on the environment and reports of changing behavioral response of mosquito vectors to the chemical (Ismail et al. 1975, Bang 1985, Chareonviriyaphap et al. 2000). Various synthetic pyrethroids have since gained general acceptance in Thailand for use in re-treatment of bed nets (permethrin) and for IRS (deltamethrin) (Patipong 2000, DDC 2010). The prospect of continuing wide-scale use of pyrethroid compounds is a major stimulus for continuing studies on the effectiveness and impact of residual deposits on treated surfaces on mosquito vector behavior in Thailand.

A better understanding of the behavioral responses of individual species within the complex will facilitate vector control by selecting and implementing the most sustainable and effective interventions possible (Chareonviriyaphap et al. 2004, Sungvornyothin et al. 2006b, Polsomboon et al. 2008). Previous studies on behavioral responses of mosquitoes to chemicals has emphasized the importance of excitation and repellency (''avoidance behavior'') as having a critical role in mosquito-borne disease prevention (Roberts et al. 2000; Chareonviriyaphap et al. 2001; Pothikasikorn et al. 2005; Thanispong et al. 2009, 2010). In general, behavioral responses can be categorized

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into at least 2 distinct types: contact stimulation ("excitation," "irritancy") and noncontact spatial repellency ("deterrency") (Roberts et al. 1997, 2000; Grieco et al. 2007). The various locomotor responses (effects) of insects to chemical stimulants have been refined further by Miller et al. (2009), but this study was restricted to contact and noncontact outcomes (escape or not) and subsequent toxicity 24 h postexposure.

Behavioral responses to insecticides have been documented with various mosquito species and populations of *Anopheles* from Thailand using the excito-repellency test box (Pothikasikorn et al. 2005, Muenworn et al. 2006, Sungvornyothin et al. 2006b, Polsomboon et al. 2008). Herein, we describe for the first time the behavioral responses of *An. minimus* and *An. harrisoni*, exposed to 3 different concentrations of α-cypermethrin using an excito-repellency test system measuring escape response over time. Alpha-cypermethrin is currently approved for public health use in interrupting malaria transmission as both an indoor residual spray for treatment or bed nets (WHO 2005).

MATERIALS AND METHODS

Mosquitoes

Anopheles minimus *(field population): Anopheles minimus* was collected from Ban Tum Sua Village, Mae Sot District, Tak Province, northern Thailand (16°41′N, 98°41′E), approximately 426 km north of Bangkok (Fig. 1). The rural collection site is surrounded by agricultural activities on the east and by the intact forest on the west. Female mosquitoes were collected off human volunteers during evening hours (1800 to 0600 h). These volunteers worked for the Ministry of Public Health. The wild population of *An. minimus* was susceptible to α-cypermethrin (Tisgratog, personal communication).

Anopheles harrisoni *(field population): Anopheles harrisoni* was collected from Ban Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14°17′N, 99°11′E). This area is surrounded by either steep mountainous terrain or agricultural fields. A 2-m-wide, slow-running stream with a vegetation margin along its margin is the primary larval habitat of *An. harrisoni* (Sungvornyothin et al. 2006b). Female mosquitoes were collected from cow-baited traps during the evening hours (1800 to 0600 h). The wild population of *An. harrisoni* was susceptible to α-cypermethrin (Tisgratog, personal communication).

Anopheles minimus *(laboratory colony):* This colony was originally collected from domesticated animal quarters in Rong Klang District, Prae Province, northern Thailand, in 1993. Since 1995, the colony has been maintained at the insectary at the Vector-Borne Disease Bureau, Department of Disease Control (DDC), Ministry of Public Health, Nonthaburi, Thailand. This same colony was obtained from DDC and was maintained in the insectary at Department of Entomology, Faculty of Agriculture, Kasetsart University, during this study. This colony was completely susceptible to α-cypermethrin (Tisgratog, personal communication).

Morphological and molecular species identification

Female mosquitoes were identified using morphological criteria and DNA analysis techniques (Rattanarithikul et al. 2006, Sungvornyothin et al. 2006a). All field-caught mosquitoes within the Minimus Complex were initially identified by the presence or absence of the humeral pale spot (HP) on the costal vein of the wings. *Anopheles minimus* lacks the HP, whereas *An. harrisoni* has the HP on at least 1 wing. Subsequently, all test specimens used in the experiment were individually subjected to DNA extraction and molecular analysis performed by the allele-specific assay–polymerase chain reaction as previously described (Garros et al. 2004, Sungvornyothin et al. 2006a).

Insecticide-treated papers

Recommended diagnostic concentration of α-cypermethrin (0.03 g/m^2) (Najera and Zaim 2002) was impregnated onto Whatman® No. 1 filter papers (Whatman International Ltd., Banbury, United Kingdom) measuring 12 × 15 cm for the World Health Organization (WHO) susceptibility test and 0.01, 0.02, and 0.04 g/m^2 concentrations applied to separate filter papers measuring 15 × 17.5 cm for the excito-repellency tests. All treated papers were prepared using acetone diluents according to WHO specifications (WHO 1998, 2006) and treated with 2 ml of prepared insecticide solution per 180-cm^2 paper surface area. Control papers were treated with carrier diluents only (acetone). All papers were allowed to air-dry for 24 h and used in tests within 48 h of preparation.

Behavioral tests

Tests were carried out to compare all 3 mosquito populations in contact and noncontact test designs using 3 different serial concentrations of α-cypermethrin (0.01, 0.02, and 0.04 g/m^2), respectively. Identical test chambers (4 per test trial) were used for all excito-repellency assays as previously described (Fig. 2) (Chareonviriyaphap et al. 2002, Thanispong et al. 2009).

Each test cycle had 2 α-cypermethrin–treated test chambers and 2 paired control boxes without active ingredient. Each assay chamber had 15 nonblooded female mosquitoes carefully introduced into each of 4 chambers using a mouth

Fig. 1. Collection sites in Kanchanaburi (Pu Teuy) and Tak (Tum Sua) provinces, Thailand.

aspirator. A receiving cage (6 × 6 × 6 cm) paper box was connected to the single exit portal to collect any escaping mosquitoes. At the beginning of the trial, a 3-min rest/acclimation period was used to permit mosquitoes to adjust to surrounding test conditions (Chareonviriyaphap et al. 2001). After 3 min, the escape portal (funnel) was opened to begin the 30-min observation period. All mosquitoes escaping from exposure chambers into the receiving cages were recorded in 1-min intervals until test completion. All assays were performed during daylight hours between 0900 and 1600 h under identical environmental conditions (ambient temperature and relative humidity) as possible. Each test series was replicated 4 times (total 60 mosquitoes each). Immediately after a 30-min exposure, the number of dead/knockdown specimens inside the chambers and those that had escaped into the receiving boxes were recorded separately for each treated and control chamber. Escaping and nonescaping mosquitoes were held separately (by test condition) for 24 h to record postexposure mortality.

Data analysis

All mean percentage escape calculations from treatment chambers were adjusted using Abbott's

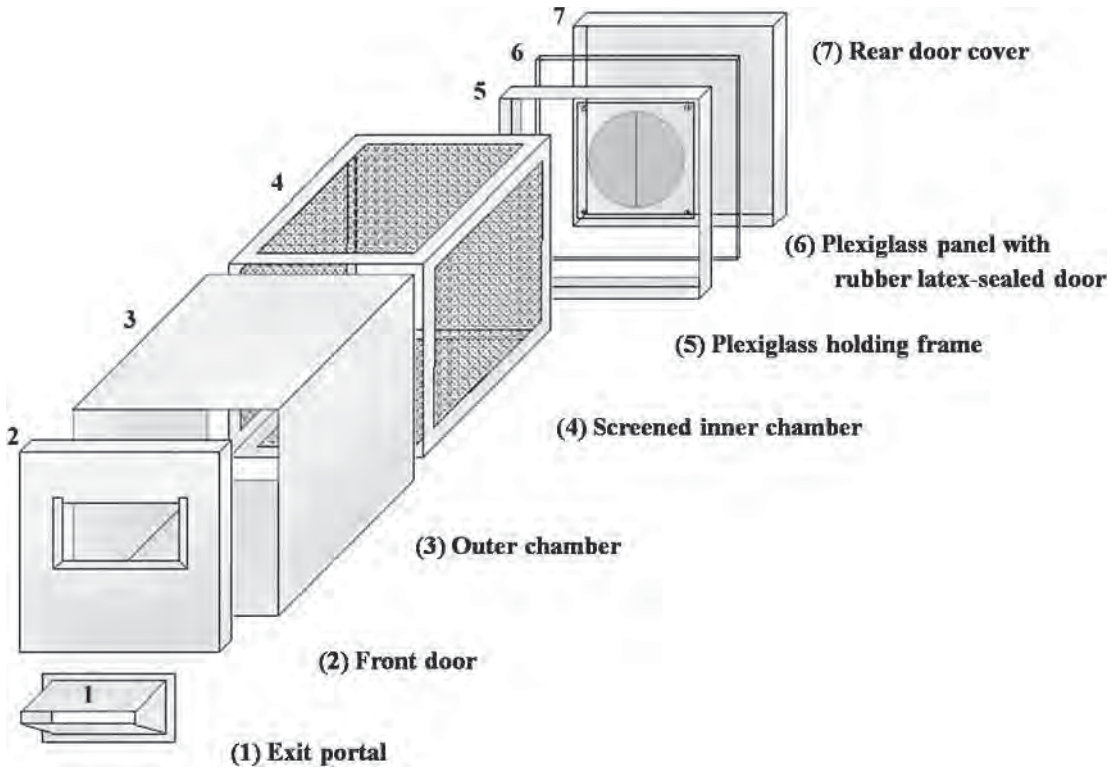

Fig. 2. Excito-repellency test chamber setup used in the excito-repellency study.

formula (Abbott 1925) based on paired control escape and then subjected to life-table survival analysis to estimate mosquito escape rates based on each test design (test population, chemical concentration, contact and noncontact). Differences in escape among the 3 test populations and 3 chemical concentrations were compared (Roberts et al. 1997). The escape time (ET) in minutes for 50% (ET_{50}), 75% (ET_{75}), and 90% (ET_{90}) of test population to escape was also calculated. A log-rank method (Mantel and Haenzel 1959) was used to compare patterns of escape behavior between tests using a SAS statistical package (Release 6.12; SAS Institute, Cary, NC). The discriminating level of significance for all tests was set at $P < 0.05$.

RESULTS

This study was designed to compare the behavioral responses of 2 closely related mosquito species within the Minimus Complex, representing 3 test populations. Two test populations belong to *An. minimus* (field and laboratory colony) and 1 field population of *An. harrisoni*. For confirmation of status, field-collected material was subjected to DNA analysis for accurate species identification.

The percentage of escaping females from each population against the 3 different concentrations of α-cypermethrin in contact and noncontact trials is presented in Tables 1 and 2, respectively. Significant escape responses were observed in all contact trials compared with matching noncontact trials across all chemical concentrations ($P < 0.05$). In contact tests, both populations of *An. minimus* females showed clear dose–response activity with significantly ($P < 0.05$) stronger escape responses as chemical concentrations increased from 0.01 g/m^2 (55.93–56.67%), 0.02 g/m^2 (67.27–92.98%), and 0.04 g/m^2 (70.9–92.45%). Yet, when viewed as a mean percent escape for all 3 populations combined, the median concentration (0.02 g/m^2) showed the greatest escape response (82.07%) compared to the lowest and highest dose. The difference in percent escape between median and highest dose was not statistically significant. The highest percent escape response (85.97%) seen in *An. harrisoni* was at the median concentration (0.02 g/m^2) and was found significantly different from that of 0.01 g/m^2 (60.35%) and 0.04 g/m^2 (73.68%) concentrations. Higher mortalities after the 24-h holding period were observed from nonescaping mosquitoes (maximum 35.29%) compared to those escaping within 30 min (maximum 9.3%). All 3 populations taken in aggregate showed a clear and significant dose–response in mean percent mortality among mosquitoes that failed to escape (1.15% at 0.01 g/m^2, 1.45% at 0.02 g/m^2,

Table 1. Mean percent escape responses at 30-min exposure and 24-h mortality of 3 test populations of the Minimus Complex exposed to α-cypermethrin in contact trials.

Population	Chemical concentration[1] (g/m^2)	No. escaped (%)[2]	% mortality	
			Escaped	Not escaped
Anopheles minimus (laboratory)	T: 0.01	56.67	0	7.69
	C: 0.01	0	0	0
An. minimus (field)	T: 0.01	55.93	0	0
	C: 0.01	1.67	0	0
An. harrisoni (field)	T: 0.01	60.35	3.45	16.13
	C: 0.01	3.33	0	0
An. minimus (laboratory)	T: 0.02	92.98	0	25
	C: 0.02	5	0	0
An. minimus (field)	T: 0.02	67.27	2.44	15.79
	C: 0.02	8.33	0	0
An. harrisoni (field)	T: 0.02	85.97	1.92	12.5
	C: 0.02	5	0	0
An. minimus (laboratory)	T: 0.04	92.45	3.57	50
	C: 0.04	11.67	0	0
An. minimus (field)	T: 0.04	70.91	2.72	25
	C: 0.04	8.33	0	0
An. harrisoni (field)	T: 0.04	73.68	9.3	35.29
	C: 0.04	5	0	0

[1] T, treatment; C, control.
[2] Adjusted rate based on control response.

and 5.20% at 0.04 g/m^2). For those mosquitoes successfully escaping, mortality varied only slightly from lowest to highest concentrations (1.92–9.3%).

In noncontact spatial repellency trials (Table 2), escape responses from all 3 populations and concentrations were significantly lower compared to the contact exposures. The highest percent escape was seen at the median dose across all 3 test populations and was the only concentration that produced an escape response significantly different from paired controls. As seen in the contact trials, when viewed as a mean percent escape for all 3 populations combined, the median concentration (0.02 g/m^2) showed the greatest escape response (11.58%) compared to the lowest and highest dose. In fact, the highest concentration had a lower adjusted mean percentage escape (2.36%) than the lowest dose (3.45%) despite a 4-fold difference in concentra-

Table 2. Mean percent escape responses at 30-min exposure and 24-h mortality of 3 test populations of the Minimus Complex exposed to α-cypermethrin in noncontact trials.

Population	Chemical concentration[1] (g/m^2)	No. escaped (%)[2]	% mortality	
			Escaped	Not escaped
Anopheles minimus (laboratory)	T: 0.01	5.08	0	0
	C: 0.01	1.67	0	1.72
An. minimus (field)	T: 0.01	5.26	0	0
	C: 0.01	5	0	0
An. harrisoni (field)	T: 0.01	0.0	0	1.72
	C: 0.01	5	0	0
An. minimus (laboratory)	T: 0.02	17.55	0	0
	C: 0.02	5	0	0
An. minimus (field)	T: 0.02	7.02	0	5.55
	C: 0.02	5	0	0
An. harrisoni (field)	T: 0.02	10.17	0	0
	C: 0.02	1.67	0	0
An. minimus (laboratory)	T: 0.04	3.57	0	0
	C: 0.04	6.67	0	0
An. minimus (field)	T: 0.04	3.51	0	0
	C: 0.04	5	0	0
An. harrisoni (field)	T: 0.04	0.0	0	0
	C: 0.04	6.67	0	0

[1] T, treatment; C, control.
[2] Adjusted rate based on control response.

Table 3. Escape time (ET) in minutes for 50% (ET_{50}), 75% (ET_{75}), and 90% (ET_{90}) of 3 test populations of the Minimus Complex to exit contact chambers treated with α-cypermethrin at 3 different concentrations.

Population	0.01 g/m²			0.02 g/m²			0.04 g/m²		
	ET_{50}	ET_{75}	ET_{90}	ET_{50}	ET_{75}	ET_{90}	ET_{50}	ET_{75}	ET_{90}
Anopheles minimus (laboratory)	29	—[1]	—	6	14	19	7	15	27
An. minimus (field)	23	—	—	10	—	—	3	—	—
An. harrisoni (field)	19	—	—	10	21	—	4	28	—

[1] Insufficient number of mosquitoes escaped from exposure chamber within 30 min.

tion (g/m²). Comparatively low mortalities after the 24-h holding period were observed from nonescaping females of the 3 populations (0.0–5.55%). Combined, all 3 populations produced a high mean mortality of only 1.85% at 0.02 g/m².

Escape times in minutes for proportions of test mosquitoes to escape from treated chambers are given in Table 3. At all 3 concentrations of α-cypermethrin, each mosquito strain had at least 50% of the females escape within 30 min of exposure and each showed a clear gradient of decreased escape time in minutes as chemical concentration increased. Collectively, ET_{50} mean time to escape was 23.7, 8.7, and 4.7 min at 0.01, 0.02, and 0.04 g/m², respectively. Numbers of escaping mosquitoes were insufficient in some cases to produce mean escape times at 75% and 90% of test populations to escape. At the median and highest concentrations, the *An. minimus* colony population produced the highest escape rate while the *An. minimus* field strain appeared the least inclined to exit the chambers. In noncontact trials, ET_{50}, ET_{75}, and ET_{90} values could not be calculated due to the low number of specimens successfully leaving the chamber within the 30-min observation time.

Using survival analysis results from escape data collected, Figs. 3 and 4 illustrate the proportions (as probabilities) of mosquitoes by test population remaining in the exposure chambers at 1-min intervals at different insecticide concentrations for contact and noncontact trials, respectively. For all pairings, significant differences in escape patterns were seen between contact trials and matched controls and noncontact trials comparing same population and chemical concentration ($P < 0.05$) (Fig. 3). The median concentration performed the best in

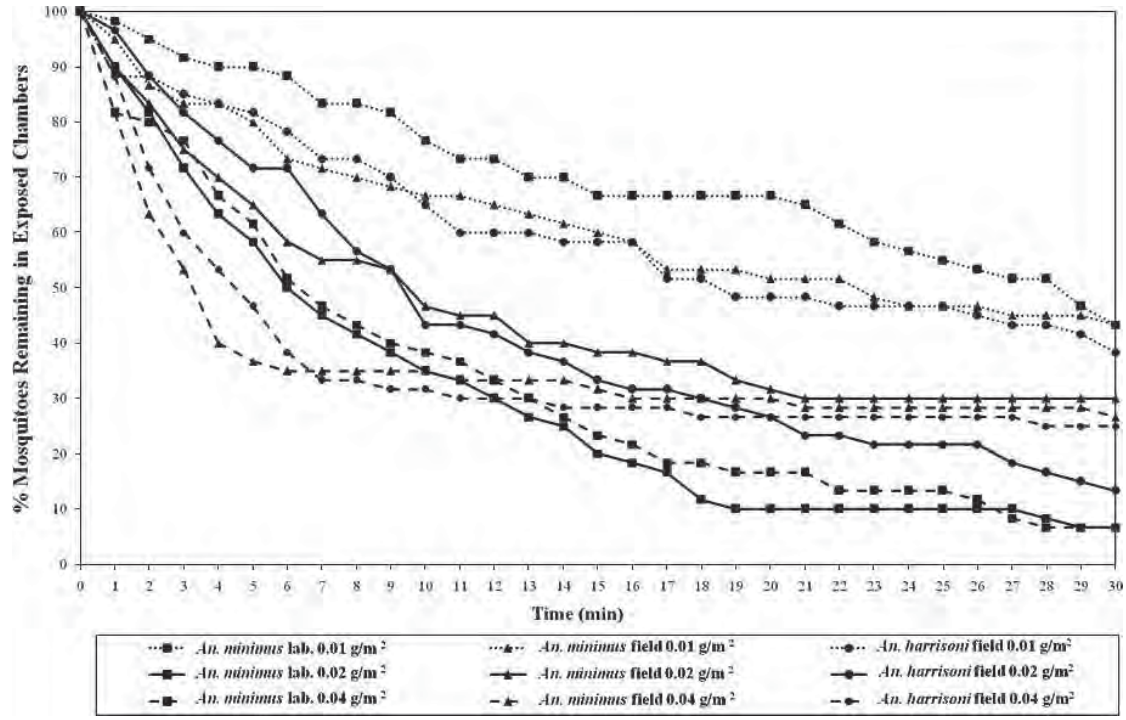

Fig. 3. Escape probability of 3 test populations of *Anopheles minimus* (laboratory), *An. minimus* (field), and *An. harrisoni* (field) in contact trials and respective paired controls against α-cypermethrin at 0.01, 0.02, and 0.04 g/m².

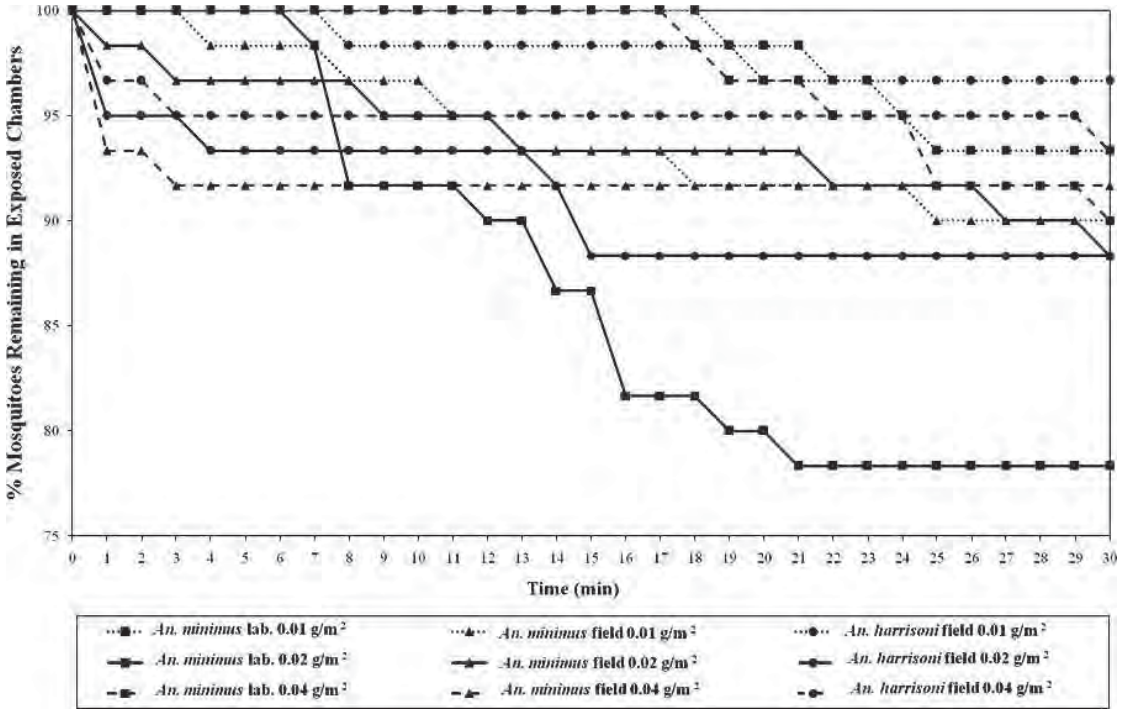

Fig. 4. Escape probability of 3 test populations of *Anopheles minimus* (laboratory), *An. minimus* (field), and *An. harrisoni* (field) in noncontact trials and respective paired controls against α-cypermethrin at 0.01, 0.02, and 0.04 g/m^2.

overall percent escape with all 3 populations. Conversely, in noncontact assays, no significant differences were evident in escape patterns among the 3 test populations and insecticide concentrations compared to paired controls, except at the median concentration (0.02 g/m^2) (Fig. 4).

DISCUSSION

The study of insect behavior is a complicated and complex endeavor. Historically, even the technical terms surrounding locomotor responses to contact with sublethal concentrations of neurotoxic insecticides have generated significant discussion to more accurately define and differentiate between behavioral outcomes (end results) and their mediating mechanisms (causes) (Dethier et al. 1960, Kennedy 1977, Roberts et al. 1997, Grieco et al. 2007, Miller et al. 2009). In this study, the behavioral responses of *An. minimus* and *An. harrisoni* with exposure to α-cypermethrin measured only outcomes (effects) of exposure and not the possible causes (both kinetic and tactic mechanisms) of the chemical stimulants on female mosquitoes. Although understanding the actual mechanisms of how chemicals elicit response outcomes is important, this study made no attempt to either define or speculate on potential mechanism(s) involved in the recorded outcomes. Therefore, we acknowledge that more broad, mechanistically neutral terms (e.g., contact and noncontact ''disengagement'') are more appropriate in the larger context of this study when describing the behavioral consequences in response to a stimulus (Miller et al. 2009).

The current use of more subjective terminology describing hyper-locomotor activity such as *avoidance*, *excitation*, *irritancy*, *deterrency*, and *excito-repellency* (e.g., studies on adult female mosquitoes exposed to insecticides) are not intended to convey either teleological connotations (intent) on the part of the insect or deliver an implied or contradictory view on the usage of more specific technical terms used in other branches of behavioral science. For purposes of an ''excito-repellency'' test system study design, we used the following terminology to distinguish 2 primary locomotor responder effects: (1) locomotor stimulation (''excitation,'' ''irritancy'') resulting from direct physical tarsal contact with chemical-treated surfaces; and (2) noncontact spatial repellency (''deterrency'') resulting from chemical emanations (i.e., vapor phase, perceived ''odors''), operating at a distance without need for an insect to make direct physical contact with a chemically treated source. Until we have a greater understanding of the behavioral stimuli, effects, and mechanism(s) involved in mosquito locomotor responses, we remain relegated and content to continue use of more general terminology.

In this study, locomotor behavioral responses of 2 species within the Minimus Complex, representing 2 field populations of *An. minimus* and *An. harrisoni* and a long-established laboratory colony of *An. minimus*, against 3 serial concentrations of α-cypermethrin (at half the recommended operational rate of 0.01 g/m^2, an operational field rate of 0.02 g/m^2, and doubled operational rate of 0.04 g/m^2) were measured using an established excito-repellency test system. At all 3 concentrations, α-cypermethrin performed as a potent contact locomotor stimulant wherein exposed mosquitoes from all 3 populations showed markedly abnormal high kinetic activity arising from initial sublethal chemical exposure. Conversely, α-cypermethrin performed very poorly as a noncontact spatial repellent. At the lower (0.01 g/m^2) and higher (0.04 g/m^2) concentrations, noncontact repellency played no meaningful role in the escape response of the 3 populations, thus indicating that α-cypermethrin applied at these rates would likely not deter mosquitoes spatially from entering a treated area. Interestingly, the median concentration (0.02 g/m^2) recommended for operational use (Najera and Zaim 2002) produced a greater escape response than a doubling of the concentration in both the contact and noncontact trials (Fig. 3). However, in the same contact tests, 24-h mortality was significantly greater at the higher than at the median concentration (36.7% and 17.7%, respectively). This inverse relationship was not seen in the noncontact tests as overall mortality was very low. We surmise that at the highest concentration, dose–response toxicity played a greater role during the 30-min exposure (contact) time, thus affecting the ability of mosquitoes to successfully escape the treated chambers.

Behavior-modifying properties of synthetic pyrethroids are known to vary depending upon the concentration used (Grieco et al. 2005, Miller et al. 2009). The mean time elapsed for 50% of the combined test populations to exit the treated chambers was inversely related to chemical concentration, i.e., as chemical concentration increased, time to escape decreased. From lowest to highest concentrations (a 4-fold difference), mean time to escape for all 3 populations showed an approximate 5-fold variance (4.7 and 23.7 min, respectively). The most striking escape rates occurred in both field populations shortly after making physical contact with the highest concentration of α-cypermethrin (ET_{50} = 3 and 4 min for *An. minimus* and *An. harrisoni*, respectively). Overall, the laboratory colony appeared to be the most responsive of the 3, and was the only population to show 90% escape at the median and highest concentrations (Table 3). Successful escape of mosquitoes at or above 75% was lower across all 3 populations. As shown in the 24-h mortality data, a clear dose–response relationship was seen in both increased escape and nonescape mosquito mortality as concentration increased. As with increased mortality, higher chemical concentration may have also resulted in greater disorientation effects on flight behavior that promoted, delayed, or prevented escape from the chambers.

In general, the laboratory colony was more sensitive to all 3 concentrations compared to the 2 field-caught populations. However, the difference seen between a long-standing laboratory strain of uniform age and genetic integrity is likely not a valid comparison with field-collected mosquitoes of mixed age and unknown physiological status. One limitation to this study design was not estimating age of wild-caught mosquitoes using standard dissection techniques to measure parity.

Grieco et al. (2007) reported that contact "irritancy" is the major action of α-cypermethrin tested against *Aedes. aegypti* (L.) and caused mosquitoes to exit the experimental hut at a much higher number than untreated controls. With our findings, we conclude that direct contact with α-cypermethrin, below, at, and above the recommended operational rate, results in a significant behavioral response (hyperexcitation and movement away from the chemical source) among all 3 mosquito populations tested. However, α-cypermethrin appears to play little or no role in spatial repellency in Minimus Complex mosquitoes tested.

It has been documented that insecticides can protect humans from the bites of blood-sucking insects using actions other than toxicity, namely direct contact locomotor stimulation and spatial repellency (Roberts et al. 2000, Grieco et al. 2007). Behavioral responses to various insecticides have been observed in several species of *Anopheles* mosquitoes in Thailand (Chareonviriyaphap et al. 2001, 2004; Pothikasikorn et al. 2005; Muenvorn et al. 2006; Polsomboon et al. 2008) but none had yet investigated the responses of individual sibling species within the Minimus Complex. Correct sibling species identification is considered an essential prerequisite for any investigation linking biological observations to specific disease vectors (Van Bortel et al. 2000, Manguin et al. 2008).

Understanding the behavior, ecology, and natural habits of mosquito vectors is of epidemiological and operational significance and inherently useful to vector control authorities in understanding the conditions and factors where a vector might be most vulnerable for controlling disease transmission (Roberts and Andre 1994). Behavioral responses of mosquito vectors exposed to insecticides used in disease control and their modes of action remain poorly understood and, in general, an underappreciated phenomenon (Grieco et al. 2007). How a chemical functions, including possible behavior-modifying properties on disease vectors, is important

information and serves as criteria for decisions on insecticide selection and operational planning for directing the most cost-efficient and sustainable vector control interventions possible.

In summary, significant behavioral avoidance responses were seen in contact trials with α-cypermethrin compared with paired controls and noncontact trials, with the highest 30-min escape response observed from *An. minimus* laboratory colony. However, minimal yet significant noncontact repellency was observed at the operational field dose in all 3 test populations; therefore, spatial repellency may play a minor role in escape from treated structures. We conclude that contact excitation is an important behavioral response in *An. minimus* complex species tested in Thailand when exposed directly to α-cypermethrin within operationally relevant concentrations. Although pyrethroids are currently the predominant insecticides of choice for use in IRS and treatment of bed nets in Thailand, the development of resistance by different mosquito vectors has increased dramatically to this general class of compounds (Chareonviriyaphap et al. 2002). Therefore, the use of α-cypermethrin and other residual pyrethroids for control of malaria vectors should be routinely evaluated for both physiological (toxicity) and behavioral responses.

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Chemically induced behavioral responses in *Anopheles minimus* and *Anopheles harrisoni* in Thailand

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ABSTRACT: Behavioral responses of female mosquitoes representing two species in the Minimus Complex exposed to an operational field dose of bifenthrin or DEET (*N,N*-diethyl-m-toluamide) were described using an excito-repellency test system. Two test populations of *An. minimus*, one from the field (Tak Province, western Thailand), the other from a long-established laboratory colony, and *Anopheles harrisoni* collected from Kanchanaburi Province, western Thailand, were used. Results showed that all test populations rapidly escaped after direct contact with surfaces treated with either bifenthrin or DEET compared to match-paired untreated controls. Greater escape response by exposed females to bifenthrin and DEET were observed in the *An. minimus* colony compared to the two field populations. Field-collected *An. minimus* demonstrated a more rapid escape response to DEET than to bifenthrin, whereas *An. harrisoni* showed a converse response. Although fewer females escaped from test chambers without direct contact with treated surfaces compared to contact tests, the spatial repellency response was significantly pronounced in all test populations compared to match-paired controls ($P < 0.05$). DEET was found to perform as both a contact stimulant and moderate spatial repellent. ***Journal of Vector Ecology* 36 (2): 321-331. 2011.**

Keyword Index: Behavioral avoidance, bifenthrin, DEET, *Anopheles minimus*, *Anopheles harrisoni*, Thailand.

INTRODUCTION

Although the incidence of malaria in Thailand has been significantly reduced during the past 50 years, malaria remains prevalent in some areas, especially in secondary scrub and forest hill environments along undeveloped stretches of the international borders with Myanmar, Cambodia, and Malaysia (Chareonviriyaphap et al. 2000, MOPH 2009). Over 70% of malaria cases have been recorded along the Thai-Myanmar border area, especially in Tak and Kanchanaburi provinces where high transmission periodically occurs (Manguin et al. 2010). The sustained malaria endemicity has been exacerbated by agricultural-based activity from uncontrolled cross-border population movements and recurring political unrest (MOPH 2009). Several important vectors of malaria are found in this area, including two members of the *Anopheles minimus* complex, collectively important malaria vectors in forest and scrub areas (Baimai 1989, Chareonviriyaphap et al. 2000, Kengluecha et al., 2005, Sungvornyothin et al. 2006a, Sungvornyothin et al. 2006b).

Anopheles minimus, Theobald 1901 is a widely distributed species complex in Southeast Asia (Subbarao 1998, Van Bortel et al. 1999, Theophil et al. 2002, Manguin et al. 2010), composed of at least two different sibling species, *An. minimus* (formerly species A) and *An. harrisoni* (formerly species C) in Thailand (Harbach 2004, Somboon et al. 2005, Garros et al. 2006, Sungvornyothin et al. 2006a), both of which can be easily separated using molecular methods of identification (Garros et al. 2004). *Anopheles minimus* is the predominant species found throughout most of Thailand, whereas *An. harrisoni* is restricted to the western Thai-Myanmar border, most notably in Kanchanaburi Province (Kengluecha et al. 2005). Although the geographical distribution of *An. minimus* and *An. harrisoni* in Thailand is known, important bionomic aspects of each species within the Minimus Complex remain poorly understood, especially feeding and resting behaviors, host preference, vector competence, and responses to insecticides (Chareonviriyaphap et al. 2004, Garros et al. 2006, Sungvornyothin et al. 2006b). Understanding the behavioral responses of a clearly defined species within the complex can facilitate vector control strategies by selecting and implementing the most appropriate interventions possible (Kongmee et al. 2004, Sungvornyothin et al. 2006b). Behavioral responses can be divided into two

distinct forms; contact stimulants or excitants (sometimes referred to as irritancy) and non-contact spatial repellency (Roberts et al. 1997). Contact irritancy results from physical contact with chemically-treated surfaces, whereas spatial repellency is a movement away (avoidance) from a chemical substance detected from a distance without making physical contact. Excito-repellency, a composite term that has been in common use for decades, is more appropriately defined in terms of an endpoint or multiple endpoints (i.e., movement away from a treated surface or area because of excitatory effects caused by chemical exposure) involving a series of responses to a stimulus and one or more combination of behavioral mechanisms. Moreover, terms with either anthropomorphic or teleological connotations, such as contact irritancy, are defined and synonymous with excitation, while avoidance is more aptly applied to deterrence or repellency.

Pyrethroids have been widely used for controlling disease vectors due to their relatively low mammalian toxicity, broad spectrum efficacy in controlling indoor mosquito populations, and affordability (Elliott et al. 1978, Roberts and Andre 1994, Hemingway and Ranson 2000). Most pyrethroids act as strong locomotor stimulants and exhibit a strong excito-repellency action in many mosquito species resulting in physical displacement (avoidance) from treated surfaces (Roberts et al. 2000, Chareonviriyaphap et al. 2004). However, the behavioral mechanisms for deterrence has not been determined and may likely vary for different species exposed to different compounds and varying concentrations.

Although behavioral responses have been recorded with various mosquito species of *Anopheles* from Thailand using the excito-repellency test chamber (Chareonviriyaphap et al. 1997, 2001, 2004, Muenvorn et al. 2006, Sungvornyothin et al. 2006b, Pothikasikorn et al. 2005, 2007, Polsomboon et al. 2008), none have observed the behavioral responses to bifenthrin, a newer generation broad spectrum synthetic pyrethroid with very low vapor pressure characteristics. DEET was selected for evaluation given this chemical's role as a strong repellent to many species of arthropods, including mosquitoes, and that it had not yet been evaluated in the excito-repellency test system. In this study, we observed the behavioral responses of two field-caught populations, *An. minimus* and *An. harrisoni*, and a long-established laboratory colony of *An. minimus*, against the recommended field concentration of bifenthrin, a synthetic pyrethroid residual insecticide used in insect control applications, and a test concentration of DEET (*N,N*-diethyl-m-toluamide), a common active ingredient used for mosquito bite protection, using an excito-repellency test system, thereby comparing the three different populations and the two chemicals.

MATERIALS AND METHODS

Mosquito populations

Anopheles minimus (laboratory colony) has been maintained in the laboratory >15 years. It was originally collected from animal quarters in Rong Klang District, Prae Province, northern Thailand, in 1993 and subsequently was maintained in the insectary at the Vector Borne Disease Bureau, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand. This colony has been maintained in the insectary at Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, since 2001.

Anopheles minimus (wild population) was collected using evening human-landing captures from the Ban Tum Sua (16°41'N; 98° 41'E), Mae Sot District, Tak Province, western Thailand. This rural area is surrounded by fruit orchards on the east and by the intact native forest on the west.

Anopheles harrisoni (wild population) was captured from Ban Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14°17'N; 99°11' E). This rural area is located within mountainous terrain surrounded by intact native forest (Sungvornyothin et al. 2006b). Female mosquitoes were collected from cow-baited traps during the evening hours (18:00-06:00). All wild-caught adult mosquitoes were transported back to the entomology laboratory in Bangkok for identification and rearing.

Morphological and molecular species identification

Mosquitoes were initially identified using morphological keys of Rattanarithikul et al. (2006). All specimens in the Minimus Complex were individually subjected to DNA extraction and molecular analysis using a restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) following published techniques (Garros et al. 2004, Sungvornyothin et al. 2006a).

Insecticide-treated papers and susceptibility tests

Bifenthrin (IUPAC: 2-methylbiphenyl-3-ylmethyl (*Z*)-(1*RS*,3*RS*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate) active ingredient was supplied by Ladda Co., Bangkok, and DEET (*N,N*-diethyl-m-toluamide or *N,N*- diethyl-3-methylbenzamide) was provided by the United States Department of Agriculture, Beltsville, MD. Filter papers, measuring 12 x 15 cm, were prepared for the WHO susceptibility test and 15 x 17.5 cm papers for the excito-repellency assay. Test papers were either impregnated with analytical grade bifenthrin at the field operational (applied) dose of 0.2% (25 mg a.i./m^2) using acetone as diluent for both susceptibility and behavioral assay systems (WHO 2001) or impregnated with 5% DEET at 182 mg/m^2 prepared with absolute ethanol in the same manner. The dose of DEET (~200 mg/m^2) was selected as the minimum effective dose to eliminate the possibility of inadequate amounts that may cause failure in mosquito response (Robbins and Cherniack 1986, Dogan and Rossignol 1999) or possibly function as an attractant at much lower concentrations (Mehr et al. 1990). All impregnated papers were prepared according to WHO specification (WHO 1998) and treated at the rate of 2.0 ml of insecticide solution per 180 cm^2. Control papers

(without active ingredient) were produced using respective diluents (acetone or ethanol). Following WHO (2006) test procedures, four independent trials (15 non-blooded, 3 to 5-day-old female mosquitoes each) with matching controls were conducted for each test population. Treated papers were used up to three tests and within one week of preparation and then discarded.

Behavioral tests

Experimental design compared two wild-caught populations (*An. minimus* and *An. harrisoni*) and a long-established laboratory colony (*An. minimus*) in contact and non-contact exposures using bifenthrin or DEET. Identical test chamber designs were use for all excito-repellency assays.

Only 3 to 5-day-old, nulliparous female mosquitoes were used in excito-repellency tests. Mosquitoes were deprived of all nutrition (sugar) for 24 h before the experiment. Excito-repellency assays were performed during daylight hours between 09:00 and 16:00 and each test was replicated four times. Each test chamber was provided 15 mosquitoes of identical age range and conditioning. Each test trial contained four test chambers, two treatment designs (either contact or non-contact configurations) each with matching controls without active ingredient. Each ER chamber was prepared with a new treated paper for each test and then discarded immediately afterwards.

Tests were conducted for 30 min with observations recorded at one-minute intervals. After each test was completed, knockdown and mortality from each chamber was recorded. Specimens that escaped and those remaining in each test chamber were separated and transferred to clean containers and held to record mortality following 24 h post-test exposure. For additional details on the excito-repellency test system, consult previous work by Noosidum et al. (2008) and Thanispong et al. (2009).

Data analysis

Kaplan-Meier survival analysis was used to analyze data stratified by replicate for differences in mosquito escape behavior between populations and concentrations of bifenthrin or DEET (Kleinbaum 1995, Roberts et al 1997). The mean time in minutes for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of the test population to escape was estimated using a life table method. A log-rank method (Mantel and Haenzel 1959) compared patterns of escape behavior using SAS Release 6.10 (SAS Institute, Cary, NC). Statistical significance for all tests was set at 5% ($P < 0.05$).

RESULTS

All three test populations exposed to an operational field dose of bifenthrin (0.025%) were found completely susceptible after 1 h contact. DEET was not tested for contact toxicity as its primary mode of action is regarded as either a spatial repellent or inhibitor. Mean percent escape and mortality to bifenthrin separated by contact irritancy (excitation) and non-contact repellency are given in (Table 1).

Table 1. Percentage escape and 24-h mortality of *Anopheles minimus* and *Anopheles harrisoni* exposed to bifenthrin (25 mg a.i./m^2) in contact and non-contact trials.

Condition	Test Population	Bifenthrin*	Number escaped (%)	% mortality	
				Escaped	Remain
Contact	AM-L	Treatment	47 (81.03)	0	1.72
		Control	7 (11.67)	0	3.40
	AM-F	Treatment	24 (40.00)	4.54	13.63
		Control	0 (0)	0	0
	AH-F	Treatment	48 (80)	2.08	83.33
		Control	5 (8.62)	0	0
Non-contact	AM-L	Treatment	40(66.67)	3.33	0
		Control	9(15.79)	0	5.26
	AM-F	Treatment	10(17.86)	0	8.33
		Control	2(3.45)	0	0
	AH-F	Treatment	13(22.41)	7.69	13.33
		Control	5 (8.62)	0	4.08

Anopheles minimus (laboratory): AM-L; *Anopheles minimus* (Tak): AM-F; *Anopheles harrisoni* (Kanchanaburi): AH-F. *four replicates, total 60 female mosquitoes.

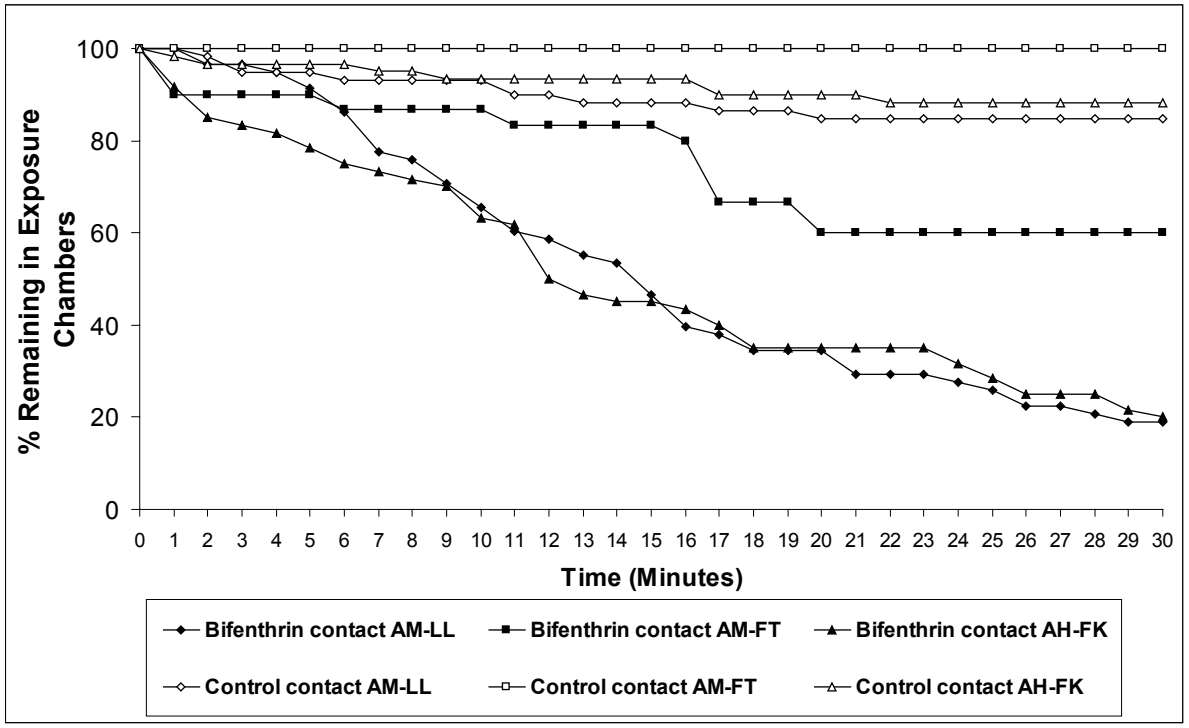

Figure 1. Escape probability of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to bifenthrin (25 mg/m^2) for treatment and control contact trials.

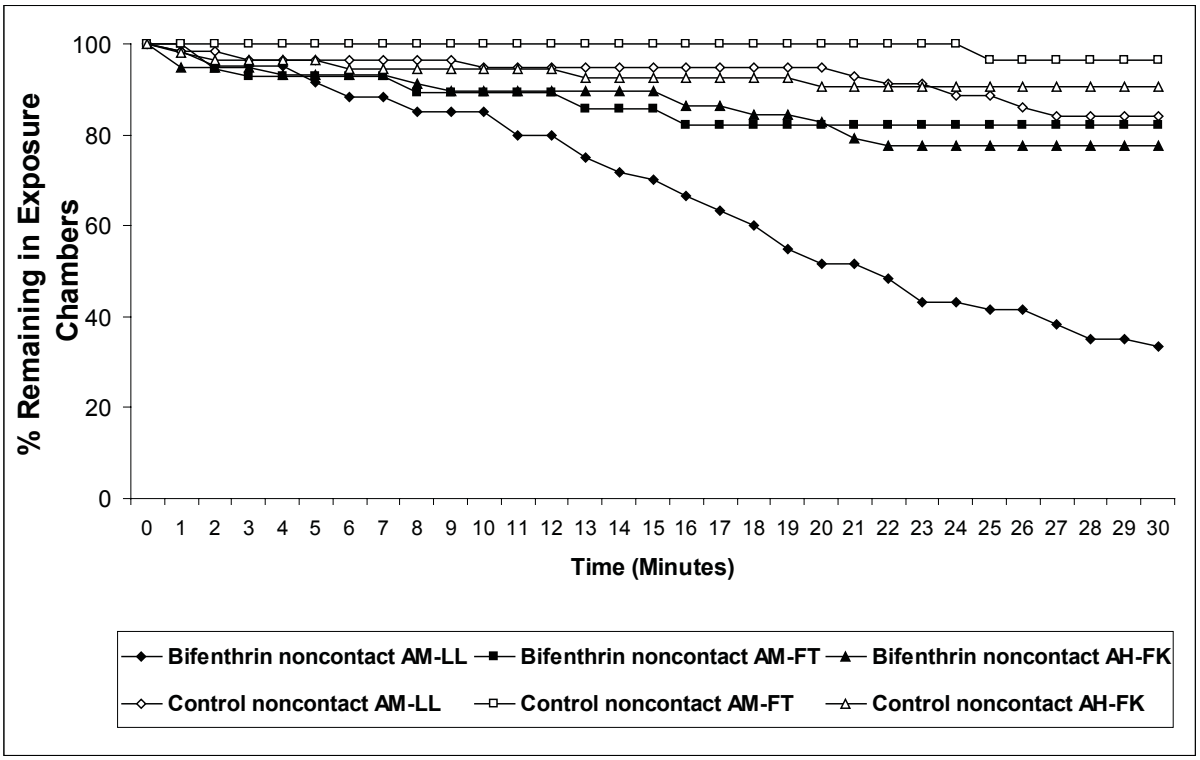

Figure 2. Escape probability of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to bifenthrin (25 mg/m^2) for treatment and control non-contact trials.

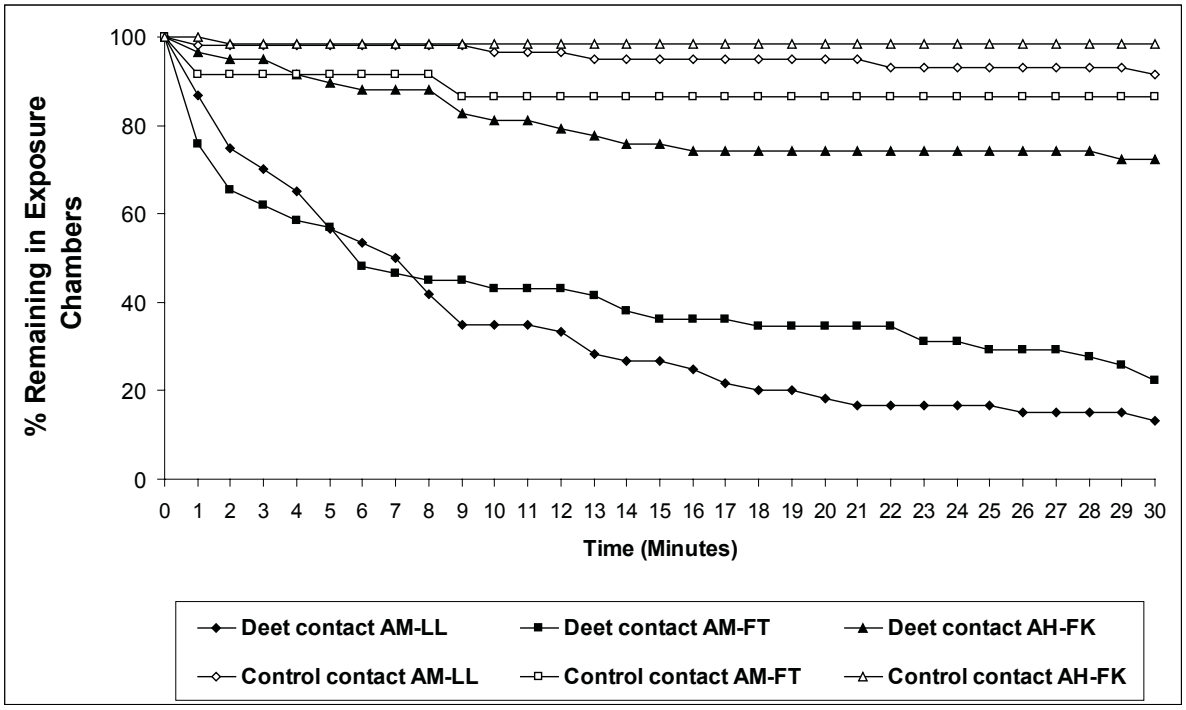

Figure 3. Escape probability of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to 5% DEET for treatment and control contact trials.

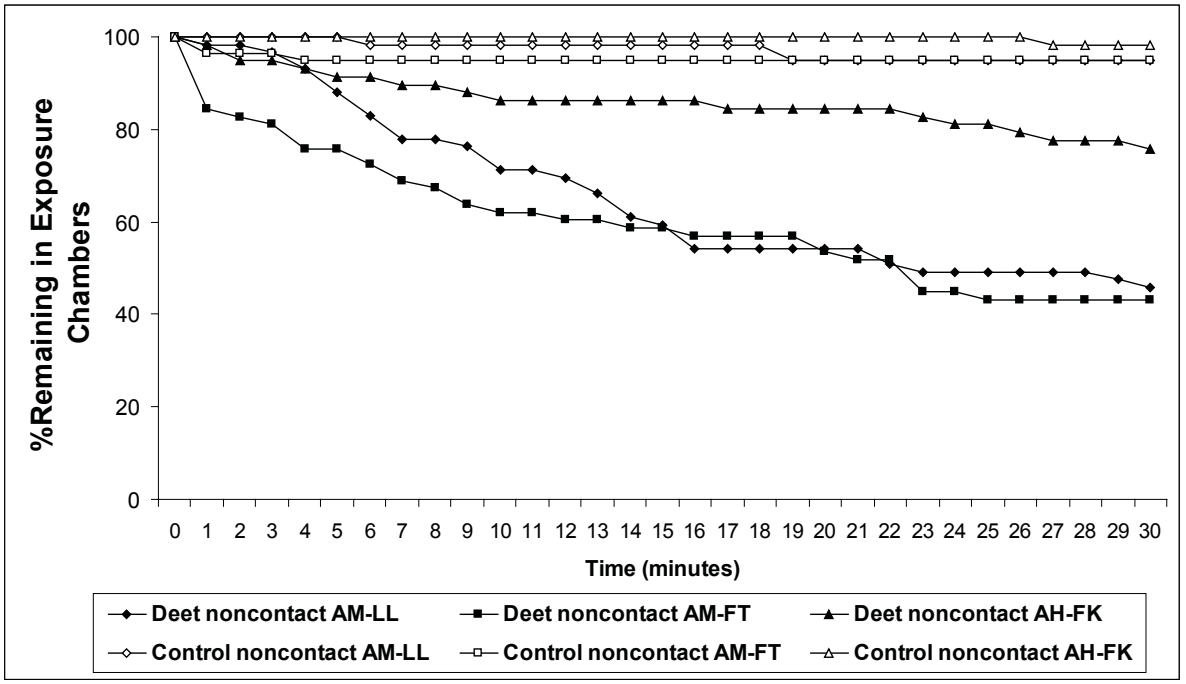

Figure 4. Escape probability of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to 5% DEET for treatment and control non-contact trials.

Table 2. Percentage escape and mortality of *Anopheles minimus* and *Anopheles harrisoni* exposed to 5% DEET (182 mg/m^2) in contact and non-contact trials.

Condition	Test Population	DEET*	Number escaped (%)	% mortality	
				Escaped	Remain
Contact	AM-L	Treatment	52 (86.67)	0	0
		Control	5 (8.62)	0	0
	AM-F	Treatment	45 (77.59)	0	3.45
		Control	5 (8.62)	5.17	5.08
	AH-F	Treatment	16 (27.59)	6.25	2.38
		Control	1 (1.72)	0	0
Non-contact	AM-L	Treatment	32(54.24)	0	0
		Control	3(5.17)	0	3.45
	AM-F	Treatment	33 (56.90)	0	0
		Control	3 (5.17)	0	3.45
	AH-F	Treatment	14 (24.14)	0	4.45
		Control	1 (1.69)	0	0

Anopheles minimus (laboratory): AM-L; *Anopheles minimus* (Tak): AM-F; *Anopheles harrisoni* (Kanchanaburi): AH-F. *four replicates, total 60 female mosquitoes.

Bifenthrin. Comparing the two field populations, the escape response in the bifenthrin contact trials was significantly stronger (P = 0.0002) in *An. harrisoni* (80%) than *An. minimus* (40%). Greater escape response in the bifenthrin contact trials was also observed from the *An. minimus* laboratory colony (81.03%) (Figure 1). Percent mortalities of escaped mosquitoes were low (0-4.5%), whereas those that remained in the bifenthrin-treated chambers produced a much higher range of lethality (1.7-83.3%). All control chambers produced low mortality (0-3.4%). Similarly, non-contact mortalities were low (0-7.7%) as were all exposed mosquitoes in control chambers (0-5.3%). In non-contact trials, a significantly stronger (P < 0.0001) escape response was observed from the laboratory colony (66.7%) than from either of the two field populations, *An. minimus* (17.86%) and *An. harrisoni* (22.41%) (Figure 2).

DEET. The escape response from contact trials was significantly (P < 0.0001) stronger in *An. minimus* (77.6%) than *An. harrisoni* (27.6%) (Figure 3). The *An. minimus* laboratory colony showed the strongest escape response in contact trials (86.7%) compared to field populations. Percent escape and mortality of the three test populations exposed to 5% concentration DEET (182 mg/m^2 equivalent) responses in both contact and non-contact tests are summarized in Table 2. Overall, percent mortalities of escaped and non-escape mosquitoes from treated contact chambers were nil except for one test series involving *An. harrisoni* (6.25%), and none were significantly different from matched controls. In non-contact trials, *An. minimus* showed a much stronger repellent response (54.2 - 56.9%) than *An. harrisoni* (24.1%) (Figure 4). Post-exposure mortality of escape and non-escape females in non-contact exposure and control chambers was low (< 5%). For all three populations, no mortality and knockdown was observed from those mosquitoes that successfully escaped from the non-contact DEET-treated chamber.

Escape time. The escape patterns by time and percent exiting generated from chemically-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 test chambers (Table 3). In contact trials, the time duration to escape was longer for the *An. minimus* laboratory colony with bifenthrin than DEET. For the *An. minimus* field population, the escape times with exposure with DEET were prolonged, whereas time estimates for *An. harrisoni* could not be calculated because of insufficient numbers of mosquitoes exiting during the 30-min test. Similarly, the ET_{50} and ET_{75} for *An. minimus* field population in the contact trial could not be estimated. For non-contact trials, DEET escape time estimates could only be generated for the *An. minimus* colony and field strains (ET_{25} and ET_{50} values only) and only for the laboratory colony exposed to bifenthrin (Table 3). The remaining non-contact trials produced insufficient numbers of escaped mosquitoes.

Probability of escape. The proportions of mosquitoes remaining in the excito-repellency test chambers treated with bifenthrin in contact (Figure 1) and non-contact

Table 3. Mean escape time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of *Anopheles minimus* and *Anopheles harrisoni* to escape from excito-repellency chambers containing bifenthrin or DEET at 30 min of exposure.

Test condition	**Bifenthrin**			**DEET**		
Population	**ET25**	**ET50**	**ET75**	**ET25**	**ET50**	**ET75**
Contact						
AM-L	8	14	25	2	7	16
AM-F	12	*	*	1	22	29
AH-F	6	12	26	*	*	*
Non-contact						
AM-L	13	21	*	9	22	*
AM-F	*	*	*	5	22	*
AH-F	*	*	*	*	*	*

*Insufficient number of mosquitoes escape from test chamber; ET: Escape Time: *Anopheles minimus* (laboratory): AM-L; *Anopheles minimus* (Tak): AM-F; *Anopheles harrisoni* (Kanchanaburi): AH-F.

Table 4. Log-rank tests of significance comparing each chemical and test format (contact and non-contact test) within and between test populations.

Species	**Bifenthrin**		**DEET**	
	Contact	**Non-contact**	**Contact**	**Non-contact**
AML vs. AMF	<0.0001	<0.0001	0.3273	0.7174
AML vs. AHF	0.4779	<0.0001	<0.0001	0.0004
AMF vs. AHF	0.0002	0.5969	<0.0001	0.0002

Chemical	**AM-L**	**AM-F**	**AH-F**
	Contact vs Non-contact	**Contact vs Non-contact**	**Contact vs Non-contact**
Bifenthrin	0.0184	0.0160	<0.0001
DEET	<0.0001	0.0118	0.6209

Significance set at $P < 0.05$.

chambers (Figure 2), and DEET-treated contact (Figure 3) and noncontact chambers (Figure 4) were used to construct probabilities of escape for the four different test formats, respectively. Stronger contact excitation was seen with *An. minimus* (lab) against bifenthrin and DEET, whereas, *An. harrisoni* demonstrated strong responses exposed to bifenthrin and *An. minimus* (field) population against DEET. As with contact test findings, the lab colony showed stronger repellent reaction in escape with both bifenthrin and DEET alone (Figures 2 and 4), while only *An. minimus* (field) presented a similar strong response when exposed to DEET (Figure 4). In all non-contact trials, there were significant differences in escape response compared with paired controls ($P < 0.05$).

Multiple comparisons using log-rank tests of significance within and between the three populations, two chemicals and contact/non-contact test designs gave varying levels of significance (Table 4). Within population comparisons, in all cases but one (DEET and *An. harrisoni*), there were significant differences between contact and non-contact escape responses. There was no difference in either excitation or repellency seen between colony and field strain of *An. minimus* exposed to DEET. All but two bifenthrin test combinations showed differences in escape response. No significant difference was seen in contact escape between *An. minimus* (lab) and *An. harrisoni* or repellency responses between the two field species.

DISCUSSION

Within the Minimus Complex, the sibling species *An. minimus* and *An. harrisoni* have been identified in Thailand as competent vectors of malaria (Green et al. 1990, Sungvornyothrin et al. 2006a, Manguin et al. 2010). Following the introduction of DDT in the late 1940s to control the vectors of malaria in Thailand (Chareonviriyaphap et al. 2000), selection pressure was reported to have modified some populations of *An. minimus* s.l. to preferentially feed outdoors in proportional greater numbers (Nutsathapana et al. 1986). As a consequence, insecticides may have a limited impact on populations that have the ability to alternate between endo- and exophagic feeding behaviors (Pothikasikorn et al. 2005, 2007). Although both species are exophagic throughout their geographic range, a few populations of the Minimus Complex remain predominately endophagic and display strong anthropophilic blood feeding preferences (Sungvornyothin et al. 2001, 2006b). Behavioral diversity and innate heterogeneity in responses to insecticidal application within the complex appear to have had a profound influence on the ability of chemicals to interrupt malaria transmission. Therefore, careful observation of insecticide behavioral responses by individual species can assist the selection of appropriate vector prevention and control strategies.

In published insecticide comparison tests, bifenthrin has shown relatively low irritant (excitant) and knockdown properties compared with permethrin and deltamethrin (WHO 2001). These same studies concluded that an excito-repellency effect was present but still provided a consistent high kill by allowing mosquitoes to rest on treated surfaces for longer periods than deltamethrin as comparison. Bifenthrin also demonstrated similar airborne knockdown effects with other pyrethroids. DEET is the most widely used active ingredient in commercial topical insect repellents, showing broad effectiveness against many insect species, including mosquitoes (Rutledge et al. 1983), yet its mechanism of action and molecular target(s) remains unknown, or at best, unclear (Ditzen et al. 2008, Pickett et al. 2008, Syed and Leal 2008). Although DEET vapor has long been regarded a repellent (i.e., a substance that causes movement away from a source), more recent studies have concluded it acts as an inhibitor vs a true repellent (Dogan et al. 1999, Dogan and Rossignol 1999). 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 chemo-attractant cues. Use of DEET in treated containers as a deterrent to induce anti-oviposition behavior on *Aedes albopictus* mosquitoes (Xue et al. 2001) may also be operating as an inhibitor of larval habitat chemical cues (attractants) rather than functioning as a true repellent.

Previous studies have demonstrated strong refractory responses of *An. minimus* s.l. populations exposed to various insecticides in Thailand (Chareonviriyaphap et al. 2001, 2004, Pothikasikorn et al. 2005, 2007). However, one of the limitations from those earlier findings was a reliance on morphological characters for identification of *An. minimus* and *An. harrisoni* that likely resulted in a certain level of sibling species misidentification (Sungvornyothin et al. 2006a, 2006b). This study overcame the limitations of using overlapping morphological characters shared between species by identifying populations using DNA (Sawabe et al. 2003, Garros et al. 2004).

Significant behavioral responses to chemical exposure were documented in both species; however, the degree of escape responses was different among the three test populations. In general, the long-standing *An. minimus* laboratory colony was the most responsive by contact excitation and non-contact repellency escape to both chemicals compared to the two field populations. Interestingly, significant differences in escape responses were observed between the two field populations, displaying more varied responses depending on the chemical and test format. *Anopheles minimus* (Tak) was less responsive in both test formats with bifenthrin compared to DEET, whereas *An. harrisoni* (Kanchanaburi) showed a much stronger contact excitation response with bifenthrin and a much lower excitation and repellency response to DEET compared to *An. minimus* (Tak) (Table 4). Repellency action was comparatively weak compared to contact tests, especially *An. harrisoni* for bifenthrin. With only one exception (*An. harrisoni* + DEET), significant differences in escape responses were seen between all paired contact and non-contact trials. All paired non-contact tests were significantly different in escape response compared to paired controls.

Contact excitation responses in this study were similar to those of previous reports of laboratory and field populations of *An. minimus* s.l. (Chareonviriyaphap et al. 2001, 2004, Sungvornyothin et al. 2001, Pothikasikorn et al. 2005, 2007). The repellency response to bifenthrin was pronounced in *An. minimus* (lab) but not the field population, whereas both lab and field populations had very similar escape patterns to DEET. Similarly, weak repellency of *An. minimus* s.l. (approx. 95% morphologically identified as *An. harrisoni* from Pu Teuy) to pyrethroids has been reported (Chareonviriyaphap et al. 2001, Pothikasikorn et al. 2005). The comparatively weak repellency response in *An. harrisoni* may be associated with biologically innate abilities of detecting chemical signals different from those in *An. minimus* (Pothikasikorn et al. 2005). Pu Teuy Village (predominately *An. harrisoni*) is considered a low risk area for malaria transmission in which indoor residual spray (IRS) with deltamethrin has been infrequently applied compared to more malaria prone areas (e.g., Mae Sot District) where *An. minimus* is abundant and a primary vector (MOPH 2008, 2009). We speculate this varying amount of previous exposure to IRS between the two field-collected populations used in this study may possibly account for the different avoidance behaviors seen between these closely related species.

Apart from insecticides used in public health control programs, other compounds, especially DEET, are well known to exhibit profound behavioral responses (Surgeoner 1995, Cox 2005). Whether DEET performs as a true repellent or inhibitor is a question of mechanism and was not addressed in this study; however, the outcome as a deterrent (escape) was measured. There has been little published information describing the two behavioral actions, contact excitation and spatial repellency, of DEET on anopheline mosquitoes. Assuming both contact excitation and spatial repellency are involved in the escape response seen in the contact chamber design, it is not therefore possible to clearly differentiate the actions of contact excitation and repellency. The differences in escape response between the two test designs (contact and non-contact) would presumably help separate and quantify the significance of either excitation or repellency actions alone. The same would apply in factoring out escape in paired controls with non-contact tests to arrive at an adjusted percent repellency. Nevertheless, neither test design used a known attractant (e.g., host cue) inside the DEET-treated chamber, a requirement to determine if inhibition was a mode of action. Those mosquitoes that escaped in the non-contact test designs appear to have been the result of repellency alone. Based on our study findings using the excito-repellency test system, DEET appears to act as both contact stimulant and a moderate spatial repellent.

The excito-repellency test system remains a useful tool for understanding how chemicals operate at sub-lethal concentrations as repellents and contact stimulants on mosquitoes (Roberts et al. 1997). Together with a probability model for analyses and interpretation of data (Roberts et al. 2000) and the development of a high throughput screening system (Grieco et al. 2007), there remains much work ahead to describe response outcomes and mechanisms of a large array of different chemical compounds and vector species. Our findings describing the clear differences in behavioral responses between the same species (*An. minimus*) show clearly that sufficient caution must be exercised extrapolating findings from more genetically homogeneous long-term laboratory colony material to making assumptions to field populations. Experimental hut studies in field settings that include accurate sampling of house-entering vector populations are thus crucial for more meaningful assessments of spatial repellents as possible vector control strategies. Our findings demonstrate clear differences in avoidance behavior between the two species of the Minimus Complex in Thailand. These differences may be innately species-dependent and influenced by ecological and geographical variations between the test populations or related to exogenous factors including previous exposure to residual insecticides between the two collection sites in Pu Teuy and Mae Sot. Despite many unanswered questions, we believe that these findings can help optimize the use of currently available public health tools and spur the development of new ones.

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Frequency of pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Thailand

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ABSTRACT: Thirty-two *Aedes aegypti* populations collected throughout Thailand and five populations of *Aedes albopictus* from southern Thailand were subjected to standard WHO contact bioassays to assess susceptibility to three commonly used synthetic pyrethroids: permethrin, deltamethrin, and lambda-cyhalothrin. A wide degree of physiological response to permethrin was detected in *Ae. aegypti*, ranging from 56.5% survival (Lampang, northern Thailand) to only 4% (Kalasin in northeastern and Phuket in southern Thailand). All 32 populations of *Ae. aegypti* were found to have evidence of incipient resistance (62.5%) or levels of survival deemed resistant (37.5%) to permethrin. Four populations of *Ae. albopictus* were found with incipient resistance (97 – 80% mortality) and one with resistance (< 80%) to permethrin. The majority of *Ae. aegypti* populations (68.7%) was susceptible (> 98% mortality) to deltamethrin, with incipient resistance (observed 97-82% mortality) in other localities. In contrast, all populations of *Ae. aegypti* were completely susceptible (100% mortality) to the recommended operational dosage of lambda-cyhalothrin. All five populations of *Ae. albopictus* were found completely susceptible to both deltamethrin and lambda-cyhalothrin. Evidence of defined incipient or resistance to synthetic pyrethroids mandates appropriate response and countermeasures to mitigate further development and spread of resistance. In light of these findings, we conclude that routine and comprehensive susceptibility monitoring of dengue mosquito vectors to synthetic pyrethroids should be a required component of resistance management policies and disease control activities. ***Journal of Vector Ecology* 36 (1): 204-212. 2011.**

Keyword Index: *Aedes aegypti*, *Aedes albopictus*, synthetic pyrethroids, susceptibility, Thailand.

INTRODUCTION

Dengue/dengue hemorrhagic fever is one of the most serious and important resurgent tropical diseases worldwide (Gubler 1998). Dengue is the most common vector-borne viral disease globally, primarily affecting developing countries of tropical and subtropical regions, with an estimated 2.5 to 3 billion people at risk of infection and resulting in approximately 50 million dengue infections annually (Guzman et al. 2010). *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) are the primary vectors of dengue viruses in Southeast Asia, a region that historically represents the epicenter of transmission and disease occurrence (Gubler 1998). Both species are primarily day-biting mosquitoes that share many of the same behavioral attributes. *Ae. aegypti* is more prevalent near and inside human dwellings and is the principal vector in urban, more densely populated zones, whereas *Ae. albopictus* is more peridomestic and more closely associated with rural and less congested suburban areas. Historically, *Ae. aegypti* is often associated with the epidemic spread of dengue viruses; *Ae. albopictus* typically provides a secondary role in outbreak transmission and maintaining rural endemic cycles in humans. Both mosquito species also play a comparable dual role in the transmission of 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).

Effective control of these two mosquito species has proven extremely difficult with both vector and virus remaining entrenched and expanding in many disease endemic areas. Lacking an effective vaccine, vector control methods attacking both larval habitats and adult mosquito populations remains the primary method for reducing risk of dengue infection (Kongmee et al. 2004, Polsomboon et al. 2008). Unfortunately, control strategies aimed at elimination of preferred vector larval habitats through source reduction or periodic application of insecticides to water sources has often met with failure to sufficiently control these two

species (Gubler 1998, Kongmee et al. 2004). Furthermore, control of adult mosquitoes using a variety of chemical means is fraught with complications including high cost, slow operational response, ineffective timing of application, low efficacy, and evolution of resistance to insecticides. However, the reduction of vector densities alone has often proven insufficient. A better understanding of the complex interplay of diverse factors, including site-specific social determinants, is critical for promoting a broader public health response leading to successful vector control management practices using routine larviciding and focal space spraying. Nevertheless, insecticides continue to play a crucial, if not indispensible, role in helping to reduce the risk of dengue transmission by complementary reduction of immature and adult vector mosquitoes (Grieco et al. 2007, Jirakanjanakit et al. 2007, Thanispong et al. 2008).

Synthetic insecticides of various chemical classes have long been used in national public health vector control programs (Roberts and Andre 1994, Reiter and Gubler 1997), the vast majority of which today are pyrethroid-based formulations. In Thailand, combinations of pyrethroids are commonly used by home owners to control mosquitoes and other arthropod pests. Applied at relatively low concentrations, pyrethroids are considered to be effective and relatively safe compounds for controlling common house-frequenting mosquitoes, especially *Ae. aegypti* and *Culex quinquefasciatus* (Chareonviriyaphap et al. 1999, Somboon et al. 2003). Since 1994, deltamethrin has been used intensively in organized public health programs in Thailand to attempt interruption of dengue transmission following reports of outbreaks. Numerous permethrin and deltamethrin-based formulations (e.g., aerosols, coils, and gels) are commercially available to the general public but much less so for lambda-cyhalothrin (Paeporn et al. 1996, Chareonviriyaphap et al. 1999). 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). The majority of published reports on pyrethroid resistance in *Ae. aegypti* and *Ae. albopictus* populations in Thailand have been restricted in geographical scope (Chadwick et al. 1977, Paeporn et al. 2004, Chareonviriyaphap et al. 1999, Yaicharoen et al. 2005, Sathantriphop et al. 2006, Jirakanjanakit et al. 2007, Thanispong et al. 2008). Without a better understanding of the temporal effects and outcomes of insecticide use on mosquito susceptibility, sustainable and successful vector control activities remain vulnerable. Routine monitoring of insecticide susceptibility integrated into control programs allow for more timely response and greater efficiency in controlling specific mosquito vectors. We report herein the susceptibility of *Ae. aegypti* collected across Thailand and *Ae. albopictus* collected in southern Thailand to three common synthetic pyrethroids used to control adult mosquitoes. Since a high level of permethrin resistance in *Ae. aegypti* was reported from Baan Suan, Nonthaburi Province (Sathantriphop et al. 2006), a decision to use a three-fold greater concentration (0.75%) was made in this study.

MATERIALS AND METHODS

Study sites

Collection sites for *Ae. aegypti* were selected based on reported dengue cases in 2008. *Ae. aegypti* larvae and pupae were collected from containers located in and around houses in 32 separate sites (29 provinces) throughout Thailand in 2008 (Figure 1). Additionally, *Ae. albopictus* was collected as immature stages in rubber plantation areas from five collections located in four provinces of southern Thailand (Figure 1). Geo-coordinates of each site are provided in Table 1.

Mosquitoes

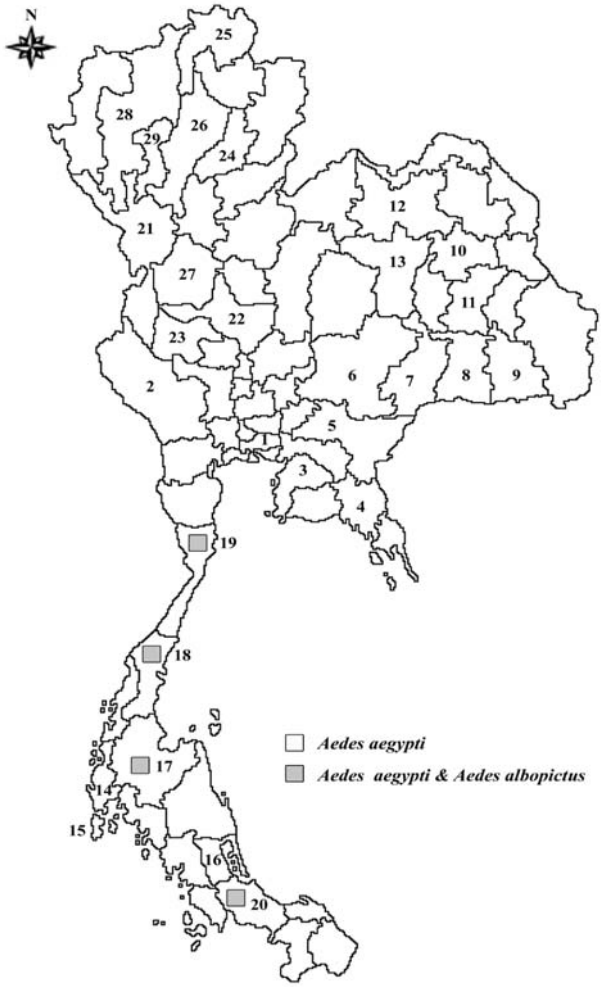
Field-collected larvae and pupae were reared in the insectary to the adult stage for morphological identification and colonization. Two fully susceptible populations of *Ae. aegypti* (USDA, USA) and *Ae. albopictus* (MOPH, Thailand) were used as a reference baseline for susceptibility to permethrin, deltamethrin, and lambda-cyhalothrin. The USDA laboratory population was provided by the Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL, U.S.A. This population has been continuously colonized for over 40 years. *Aedes albopictus* was originally collected from Nonthaburi Province and has been maintained in a colony at the MOPH for approximately ten years.

All mosquito populations were carefully segregated to prevent contamination from cross-mating and reared under identical laboratory-controlled conditions at 25$\pm$5° C and 80$\pm$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 a live guinea pig beginning on the fourth day post-emergence. Moistened oviposition substrates were provided for females to deposit eggs. Following conditioning, eggs were rehydrated and allowed to hatch in pans reserved for each population. Subsequent susceptibility tests were restricted to F1-F3 generation female mosquitoes.

Insecticides

Three synthetic pyrethroids (without addition of piperonyl butoxide or other synergists) were used in susceptibility testing. 1. Permethrin 92% pure active ingredient (a.i.) [IUPAC (3-phenoxybenzyl (1 *RS*, 3 *RS*, 1*RS*, 3 *SR*)-3 -(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate)] provided by Ladda Company, Bangkok, Thailand (June, 2008). 2. Deltamethrin 98% a.i. [IUPAC (*S*)-alpha-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2 dimethylcyclopropanecarboxylate] obtained from BASF (August, 2008), and 3. Lambda-cyhalothrin 98% a.i. [IUPAC (*S*)-alpha-cyano-3-phenoxybenzyl (*Z*)-(1*R*,3*R*)-3-(2-chloro-3,3,3-trifluropropenyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-alpha-cyano-3- phenoxybenzyl (*Z*)-

Figure 1. *Aedes aegypti* and *Aedes albopictus* collection sites by province in Thailand.



1 = Bangkok
2 = Kanchanaburi
3 = Chonburi
4 = Chanthaburi
5 = Prachinburi
6 = Nakhon Ratchasima
7 = Buri Ram
8 = Surin
9 = Si Sa Ket
10 = Kalasin
11 = Roi Et
12 = Udon Thani
13 = Khon Kaen
14 = Phang Nga
15 = Phuket
16 = Phatthalung
17 = Surat Thani
18 = Chumphon
19 = Prachuap Khiri Khan
20 = Songkhla
21 = Tak
22 = Nakhon Sawan
23 = Uthai Thani
24 = Phrae
25 = Chiang Rai
26 = Lampang
27 = Kamphaeng Phet
28 = Chiang Mai
29 = Lamphun

(1*S*,3*S*)-3-(2-chloro-3,3,3-trifluropropenyl)-2,2-dimethyl cyclopropanecarboxylate] received from Syngenta Crop Protection, Thailand (June, 2008).

Insecticide-treated paper

Insecticide treated papers were produced at the Department of Entomology, Kasetsart University according to WHO specifications (WHO 1998). Test papers (Whatman® No. 1) 12 × 15 cm in size were individually impregnated with one of the following concentrations: 0.75% (equivalent 294 mg a.i./m^2) permethrin, 0.05% (18.3 mg a.i./m^2) deltamethrin, or 0.05% (19.4 mg a.i. /m^2) lambda-cyhalothrin with silicon oil and acetone serving as diluents. Papers were treated at the rate of 2 ml of insecticide solution per sheet. Control papers were prepared in the same manner but impregnated with only 2 ml acetone solvent mixed with the non-volatile carrier silicon oil at 3.6 mg/cm^2 of the carrier, i.e., 648 mg/paper or 0.66 ml/paper for silicon oil.

Insecticide susceptibility test

Only three to five-day-old, non-blood fed adult female mosquitoes (the gender of interest as potential transmitter of dengue viruses) were used in tests. Test kits, procedures, data analysis and interpretation followed provided instructions (WHO 1998). Each test consisted of five clear plastic tubes lined inside with treated papers (two controls and three treatments). Twenty-five mosquitoes were introduced into each holding tube lined with clean (untreated) paper for 60 min to establish the vitality of mosquitoes before insecticide exposure. Dead and moribund mosquitoes were removed before beginning each test. Mosquitoes were exposed for 60 min with all tubes placed in the vertical (standing up) position for the duration of the test period. All tests took place during daylight hours (between 09:00 and 16:00) under identical environmentally controlled conditions (25$\pm$5° C and 80$\pm$10% RH). At the end of the test exposure, the number of knockdown (KD) mosquitoes was recorded. All mosquitoes (live and moribund) were carefully transferred to separate clean holding tubes and provided with 10% sugar solution applied to cotton wool. Mortality was recorded at 24 h post-exposure**.** Four test replicates (~100 female mosquitoes total per test series) were conducted for each mosquito population and insecticide combination.

Data analysis

Post-test KD and final (24 h) mortality were averaged among each test series. Analysis and interpretation of bioassay results were based on established criteria (WHO 1998). Mosquitoes were considered susceptible to insecticide if the percent mortality was >98%. Incipient insecticide resistance (also referred to as tolerance) is defined as final mortality ranging between 80 and 97%. Test populations were considered resistant if percent mortality was below 80%. Because only one concentration of each chemical was used in the assays, it was not possible to estimate varying percent lethal concentrations using a probit analysis.

Table 1. Location and geo-coordinates of *Aedes* species collection sites.

Region	Province	District	GPS coordinates
		Aedes aegypti	
Central	Bangkok	Kannayaw	13° 50′ N, 100° 40′ E
Western	Kanchanaburi	Sai Yok	14° 20′ N, 98° 59′ E
Eastern	Chanthaburi	Mueang	14° 17′ N, 100° 55′ E
	Chonburi	Mueang	13° 19′ N, 100° 55′ E
	Prachinburi	Mueang	15° 11′ N, 100° 55′ E
Northeastern	Buri Ram	Lam Plai Mat	15° 00′ N,102° 50′ E
	Kalasin	Huaypung	16° 38′ N,103° 54′ E
	Khon Kaen	Mueang	16° 19′ N,102° 47′ E
	Nakhon Ratchasima	Wang Nam Kheow	15° 00′ N,102° 06′ E
	Roi Et	Suwannaphum	15° 40′ N,103° 49′ E
	Si Sa Ket	Uthumpronpisai	15° 08′ N,104° 12′ E
	Surin	Dontoom	15° 14′ N,103° 30′ E
	Udon Thani	Wungsammor	16° 54′ N,103° 28′ E
Southern	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
Northern	Chiang Mai	Mueang	18° 46′ N,98° 56′ E
	Chiang Rai	Mae Chun	20° 08′ N,99° 51′ E
	Kamphaeng 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° 40′ N,98° 10′ E
	Uthai Thani	Ban Rai	15° 23′ N, 100° 55′ E
		Ae. albopictus	
Southern	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
	Surat Thani	Mueang	09° 02′ N,99° 22′ E

RESULTS

The summary findings of KD and mortality for *Ae. aegypti* and *Ae. albopictus* exposed to permethrin, deltamethrin, and lambda-cyhalothrin are provided in Table 2. For all tests, control mortalities for *Ae. aegypti* and *Ae. albopictus* were nil, thus indicating that colonized mosquitoes used in this study were healthy at time of testing. For all three compounds, knockdown response after 60 min was a highly accurate predictor of final recorded mortality at 24 h post-exposure. In many instances, the KD and mortality percentages were identical.

The USDA *Ae. aegypti* control population was completely susceptible to all three chemicals along with 100% KD after 60 min. All 32 populations of *Ae. aegypti* had evidence of incipient resistance (62.5%) or levels of survival deemed resistant (37.5%) to permethrin. In general, a higher level of physiological tolerance/resistance to permethrin was seen in northern populations compared to other geographical regions in Thailand. The highest resistance to permethrin was seen in populations from Lampang (43.54% mortality), followed closely by Chiang Mai (44.59% mortality) and Kamphaeng Phet (50.00% mortality). In all, five populations (55.6%) in northern Thailand were resistant. Physiological resistance was also observed in two populations from the eastern region, Chonburi and Chanthaburi, and in five populations (50% of tested populations) from the south [Surat Thani, Chumphon, Prachuap Khiri Khan, Namom (Songkhla), and Sadao (Songkhla)]. Incipient resistance to permethrin (80-97% mortality) was seen in all remaining populations and within all six regions.

With deltamethrin, incipient resistance was seen in eleven of 32 populations (34.4%); six (60%) from the southern region, two populations each from eastern (67%) and northern (22.2%) regions, and one population from the northeastern region (12.5%). The remaining majority of *Ae. aegypti* tested were considered as susceptible to deltamethrin (98-100% mortality). All populations of *Ae. aegypti* were found completely susceptible (100% KD and mortality) to lambda-cyhalothrin.

Among the five populations of *Ae. albopictus* tested, only one population (Songkhla-Sadao, 54% mortality) was considered permethrin-resistant, whereas the other four had evidence of moderate to light incipient resistance (84-96% mortality). In contrast, *Ae. albopictus* was found completely susceptible to deltamethrin and lambda-cyhalothrin.

DISCUSSION

Preliminary studies using the recommended operational concentration of 0.25% permethrin for *Ae. aegypti* found that all samples were resistant to this dose and 0.5% (data not presented). Sathantriphop et al. (2006) reported using a 0.75% concentration of permethrin and finding high levels of resistance in *Ae. aegypti* from Baan Suan, Nonthaburi Province. Therefore, a decision was made to use a three-fold greater concentration (0.75%) for all tests used in this study.

First to third generation field-collected populations of *Ae. aegypti* and *Ae. albopictus* were either tolerant or resistant to a three-fold higher concentration of permethrin above the normal recommended operational concentration. All 32 populations of *Ae. aegypti* had evidence of incipient resistance (62.5% of populations) or levels of survival deemed resistant (37.5%) to permethrin. A wide degree of physiological response to permethrin was detected in *Ae. aegypti*, ranging from 56.5% survival (Lampang in northern Thailand) to only 4% (Kalasin in northeastern and Phuket in southern Thailand). Four populations of *Ae. albopictus* were found with incipient resistance (97 to 80% mortality) and one with resistance (< 80%) to permethrin. The majority of *Ae. aegypti* populations (68.7%) were susceptible (> 98% mortality) to 0.05% deltamethrin, with incipient resistance (observed 97 to 82% mortality) in other localities. In contrast, all populations of *Ae. aegypti* were completely (100%) susceptible to 0.05% dosage of lambda-cyhalothrin. All five populations of *Ae. albopictus* were completely susceptible to both deltamethrin and lambda-cyhalothrin. Geographically, collection sites in northern and southern provinces showed the highest percent resistance to permethrin by *Ae. aegypti*, 55.6% and 50% of populations tested, respectively. Deltamethrin tolerance (60% of populations) was more common in the south.

In Thailand, synthetic pyrethroids, particularly permethrin and deltamethrin, were introduced in the last three decades and have been widely available for the control of mosquitoes and other insects (Chareonviriyaphap et al. 1999). Historically, pyrethroids have demonstrated great promise for effective mosquito control due to their low toxicity to humans and high potency at low doses quickly immobilizing and killing insects. However, the continuous use of particular insecticides for long periods of time has been associated with development of resistance in mosquitoes and thus reduced effectiveness controlling vectors (Hemingway and Ranson 2000).

From the 1960s, investigations examining patterns of mosquito resistance to insecticides in Thailand began reporting varying and increased levels of tolerance/resistance to various synthetic chemicals used for control of adult and larval *Ae. aegypti* (Neely 1964, Bang et al. 1969, Chadwick et al. 1977, Malcolm and Wood 1982). Only recently have a few studies made similar assessments of *Ae. albopictus* (Somboon et al. 2003, Ponlawat et al. 2005, Jirakanjanakit et al. 2007). During the previous decade, increasing tolerance or resistance to permethrin and/or deltamethrin has been documented in adult and larval *Ae. aegypti* in Thailand (Chareonviriyaphap et al. 1999, Somboon et al. 2003, Paeporn et al. 2004, Yaicharoen et al. 2005, Ponlawat et al. 2005, Sathantriphop et al. 2006, Jirakanjanakit et al. 2007, Thanispong et al. 2008). Although significantly high levels of permethrin resistance were reported by Jirakanjanakit et al. (2007) (5% mortality) and Thanispong et al. (2008) (2-9% mortality) exposed to 0.25% a.i., not surprisingly, our study showed relatively lower degrees of resistance (43-96% mortality) when exposed to a three-fold higher concentration. Additionally, these

Table 2. Percent knockdown (KD) and mortality of *Ae. aegypti* and *Ae. albopictus* following 60-min exposure to operational concentrations of three pyrethroid insecticides.

Species (Province)	Permethrin (0.75%)		Deltamethrin (0.05%)		Lambda-cyhalothrin (0.05%)	
	%KD	%Mortality±SE	%KD	%Mortality±SE	%KD	%Mortality±SE
			Aedes aegypti			
USDA	100	100	100	100	100	100
Central						
Bangkok	82.00	82.00 ± 0.43	98.00	98.00 ± 0.22	100	100
Eastern						
Chanthaburi	75.86	76.55 ± 0.50	94.00	94.00 ± 0.22	100	100
Chonburi	77.33	78.00 ± 0.43	94.66	95.33 ± 0.31	100	100
Prachinburi	90.66	92.00 ± 1.17	100	100	100	100
Northeastern						
Buri Ram	88.00	88.00 ± 0.58	100	100	100	100
Kalasin	96.00	96.00 ± 0.58	100	100	100	100
Khon Kaen	82.43	82.43 ± 0.52	96.00	96.00 ± 0.26	100	100
Nakhon Ratchasima	93.33	95.33 ± 0.60	100	100	100	100
Si Sa Ket	84.46	84.46 ± 0.40	100	100	100	100
Surin	89.93	92.62 ± 0.36	100	100	100	100
Roi Et	94.00	94.00 ± 0.43	100	100	100	100
Udon Thani	84.67	86.00 ± 0.56	98.00	98.00 ± 0.22	100	100
Northern						
Chiang Mai	44.59	44.59 ± 0.36	100	100	100	100
Chiang Rai	82.00	82.00 ± 0.43	100	100	100	100
Kamphaeng Phet	50.00	50.00 ± 0.43	100	100	100	100
Lamphun	68.00	68.00 ± 0.36	100	100	100	100
Lampang	43.54	43.54 ± 0.33	100	100	100	100
Nakhon Sawan	70.00	70.00 ± 0.43	84.00	84.00 ± 0.48	100	100
Phrae	90.60	90.60 ± 0.43	100	100	100	100
Tak	80.13	81.51 ± 0.31	96.00	96.00 ± 0.26	100	100
Uthai Thani	93.33	93.33 ± 0.33	100	100	100	100
Southern						
Chumphon	70.00	70.00 ± 0.43	85.14	86.47 ± 0.21	100	100
Phuket	96.00	96.00 ± 0.43	100	100	100	100
Phang Nga	93.33	94.00 ± 0.43	100	100	100	100
Prachuap Khiri Khan	78.00	78.00 ± 0.43	96.00	96.00 ± 0.26	100	100
Phatthalung	87.33	87.33 ± 0.45	100	100	100	100
Songkhla-HadYai	81.33	82.00 ± 0.43	90.00	90.00 ± 0.22	100	100
Songkhla-Namom	64.43	64.43 ± 0.58	84.00	84.00 ± 0.36	100	100
Songkhla-Ranode	89.19	89.19 ± 0.58	100	100	100	100
Songkhla-Sadao	54.00	54.00 ± 0.43	81.33	82.00 ± 0.22	100	100
Surat Thani	73.33	74.00 ± 0.50	88.00	88.00 ± 0.36	100	100
Western						
Kanchanaburi	82.00	84.00 ± 0.26	100	100	100	100
			Aedes albopictus			
MOPH population	100	100	100	100	100	100
Southern						
Chumphon	95.33	95.33 ± 0.60	100	100	100	100
Prachuap Khiri Khan	96.00	96.00 ± 0.43	100	100	100	100
Songkhla-Namom	84.00	84.00 ± 0.26	100	100	100	100
Songkhla-Sadao	78.00	78.00 ± 0.43	100	100	100	100
Surat Thani	96.00	96.00 ± 0.58	100	100	100	100

variations in permethrin susceptibility may partially reflect differences in collection sites and previous levels of exposure to permethrin or related compounds. It is suspected that household insecticide products, i.e., coil, mat, liquid, lotion, and cream formulations containing permethrin, play a major role for permethrin resistance in *Ae. aegypti* populations in Thailand as reported by Paeporn et al. (1996) and Thanispong et al. (2008). The increased use of materials treated with permethrin (blanket, cloths) for disease control in many parts of Thailand and the use of pyrethriods for control of agricultural pests have likely accelerated the development of physiological resistance in this mosquito. Incipient resistance (tolerance) to deltamethrin was detected in 11 of 32 *Ae. aegypti* populations. Like permethrin, the decreased susceptibility to deltamethrin over time is the consequence of widespread use of this chemical and related pyrethroid compounds. Several pyrethroids are also commercially available to the general public for use in homes for protection against indoor biting mosquitoes (Kongmee et al. 2004). Moreover, ultra-low-volume (ULV) applications of deltamethrin have been commonly used to combat dengue outbreaks for many years.

Incipient or outright resistance (WHO 1998) to permethrin was detected in several populations of *Ae. albopictus* from the south. Permethrin-treated materials have been commonly used for protection against outdoor biting mosquitoes like *Ae. albopictus*, e.g., workers in rubber plantations and fruit orchards. Since 2004, routine indoor residual spray (IRS) for malaria control was suspended because of civil unrest in areas nearer the southern border with Malaysia. Tolerance or resistance may also have arisen because of exposure to household or agricultural use of permethrin or other pyrethriods. Likewise, half of the populations of *Ae. aegypti* tested from the southern districts exhibited resistance to permethrin due to more indoor exposure or to cross-resistance. Six populations (60%) of *Ae. aegypti* also demonstrated incipient resistance to deltamethrin. In contrast, all populations of *Ae. albopictus* remain completely susceptible to deltamethrin and lambda-cyhalothrin. However, this does not preclude the rapid development cross-resistance to these two chemicals as a consequence of the extensive exposure to related compounds (Ponlawat et al. 2005).

In addition to differing exposures to these three chemicals over time, the varying degrees of *Aedes* susceptibility seen between them may be related to differences in attributes and chemistry. Permethrin is grouped together with other so-called third generation pyrethroids that exhibit enhanced insecticidal activity and photostability over their first and second generation chemical predecessors. Deltamethrin and lambda-cyhalothrin are more advanced fourth generation pyrethroids with active ingredients displaying greater toxicity, enhanced photostability, and lower volatility for extended residual effectiveness than permethrin. The chemistry and symptomology also differs between permethrin on the one hand and deltamethrin and lambda-cyhalothrin on the other (Bloomquist 1996). Pyrethroids are potent axonic poisons in susceptible insects, acting as sodium channel blockers or modulators (Ware 2000). Their mode of action is similar to DDT, by keeping open the sodium channels in neuronal membranes and thus producing a hyper-excitatory effect stimulating repetitive nerve discharges. For insects susceptible to pyrethroid intoxication, the outcome is eventual paralysis and death. Pyrethroids are typically divided into two classes based on differences in specific responses and differential poisoning syndromes produced. Type 1 compounds (e.g., permethrin) are associated with more rapid decay of modified sodium currents that is consistent with their lower toxicity and less intense effects on nerve firing (Bloomquist 1996). Type 2 compounds (deltamethrin and lambda-cyhalothrin) are approximately ten-fold more potent than Type 1 pyrethroids for depolarizing motor nerve terminals, an effect that is correlated with acute toxicity. Another differing physiological response is that Type 1 compounds have a negative temperature coefficient for toxicity action, while Type 2 show a positive temperature toxicity producing increasing kill with increased in temperature (Ware 2000). Although speculative as a mechanism for selection of resistance, the higher ambient temperatures associated with the typical tropical/subtropical climate in Thailand may actually decrease the potential toxicity of permethrin to sublethal levels while enhancing potency of the other two.

One limitation to this study was not including a component to investigate the possible mechanisms involved in permethrin or deltamethrin resistance. Mosquito resistance to pyrethroids can involve one or more mechanisms including target-site (sodium ion channel) insensitivity (knock-down resistance) and/or metabolic detoxification of the active ingredient. The genetic mutation expressing knock-down resistance (*kdr*) results in reduced nerve sensitivity to pyrethroids. A range of mosquito species, including *Ae. aegypti* (Hemingway et al. 1989), have had *kdr*-like mechanisms identified that can either involve a broader pattern of DDT- pyrethroid cross-resistance or be limited to a subset of pyrethroids alone (Hemingway and Ranson 2000). Earlier studies indicate a *kdr*-type mechanism may have been involved in a DDT-resistant population of *Ae. aegypti* from Bangkok that showed concurrent resistance to permethrin and bioresmethrin (Malcolm and Wood 1982). Alternatively, metabolic mechanisms involving enzymatic detoxification of pyrethroids typically involve one or more non-specific esterases and/or monooxygenases (cytochrome P_{450}). More recently, biochemical analysis had shown a significant metabolic elevation of monooxygenase and esterase activities, together with alterations to the sodium ion channel as evidence of target site insensitivity associated with deltamethrin-resistant *Ae. aegypti* in Thailand (Yaicharoen et al. 2005). Paeporn et al. (2004) selected for permethrin and deltamethrin resistance in separate laboratory populations of *Ae. aegypti* and detected significant elevated esterase and monooxygenase activity in addition to increases in glutathione-*S*-transferase (GST) activity in the permethrin-selected population. Only metabolic mechanisms were investigated, so that a *kdr*-type mechanism may also have been involved. Moreover,

metabolic degradation mechanisms appear to play a relatively minor role, if any, in pyrethroid resistance in *Ae. aegypti* compared to *kdr* resistance alleles (Hemingway et al. 1989).

If *kdr* resistance is the primary mechanism behind resistant populations identified in our study, genotypic selection may have resulted from wide spread use of pyrethroids in organized space spray operations or the ubiquitous presence of these active ingredients in commercial household insect control products. Alternatively, the persistence of genetic resistance to DDT years after being discontinued may have perpetuated the *kdr* mechanism following its replacement with pyrethroids (Chadwick et al. 1977). Close monitoring of the resistance gene frequency over one or multiple spray seasons would indicate whether this mechanism is being selected and maintained in natural populations.

Insecticides will continue to play a crucial, if not indispensible, role in the control of most vector-borne diseases. This is particularly true for curbing transmission of dengue and chikungunya viruses. Vector control by attacking both larval habitats and adult mosquitoes remains the principal method for reducing risk of dengue infection (Reiter and Gubler 1997). Despite the infrequency and incomplete coverage in monitoring and reporting, there is sufficient evidence to show an increasing trend in vector resistance in Thailand. It is imperative that investigations be broadened geographically to also include increased inquiry on *Ae. albopictus* and the identification of the physiological and genetic mechanisms of resistance in the country.

In this study, despite use of a three-fold increase in permethrin concentration, tolerance or resistance was manifest in all populations of *Ae. aegypti* and *Ae. albopictus* tested, representing a wide geographical coverage within Thailand. Increased emergence and frequency of either direct or cross-resistance to a wider array of different pyrethroid chemicals and formulations would have serious and long-term implications for future dengue control strategies using insecticides. Current and on-going monitoring of mosquito vector populations will remain the primary determinant to guide timely and practical decisions as to the continued effectiveness and use of certain insecticides under relevant circumstances (Roberts and Andre 1994). Evidence-based decisions will further help select appropriate countermeasures (e.g., rotation or mixtures of effective insecticides) to control or mitigate the impact of increased resistance. Establishing a quality controlled, comprehensive program for routine monitoring of susceptibility and early detection of insecticide resistance to include the identification of those mechanism(s) responsible for resistance should be an integral part of an insecticide evaluation program aimed at effective integrated vector management practices.

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Evaluation of a Peridomestic Mosquito Trap for Integration into an *Aedes aegypti* (Diptera: Culicidae) Push-Pull Control Strategy

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

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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).

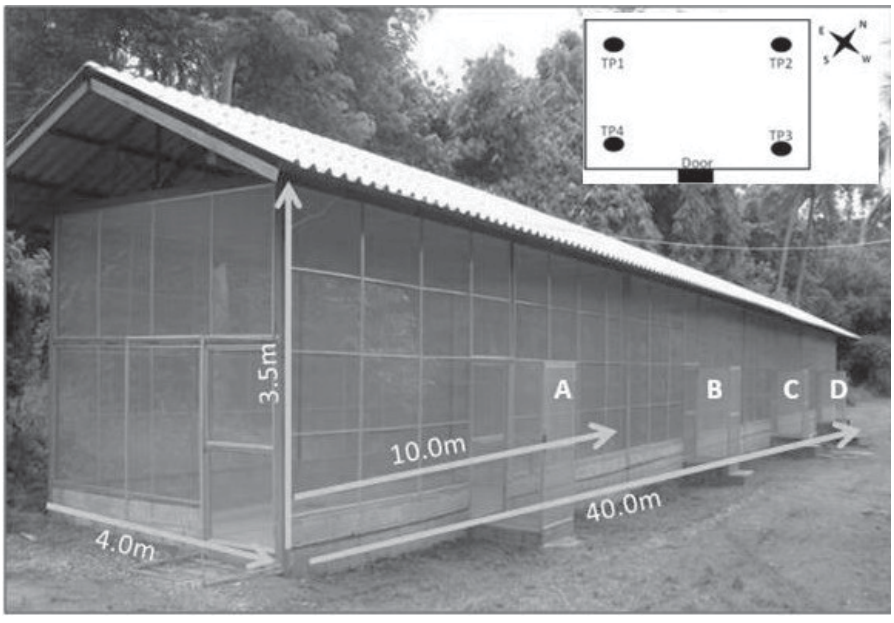

Figure 1. The screen house facility at Pu Teuy, Kanchanaburi, Thailand. All sides are screened and the cement floor is covered with white plastic to detect knocked-down mosquitoes. Collapsible walls are used to partition the screen house into four separate 10m long cubicles (A-D). Top insert: designated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

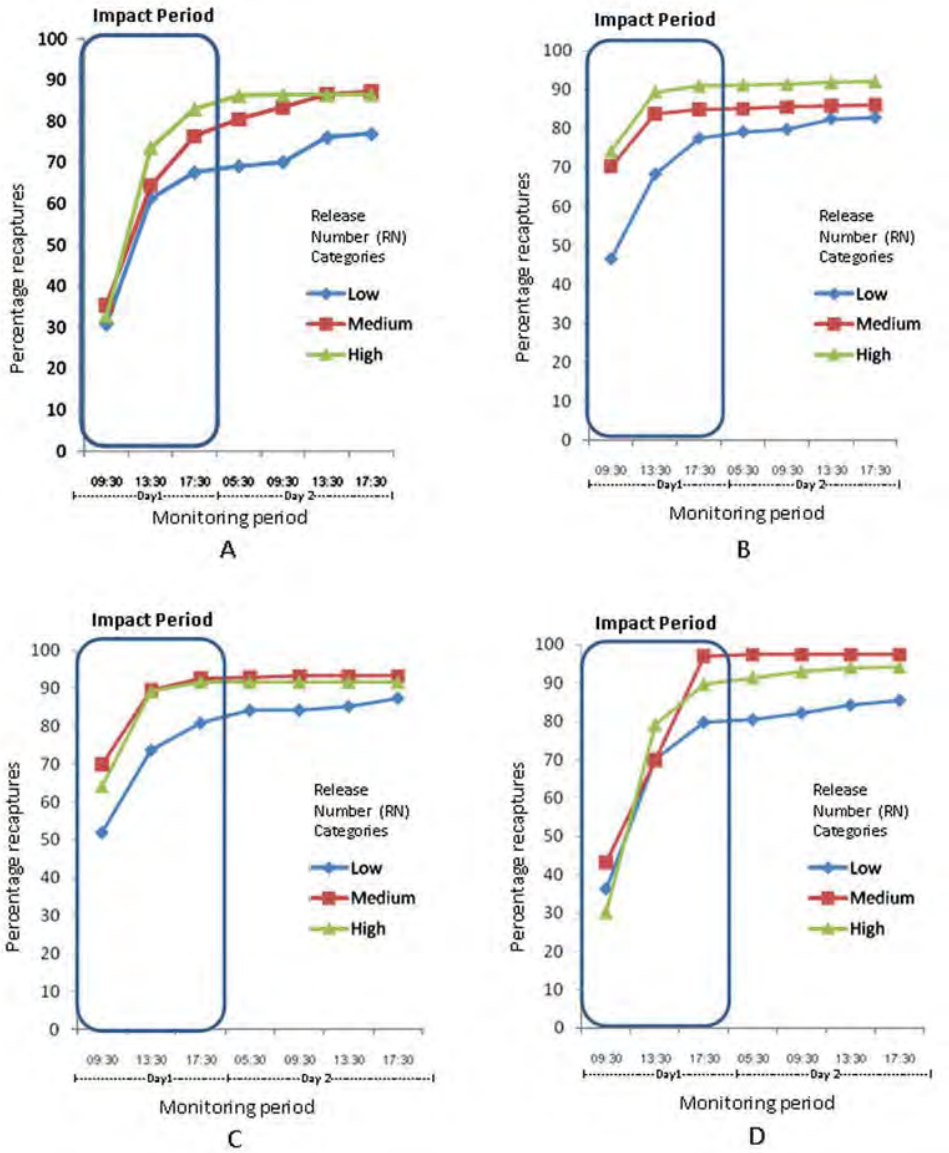

Figure 2. Cumulative percentage recpature of marked *Ae. aegypti* females using: A) one, B) two, C) three, and D) four BGS traps against low, medium, and high mosquito release densities . The Impact Period designates the monitoring interval when the greatest recapture occurred.

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 ethal Concentrations and Efficacy of Si 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]		Kanchanaburi		Khon Kaen		Nong Khai	
		No. tested	Dead (%)	No. tested	Dead (% ± SE)	No. tested	Dead (% ± SE)	No. tested	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).

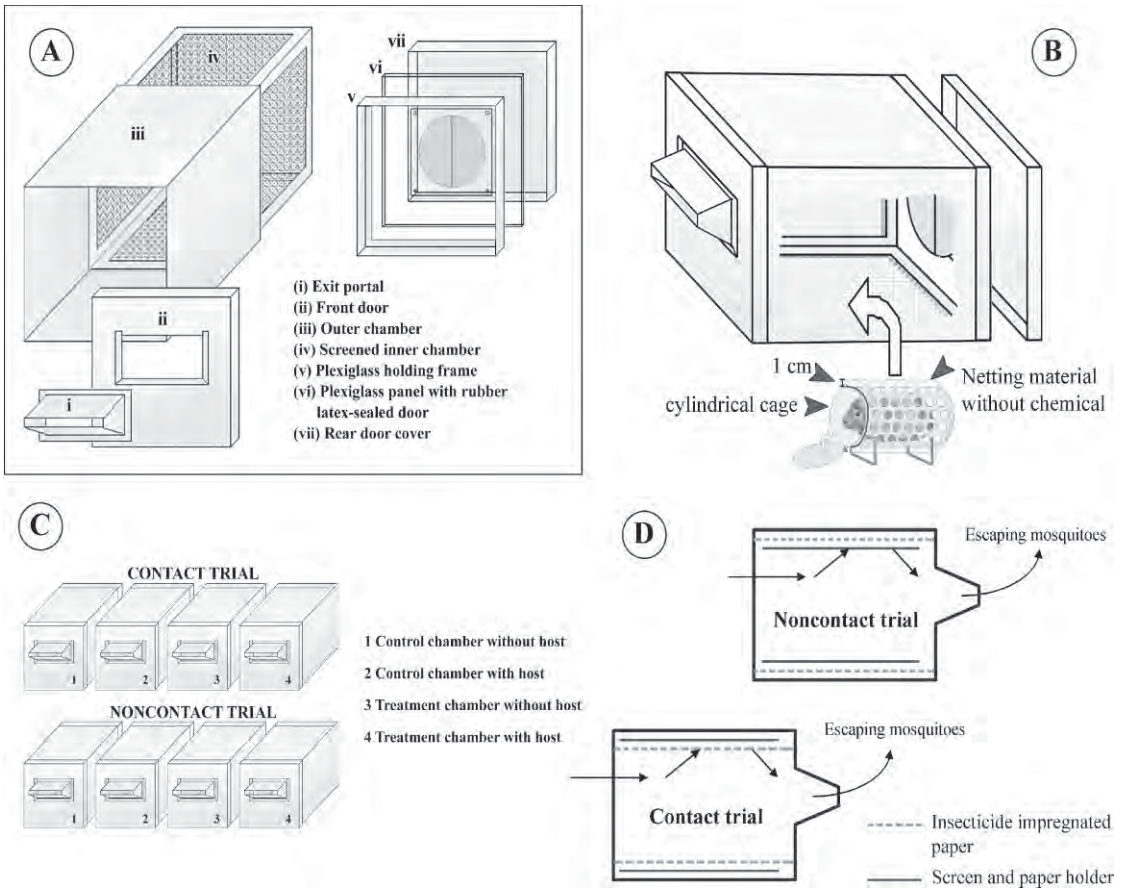

Fig. 1. (A) Excito-repellency test chamber, (B) the chamber with live host (guinea pig), (C) composition of excito-repellency test chamber for behavioral studies, and (D) side view of test chamber for contact and noncontact trials.

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.

Trial	Insecticides	Conditions	*An. harrisoni*		*An. minimus*	
			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.

Species	Conditions	Deltamethrin			Bifenthrin		
		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.

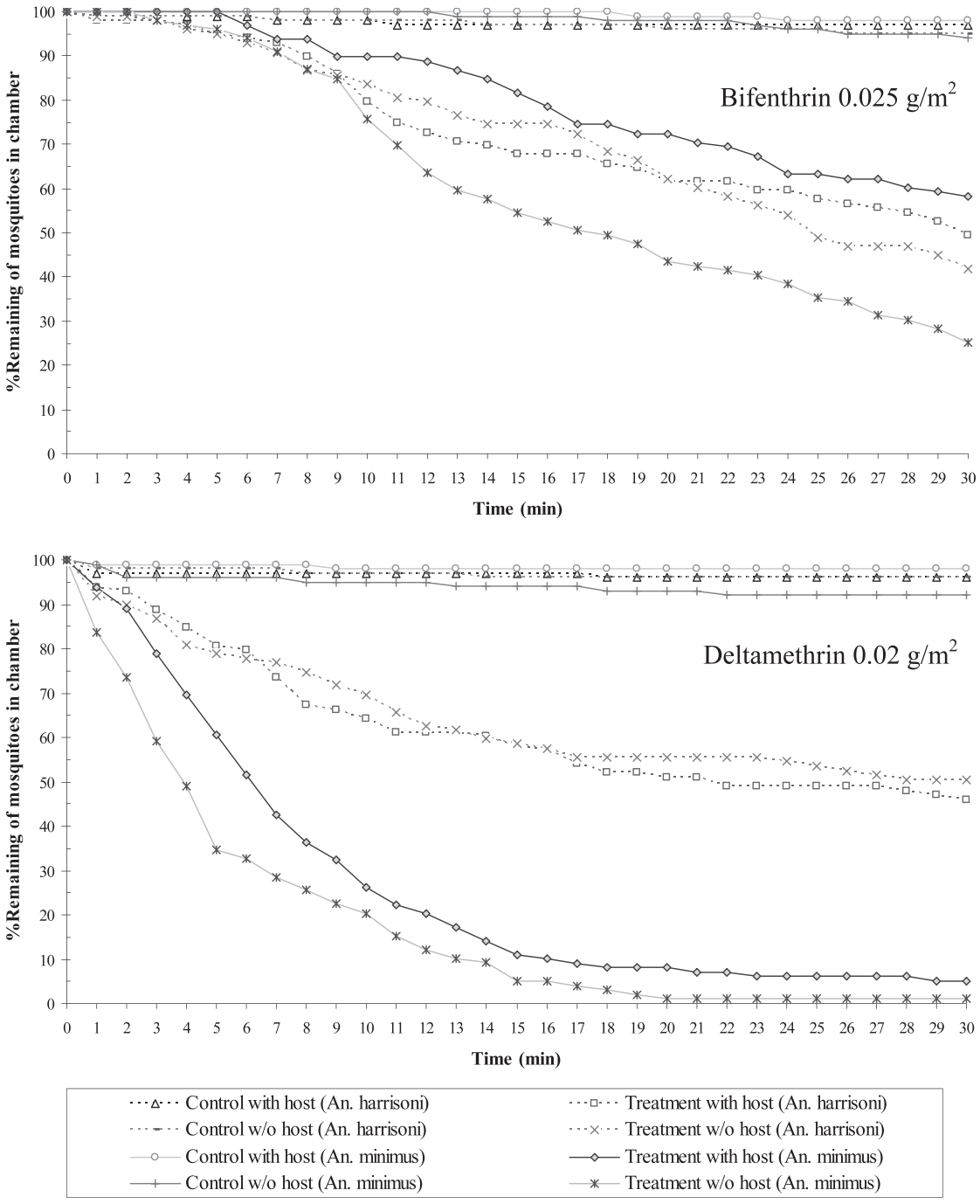

Fig. 2. Escape patterns of *Anopheles harrisoni* and *An. minimus* to bifenthrin 0.025 g/m² and deltamethrin 0.02 g/m² in contact trial.

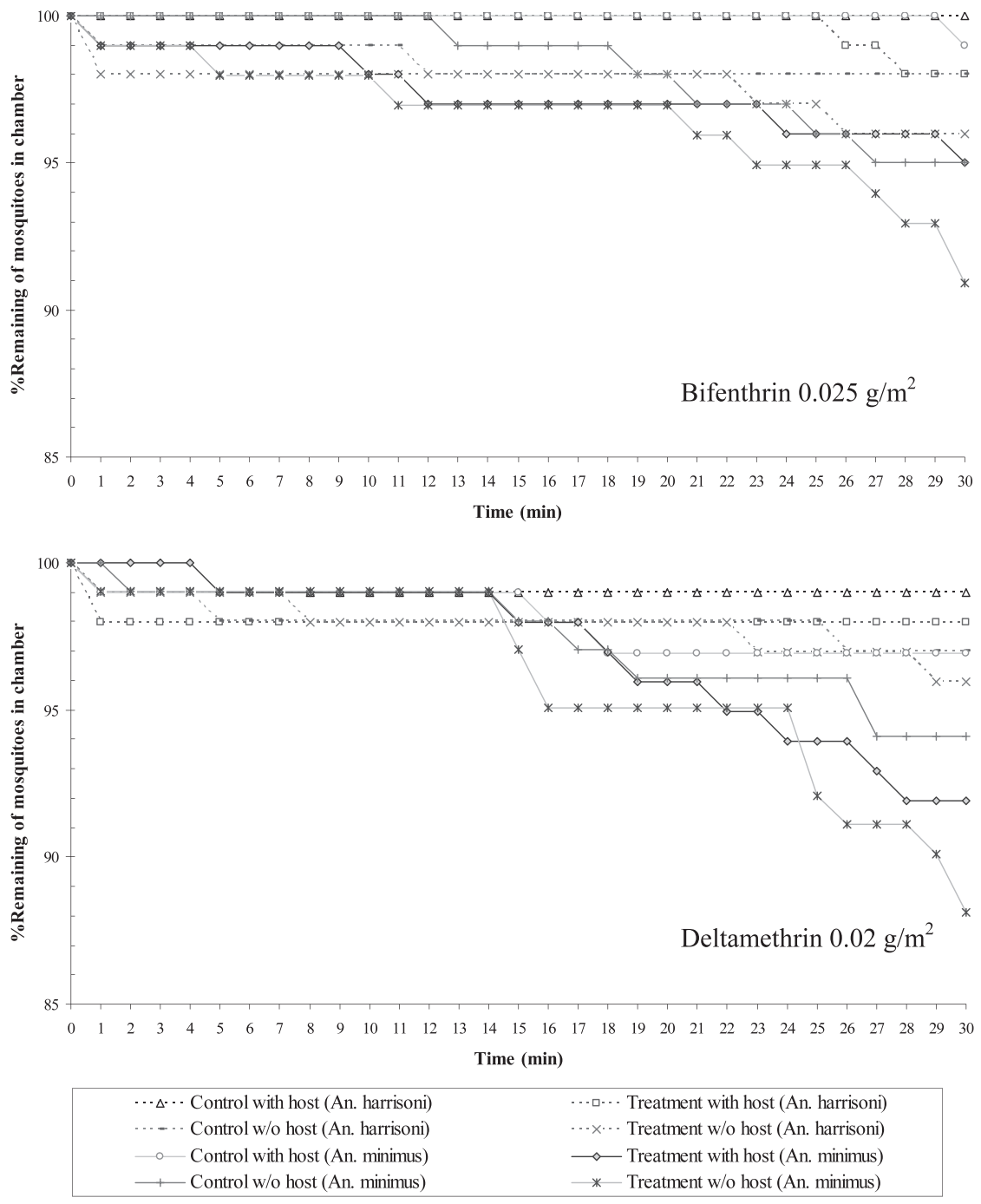

Fig. 3. Escape patterns of *Anopheles harrisoni* and *An. minimus* to bifenthrin 0.025 g/m^2 and deltamethrin 0.02 g/m^2 in noncontact trial.

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-phenoxyphenyl)methyl (1*S*,3*S*)-*rel*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) (BASF Aktiengescllshaft, Bangkok, Thailand), 3) λ-cyhalothrin (LAM) (Cyano-3-phenoxybenzyl (1s+1r)-cis-3-(z-2-chloro-3, 3, 3-trifluoroprop-1-enyl)-2, 2-dimethyl cyclopropanecarboxylate) (Syngenta Crop Protection (Thailand) Ltd, Bangkok, Thailand), and 4) DEET (*N,N*-diethyl-m-toluamide or *N,N*- diethyl-3-methylbenzamide) (United States Department of Agriculture, 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*

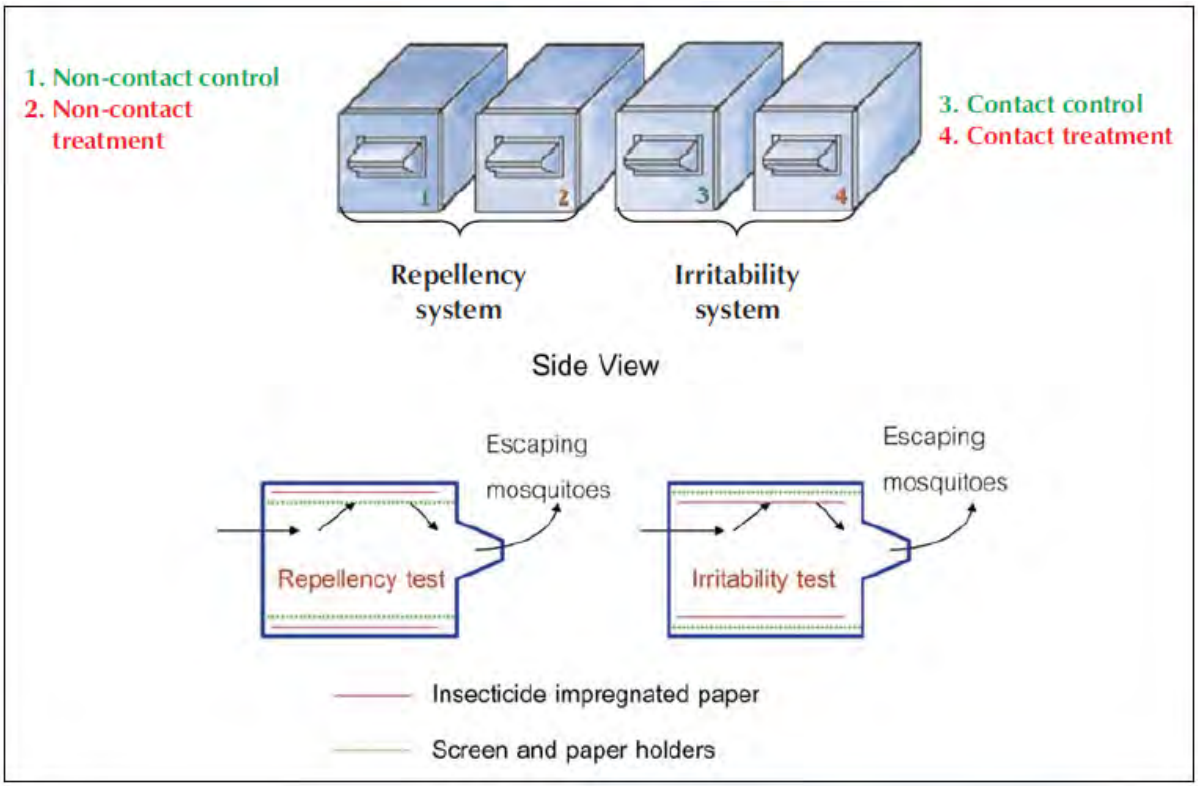

Figure 1. Excito-repellency test chamber used to study insecticide behavioral responses.

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

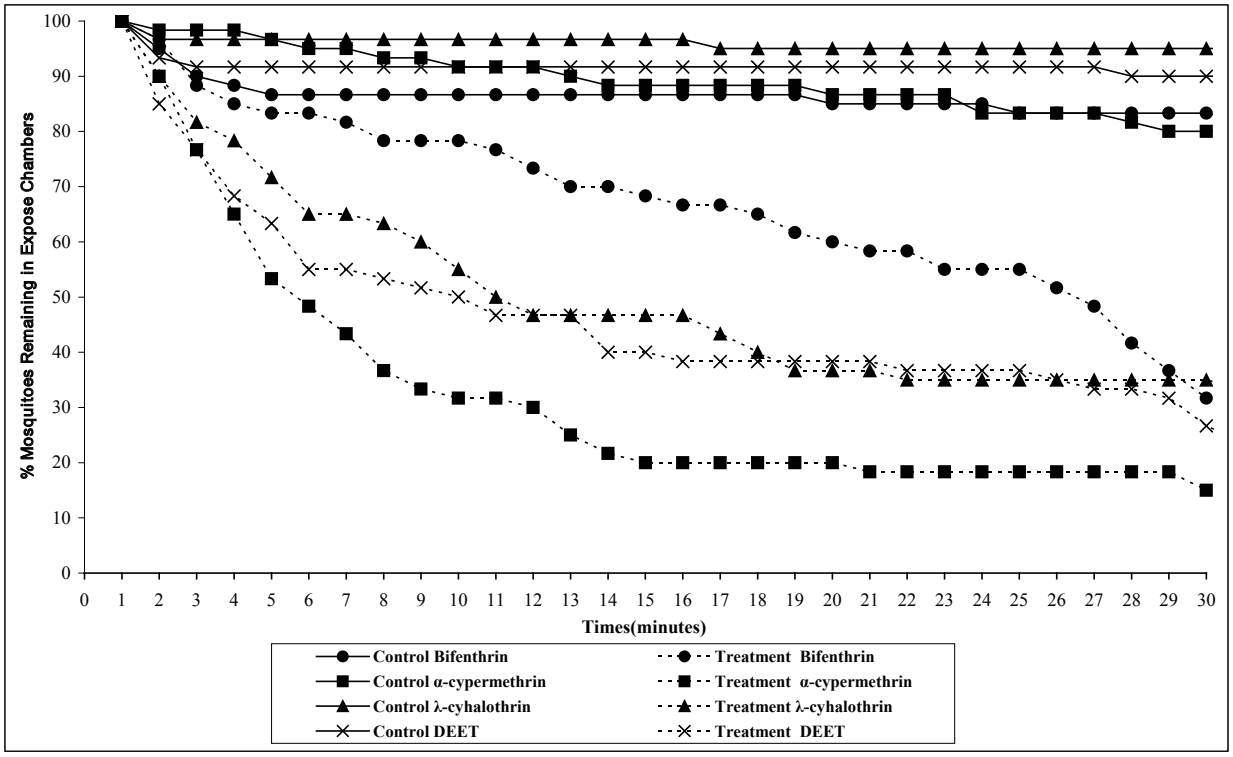

Figure 2. Escape probability of *Anopheles dirus* exposed to bifenthrin =0.025 g/m², α-cypermethrin=0.03 g/m², λ-cyhalothrin =0.02 g/m² and DEET =2 g/m² for treatment and control contact trials.

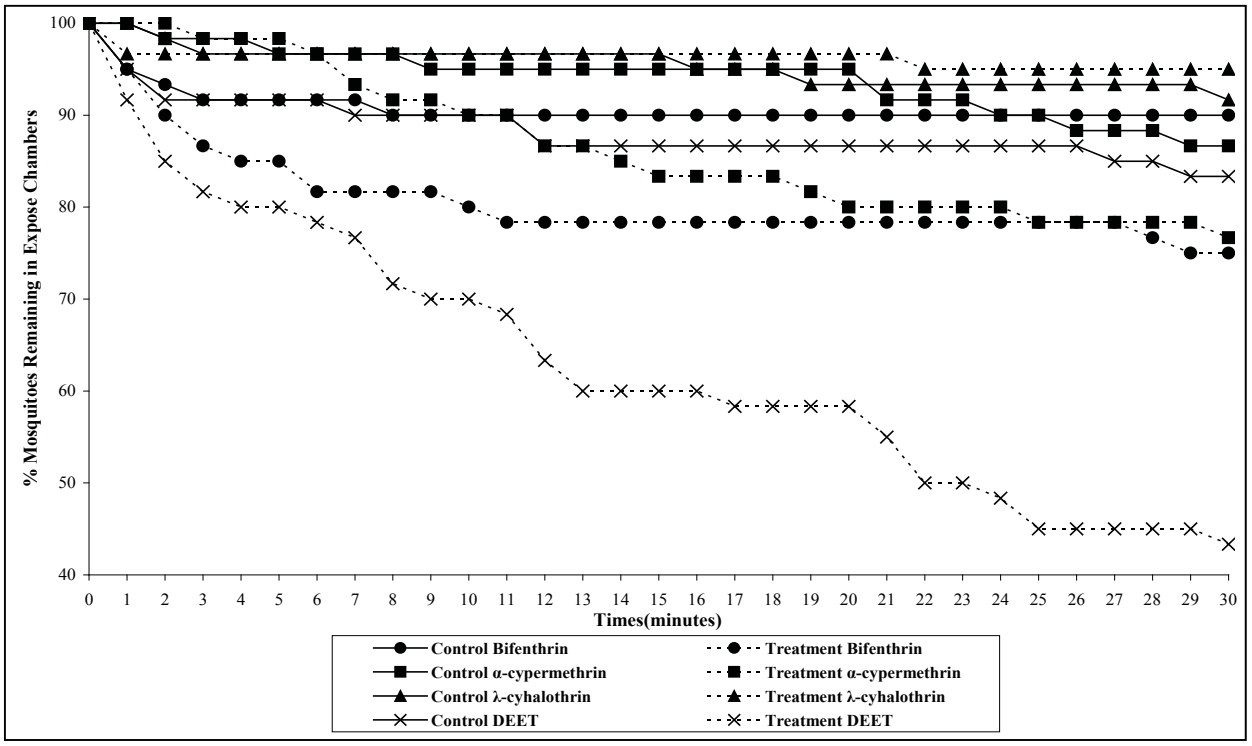

Figure 3. Escape probability of *Anopheles dirus* exposed to bifenthrin =0.025 g/m², α-cypermethrin=0.03 g/m², λ-cyhalothrin =0.02 g/m² and DEET =2 g/m² for treatment and control noncontact trials.

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.