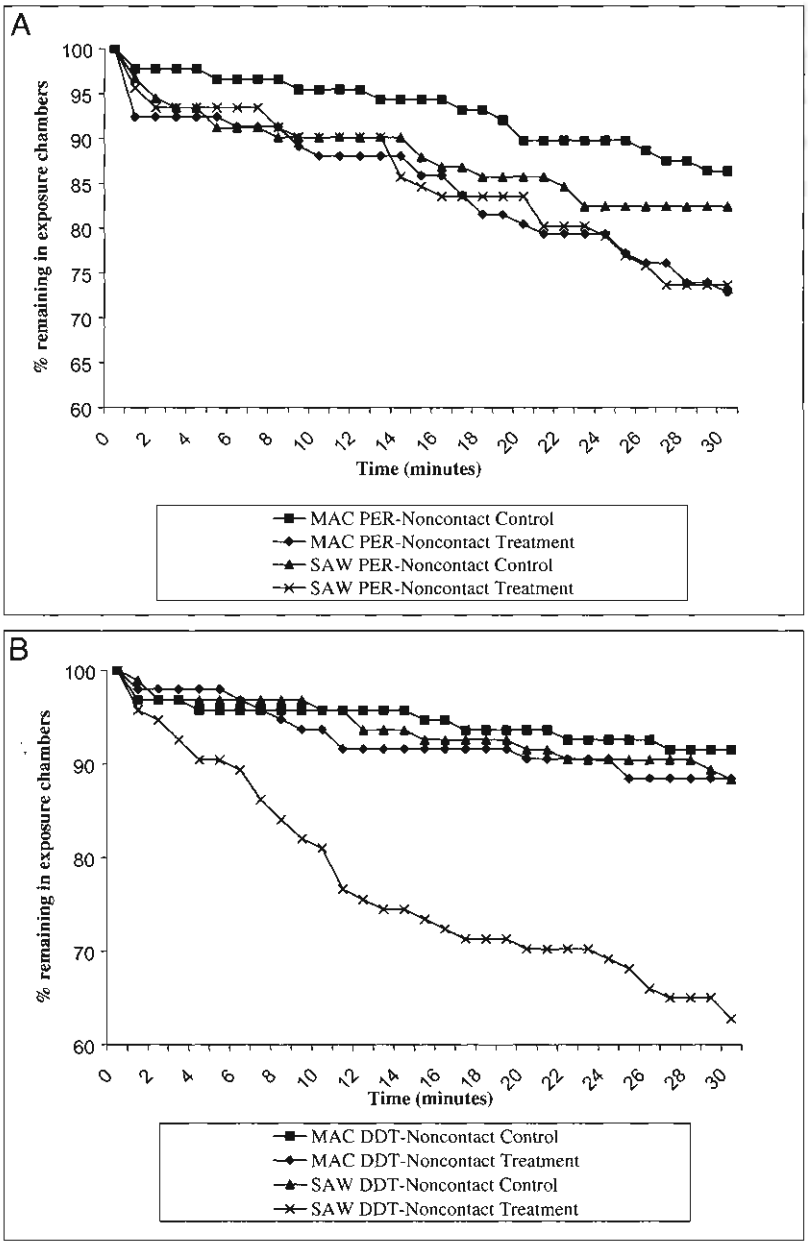

Fig. 3. Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in paired control versus noncontact trials after exposure to either (A) 0.5 g/m^2 permethrin or (B) 2 g/m^2 DDT.

demonstrated that the combined responses of irritancy and repellency exerted the most dominant force on vector mosquitoes in reducing indoor human–vector contact. Other related entomological studies have similarly shown that both ER and toxicological roles of insecticides should be carefully evaluated on vector populations from different geographic locations (Sungvornyothin et al. 2001, Chareonviriyaphap et al. 2004, Potikasikorn et al. 2005). Pyrethroids also elicit profound behavioral responses in insects (Threlkeld 1985). Malaria transmission control strategies by wide-scale use of permethrin-treated bed-nets (inseciticide-treated nets; ITN) were initiated in Thailand in 1997 (Chareonviriyaphap et al. 1999) and have been a major reason for

extensive laboratory evaluations and field studies on pyrethroid avoidance behavior in mosquito vectors in Thailand.

This study observed the behavioral responses of 2 important malaria vectors to past and present chemicals used for malaria control. This investigation further supports the need for optimization and standardization of an ER test system to assess behavioral responses of mosquitoes considered significant and fundamental functions of public health insecticides in disease control. Significant behavioral avoidance responses were observed in contact trials compared with paired controls and were very similar for both species. The most striking escape response after physical contact with permethrin was observed in *An. maculatus*. More moderate irritancy with DDT was observed with both species, but responses remained significantly different from the controls. Noncontact repellency to DDT played a significant role in the escape response of *An. sawadwongporni*, an observation in agreement with previous findings against other vector species (Chareonviriyaphap et al. 1997, 2001; Sungvornyothin et al. 2001; Potikasikorn et al. 2005). Postexposure mortality was low in mosquitoes escaping the treated chambers in virtually all contact and noncontact trials, suggesting that behavioral avoidance to test compounds, not their toxicity, was the primary outcome when allowing mosquitoes a free choice.

Behavioral response results represent an important, but often overlooked, component of the chemical-disease control equation. DDT is a poignant example. After decades of proven success in dramatically reducing the burden of malaria worldwide, DDT fell into political disfavor more out of concern for environmental degradation than loss of effectiveness. Interestingly, the development of resistance to DDT by various malaria vectors was not documented in many countries, including Thailand (Roberts and Andre 1994, Chareonviriyaphap et al. 1999); therefore, the decision to withdraw the use of this valuable chemical from the control arsenal seemed premature from an operational standpoint. DDT continues to prove effective in malaria suppression in countries that have either maintained or resumed its routine use for indoor vector control (Roberts et al. 1997b, Curtis and Mnzava 2000, Curtis 2002, Romi et al. 2002). Historically, the purported failure of DDT seems to have been more a reflection of failed commitment by governments to properly sustain control programs and adequate spray coverage than any inherent failure of the chemical itself (Farid 1991).

Prevailing arguments maintaining a chemical's efficacy based solely on toxic properties notwithstanding, we alternatively suggest that the lack of physiological resistance in mosquito populations would provide evidence for insufficient selection pressure based on contact toxicity, implicating behavioral avoidance as an important mechanism of effective transmission control. Most recent studies remain focused on toxicity as the explanation and principal measure of vector control success, when ER alone could explain the continued efficacy of an insecticide despite resistant vector populations (Henry et al. 2005). Even in the face of resistance, if an insecticide remains effective in the control of malaria incidence, by inference alone, its true value could be measured in its ability to continue to evoke strong avoidance behavior in vector populations, thereby reducing in human–vector contact.

The present findings are compatible with previous studies examining a wide range of species from varying locations (Ree and Loong 1989; Evans 1993; Chareonviriyaphap et al. 1997, 2001, 2004; Bangs 1999; Potikasikorn et al. 2005). The strong repellent action of DDT to *An. sawadwongporni* could be partly a consequence of previous exposure to DDT, or more likely, an innate response characteristic of the test population. Wild-caught mosquitoes are heterogeneous in age and nutritional/physiological status. Previous work has demonstrated that physiological and nutritional conditions can influence avoidance behavior; therefore, interpretation of avoidance responses in our wild-caught populations should be taken with some measure of caution compared with findings from laboratory studies (Roberts et al. 1984, Sungvornyothin et al. 2001).

Anopheles maculatus and *An. sawadwongporni* are recognized as important vectors of malaria in southern Thailand and of areas along the Thai–Myanmar border (Baimai 1989, Rattanarithikul et al. 1996, Chareonviriyaphap et al. 2004). Southern Thailand is a significant producer of natural rubber. Hilly rubber plantations provide good habitats for *An. maculatus* and *An. sawadwongporni*. Local people wear long-sleeved clothing during evening work for both warmth and personal protection against biting mosquitoes. Thailand recently launched a revised malaria control strategy by using permethrin ITN technology with impregnated bed-nets (Chareonviriyaphap et al. 2004, Department of Disease Control 2005). It is widely accepted that many pyrethroids stimulate mosquitoes to avoid (escape) sprayed surfaces, especially upon direct contact (Miller 1990, Lindsay et al. 1991). Our results clearly showed that permethrin produces strong and unmistakable behavioral escape responses. Appreciating the effects that some insecticides have on the intrinsic behavioral patterns and responses of mosquitoes that can ultimately disrupt or interfere with bloodfeeding success must be considered when assessing the full impact of a chemical's usefulness on reduction of disease transmission. During comprehensive eval-

uations of insecticides, it would be prudent to document the full range of responses by vectors to a chemical before expending significant resources and initiating large-scale use of particular compounds in control programs.

ACKNOWLEDGMENTS

We thank the Armed Forces Development Command, Sai Yok District, Kanchanaburi Province, for supporting activities in the study areas. This project was jointly supported by the Thailand Research Fund and the Kasetsart University Research and Development Institute, Thailand.

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Behavioral Responses of Malaria Vectors, *Anopheles minimus* Complex, to Three Classes of Agrochemicals in Thailand

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J. Med. Entomol. 44(6): 1032–1039 (2007)

ABSTRACT Behavioral responses of two wild-caught populations of *Anopheles minimus* complex, species A and C, exposed to operational field doses of three commonly used agricultural insecticides, carbaryl (carbamate), malathion (organophosphate) and cypermethrin (pyrethroid), were characterized using an excito-repellency test system. Test populations were collected from different localities in Kanchanaburi Province, western Thailand. Both populations showed strong irritancy by quickly escaping test chambers after direct contact with individual surfaces treated with each insecticide compared with match-paired untreated controls. Noncontact repellency response to cypermethrin and carbaryl was significantly pronounced in both A and C populations, but comparatively weak when exposed to malathion. Noncontact repellency produced much weaker escape response in both populations, but in some species–chemical combinations, it remained significant compared with controls. We conclude that contact irritancy is a major behavioral response of both A and C when exposed directly to any of the three compounds, whereas only cypermethrin produced a significant repellency response in species A.

KEY WORDS behavioral avoidance, carbaryl, cypermethrin, malathion, *Anopheles minimus* complex

The development of pesticide resistance by arthropods is a primary concern for management of agricultural and human pests and disease vectors. Over 50 yr of extensive use of a variety of synthetic organic compounds used to control arthropods has resulted in the selection of insecticide resistance in >500 species, of which >100 are mosquitoes (Culicidae) (Brown and Pal 1971, Georghiou 1990, Roberts and Andre 1994). Over a decade ago, the World Health Organization estimated that 40% of the 506 insect species of medical importance had evidence of resistance to various insecticides (WHO 1992). Most documented cases of resistance have involved organochlorine, organophosphate, and carbamate class compounds compared with the relatively more recent introduction of broad-spectrum pyrethroids (Brogdon and McAllister 1998).

Although resistance to insecticides in mosquitoes has been reported in many areas of the world, some mosquito species have not developed resistance in spite of the apparent heavy and pervasive exposure to pesticides used in public health and agriculture (Roberts and Andre 1994). One plausible explanation for these findings is the role of avoidance behavior, i.e., the innate response by an insect after exposure to compounds that can elicit profound irritant and/or repellent reactions, thereby limiting direct contact with lethal toxicants. By avoidance alone, selection pressure to increase frequency of resistant genotypes in the mosquito population is slowed or mitigated (Chareonviriyaphap et al. 1997).

Behavioral avoidance can be separated into two distinct responses: contact irritability and noncontact repellency, collectively termed excito-repellency (Davidson 1953, Rawlings and Davidson 1982, Roberts et al. 1997). Irritability occurs when an insect is stimulated to move away from an insecticide after direct physical contact with the chemical residue, whereas repellency occurs when the insect detects chemicals from a distance and avoids treated surfaces before making physical contact (Roberts et al. 1997, Potikasikorn et al. 2005). The relative importance of either form of behavioral avoidance is more clearly demonstrated when using a specially designed excito-repellency test system (Roberts et al. 1997, Chareonviriyaphap et al. 2002). This test system has repeatedly proven useful for quantitative evaluation of excito-repellency in mosquito species against various insecticidal compounds in Thailand (Sungvornyothin et al. 2001; Chareonviriyaphap et al. 2003, 2004; Kongmee et al. 2004; Potikasikorn et al. 2005).

Thailand is an important commercial producer of natural rubber, rice, corn, palm oil, cassava and oranges, all major export-earning crops. Most crop pro-

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0022-2585/07/1032–1039$04.00/0

duction at some point involves the use of various pesticides to control destructive and disease-carrying pests (Jungbluth 1996). Ecologically, many areas of intensive agricultural are potential, if not, preferential larval habitats and adult resting places for certain disease vectors. The widespread use of pesticides to protect agricultural crops may inadvertently select for resistance in those vectors having frequent contact with these areas (Georghiou et al. 1972, Bown 1987, Brogdon et al. 1988, Georghiou 1990). In some cases, even the separation of mosquito populations between agricultural spray and nonagricultural environments has been shown to have minimal effect limiting the extent of uniform patterns of resistance and cross-resistance due to genetic intermixing (Bailey et al. 1981). More recently, pyrethroids have gained a substantially larger share of the agricultural pest control and public health market, replacing many of the other common class chemicals, primarily organochlorines and cyclodienes, along with various organophosphates and carbamates (Roberts and Andre 1994). Pyrethroids, with their high insecticidal activity, combined with relatively low mammalian toxicity and rapid environmental biodegradation, are more desirable for broad-spectrum use.

Under the right conditions, agricultural development and cultural practices can have a profound influence on vector populations (Hobbs 1973, Mulla et al. 1987, Bown 1987). There have been strong associations between agricultural production and malaria (Chapin and Wasserstrom 1981, 1983), including the development of insecticide resistance in anopheline vectors as a result of exposure to agrochemicals (Georghiou et al. 1972, Brogdon et al. 1988, Lines 1988). Understanding the behavioral responses of malaria vectors exposed to agrochemicals can assist a mosquito abatement program's monitoring efforts for detection of resistance and help guide the most appropriate interventions to mitigate or counter resistance before it occurs.

The *Anopheles minimus* complex, Theobald 1901, is an example of a malaria vector species that has a close association with agriculturally developed areas (Rongnoparut et al. 2005, Garros et al. 2006). Because *An. minimus* is a rural mosquito that can breed in and around cultivated areas, it has potentially ample exposure to a variety of insecticides. In rural northern Thailand, the density of malaria vectors was shown to decrease proportionally with an increase in the development of fruit orchards, presumably the result of insecticide spraying to protect production (Overgaard et al. 2003). However, this and subsequent observations on the relative sustained susceptibility of *An. minimus* to agrochemicals indicated that behavior avoidance was a possible mechanism limiting exposure and suppressing selection pressure favoring resistant genotypes (Overgaard et al. 2005).

Although other studies have shown clear behavioral avoidance by anophelines, including some member of the *An. minimus* complex, to various public health insecticides (Chareonviriyaphap et al. 2001, 2004; Potikasikorn et al. 2005), no investigation has been performed on anopheline response to agrochemicals. Described herein is the response of *An. minimus* species A and C to recommended field concentrations of three commonly applied agrochemicals (carbaryl, malathion, and cypermethrin) in Thailand.

Materials and Methods

Test Populations. *Anopheles minimus* species A was collected by human bait capture in Mae Nam Noi Village, Thong Pha-Phoom District, Kanchanaburi Province (14° 35′ N, 98° 36′ E), and *An. minimus* species C was collected by cow bait capture in Pu Teuy Village, Sai Yok District, Kanchanaburi (14° 20′ N, 98° 59′ E). Kanchanaburi Province is located in western Thailand bordering Myanmar. Mosquitoes were held in plastic cups, provided with cotton pads soaked with 10% sugar solution, and they were transported to the field laboratory for morphological identification. During transport and holding, mosquitoes were kept in larger Stryofoam containers and covered with damp cotton towels to reduce desiccation. The *Anopheles minimus* complex represent closely related sibling species that are difficult to definitively distinguish from one another using morphological characters alone (Green et al. 1990, Garros et al. 2006, Harbach et al. 2006); thus, molecular methods were used to establish the correct identity of both populations (Sharpe et al. 1999). Each collection site harbored only one sibling species.

Insecticide-Treated Papers. Papers were impregnated using technical formulation grade insecticides at applied operational field concentrations as recommended on the product label. The final dosage rates were 0.4 g/m^2 carbaryl (1-naphthalenyl methylcarbamate), 0.19 g/m^2 malathion {diethyl [(dimethoxyphosphinothioyl)thio]butanedioate}, and 0.04 g/m^2 cypermethrin [cyano(3-phenooxyphenyl)methyl 3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylate]. All papers were treated at the rate of 12.5 ml of insecticide solution per 0.0928 m^2 (26.5- by 35-cm) paper as described previously (Chareonviriyaphap et al. 2002).

Excito-Repellency Tests. Tests were designed to compare the behavioral response of both field populations during contact and noncontact exposures by using insecticide-treated papers inside excito-repellency test chambers (Chareonviriyaphap et al. 2002). The tests were undertaken within 48 h of mosquito capture. Only nonblood-fed/nongravid females were used in the tests. Mosquitoes were deprived of all nutritional energy sources, and they were provided access to water only for a minimum of 12 h before test exposure. All trials were performed in a field laboratory during daylight hours, and each test series was replicated three times. Temperature and relative humidity were recorded during tests. Escaping mosquitoes were observed at 1-min intervals for 30 min. The escape times (ETs) for 30, 50, and 70% (ET_{30}, ET_{50}, and ET_{70}, respectively) of the test population were recorded. The number of dead or knockdown specimens was recorded separately for each exposure

Table 1. Escape response and 24-h mortality of *An. minimus* A and C to carbaryl, malathion, and cypermethrin in contact and noncontact trials with paired controls (Ct)

Test	Pop	Chemicals (no. tested)	No. escaped (%)	% Mortality	
				Escaped	Remained
Contact	A	Carbaryl (77)	35 (45)	8.6	16.7
		Carbaryl-Ct[a] (76)	14 (19)	0	0
		Malathion (65)	55 (85)	0	
		Malathion-Ct (60)	13 (22)	0	0
		Cypermethrin (72)	42 (58)	21	0
		Cypermethrin-Ct (76)	19 (25)	0	0
	C	Carbaryl (78)	53 (68)	0.2	0.1
		Carbaryl-Ct (77)	21 (27)	0	0
		Malathion (78)	18 (23)	0	0.1
		Malathion-Ct (80)	10 (13)	0	0
		Cypermethrin (87)	58 (67)	0.1	0.1
		Cypermethrin-Ct (84)	19 (23)	0	0
Noncontact	A	Carbaryl (71)	35 (49)	0	5.6
		Carbaryl-Ct (75)	15 (20)	0	0
		Malathion (65)	34 (52)	2.9	0
		Malathion-Ct (60)	14 (23)	0	0
		Cypermethrin (73)	20 (27)	10	1.9
		Cypermethrin-Ct (77)	9 (12)	0	0
	C	Carbaryl (76)	61 (80)	0	0
		Carbaryl-Ct (76)	15 (20)	0	0
		Malathion (80)	30 (38)	0.1	0.1
		Malathion-Ct (78)	3 (4)	0	0
		Cypermethrin (85)	54 (64)	0.1	0.1
		Cypermethrin-Ct (83)	29 (35)	0	0

[a] Ct, control.

chamber, external escape cage, and control chambers (without insecticide). Escaped specimens and those remaining inside chambers, for both treatment and paired controls, were held separately in small holding containers under controlled conditions (25 ± 5°C and 80 ± 10% RH), and they were provided with 10% sugar solution until recording post-24-h mortality.

Data Analysis. A Kaplan–Meier survival analysis method was used to analyze and interpret the behavioral response data (Kleinbaum 1995). Survival analysis provides a robust statistical treatment of sequential excito-repellency data, and relative to other quantitative methods describing behavioral avoidance, survival curves minimize loss of valuable information while estimating temporal mosquito escape probability (Roberts et al. 1997). Patterns of escape response between treatment groups were compared using the log-rank method (Mantel and Haenzel 1959), and SAS Release 6.10 (SAS Institute, Cary, NC) was used in the analysis. The discriminating level for all significant tests was 0.05%.

Results

Table 1 shows percentage of escape and percentage of mortality to tested chemicals separated by contact irritancy and noncontact repellency (Table 1). In the carbaryl contact trial, the escape response was significantly stronger ($P = 0.001$) in species C (68%) than in species A (45%), but less so for cypermethrin, wherein 67% of C and 58% of A escaped ($P = 0.054$) (Table 1). The opposite pattern was observed when different cohorts were exposed to malathion; species A showed an escape response significantly stronger (85%) than C (23%) ($P = 0.0001$). Similar escape patterns were observed in the noncontact trials. Repellency was significantly stronger ($P = 0.001$) in C (80 and 64%) than A (49 and 27%) when exposed to carbaryl and cypermethrin, respectively. As in the contact trial, the opposite pattern was observed for malathion where escape response was significantly stronger ($P = 0.001$) in A (52%) than in C (38%). Comparison between contact and noncontact responses showed significant differences in escape response *within* populations across all three compounds ($P = 0.001$–0.0001), with exception for carbaryl exposure with A ($P = 0.105$) and cypermethrin with C ($P = 0.205$). Species A escaped in higher numbers in contact versus noncontact trials involving malathion and cypermethrin, whereas species C escaped in greater numbers in noncontact versus contact trials with malathion and carbaryl.

Mortalities after 24 h were generally higher in A (0–21%) than in C (0–0.2%) for mosquitoes that either remained inside the chambers or those that had escaped (Table 1). The highest mortalities were observed in species A that had successfully escaped in contact and noncontact cypermethrin trials. For species A mosquitoes that remained in the chambers after 30 min, the 24-h mortalities were highest with carbaryl (contact, 16.7%; noncontact, 5.6%). Lower mortality was seen in other tests, and there were no deaths recorded in any of the controls.

Some escape times (ET_{30}, ET_{50}, and ET_{70}) could not be derived because of insufficient number of escaping mosquitoes after 30 min (Table 2). An ET_{70} could only be measured in malathion contact trials with species

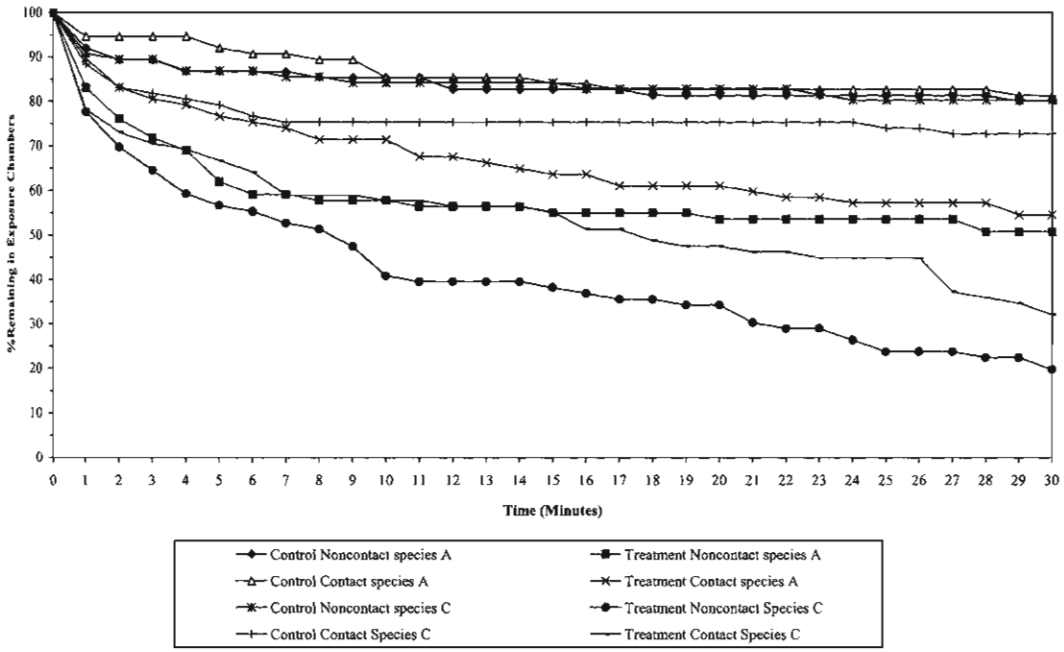

Fig. 1. Escape probability of *An. minimus* species A and C exposed to carbaryl and paired control for contact and noncontact trials.

A. All other contact trails with carbaryl and cypermethrin were not able to elicit >70% escape response in either population. In noncontact trials, only carbaryl forced >70% of C to escape (21 min). In the same test only 30% of A managed to escape within 4 min with no subsequent activity up to 30 min.

Figures 1–3 show the proportions of mosquitoes remaining in the excito-repellency test chambers

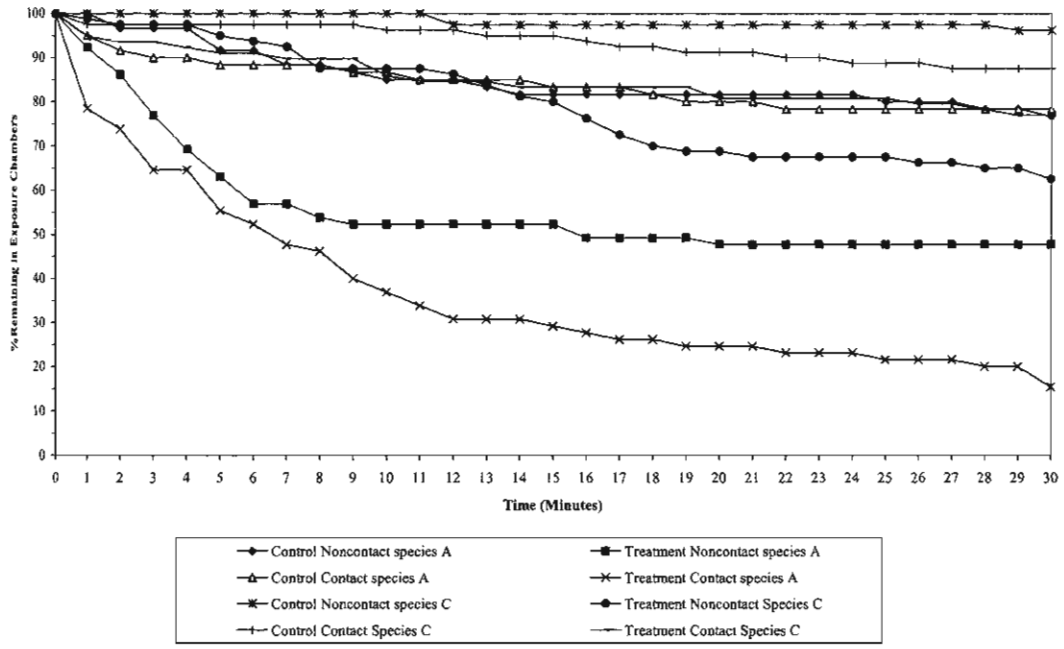

Fig. 2. Escape probability of *An. minimus* species A and C exposed to cypermethrin with paired control for contact and noncontact trials.

Table 2. Escape time in minutes for 30% (ET30), 50% (ET 50), and 70% (ET70) of *An. minimus* A and C to escape insecticide-treated chambers

Test/pop	Carbaryl			Malathion			Cypermethrin		
	ET30	ET50	ET70	ET30	ET50	ET70	ET30	ET50	ET70
Contact									
A	10	—	—	2	7	14	4	21	—
C	4	18	—	—	—	—	12	20	—
Noncontact									
A	4	—	—	4	16	—	—	—	—
C	2	8	21	18	—	—	9	18	—

—, insufficient number of mosquitoes escape from test chamber.

treated with each insecticide. Proportions were used to analyze and develop probabilities of escape from test chambers in the different formats. In both contact and noncontact trials, the escape rate of population A exposed to carbaryl and cypermethrin were significantly lower than those for C ($P < 0.05$). However, the opposite relationship was observed in trials with malathion, wherein the contact and noncontact escape rates were significantly greater ($P < 0.05$) for A compared with C (Fig. 3).

Discussion

This study represents the first measurement of behavioral responses of populations of *An. minimus* complex, species A and C, to common agrochemicals used in Thailand. The two primary avoidance responses, irritancy and repellency, were documented in both populations; however, depending on the chemical, significant differences in rate of escape were observed between species. For carbaryl and cypermethrin, greater irritancy and repellency were seen in C, whereas malathion produced stronger responses in species A. Although differences in degree and patterns of escape were noted between irritancy and repellency, both seem to play a role in *An. minimus* escape responses. Both species used in this study were nonsympatric and collected from substantially different land use areas and geographically separated at a direct linear distance of ≈60 km. Species A was collected from a village and forest fringe setting, an area that is considered a high-risk malarial zone (designated A1), but having a relatively low level of agricultural insecticide use (primarily rubber plantations and mixed farming). Species C was obtained from a low malaria risk zone (A2) in an area of low hills near the margins of villages with relatively high levels of agrochemical use (assorted fruit orchards, corn, and other row

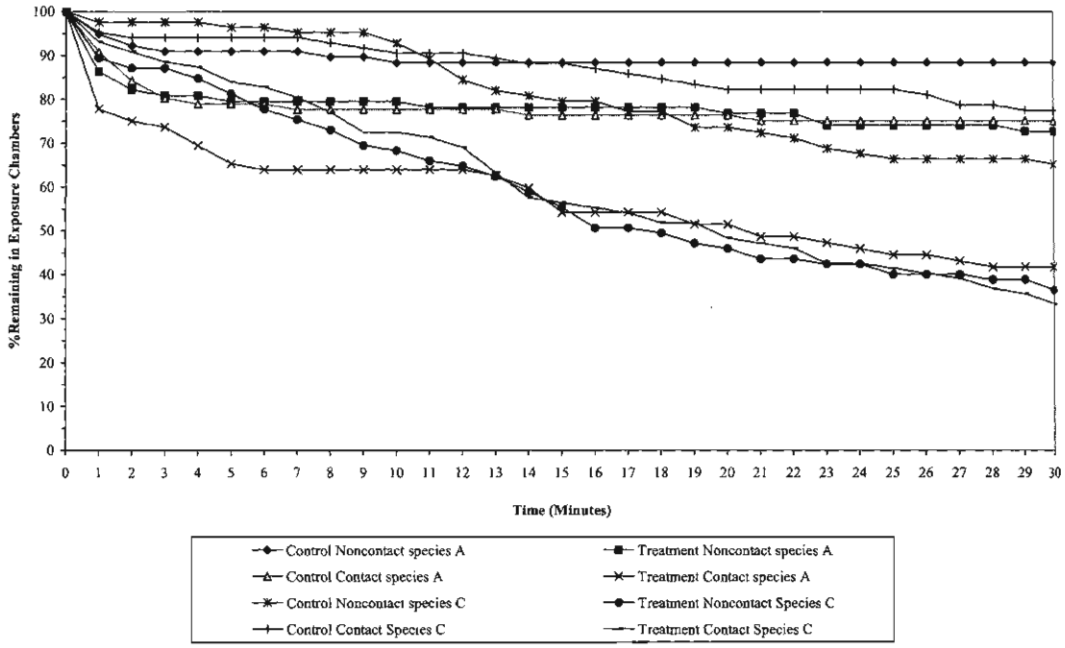

Fig. 3. Escape probability of *An. minimus* species A and C exposed to malathion with paired control for contact and noncontact trials.

crops). In addition, both deltamethrin and malathion have been routinely used in both areas to control malaria and dengue vectors, respectively, with a greater amount of public health spray activity in the A1 area (MOPH 2005).

Although behavioral responses to test compounds by anopheline malaria vectors have long been recognized, the true significance of behavioral avoidance for disease transmission and control remain complex and controversial. In fact, the full effect of resistance itself on control efforts is not clearly known (Brogdon and McAllister 1998). A mathematical framework for better understanding of the repellent, irritant, and toxic functions of chemicals to control diseases has been quantified (Roberts et al. 2000). Since this framework was developed, studies showing strong behavioral responses by mosquitoes to public health insecticides intended to control vectors have been progressively reported in Thailand (Chareonviriyaphap et al. 2001, 2002, 2003, 2004; Sungvornyothin et al. 2001; Kongmee et al. 2004; Potikasikorn et al. 2005; Chareonviriyaphap et al. 2006; Sathantriphop et al. 2006). In all these studies, irritability and repellency responses were quantitatively assessed using the same excito-repellency test system developed and modified by Chareonviriyaphap et al. (2002) as used in this investigation. Apart from common chemicals used in public health, numerous agrochemicals are known or suspected to exert profound behavioral responses in agricultural insect pests (Roberts and Andre 1994). However, there has been relatively little information on how malaria vectors respond to common insecticidal chemicals used to protect crops. Some of the most detailed information has been derived from field studies in the Americas on *Anopheles albimanus*, one of the first vectors to demonstrate multiple resistance to different classes of compounds in relation to exposure to agricultural chemicals (Brown and Pal 1971; Georghiou et al. 1972, 1973; Bailey et al. 1981; Bown 1987; Brogdon et al. 1988).

There is no clear explanation to account for differences in rate of escape responses to particular agrochemicals and the two vector populations. For insecticide susceptibility, populations of the same species separated by just a few kilometers may not only show significant focal variation in presence or absence of resistance but also in level of resistance and dominant mechanism responsible (Brogdon et al. 1998). In our study, focal distribution of innate behavioral variation might account for the differences, because the two populations were separated from one another by nearly 60 km. Much remains unknown about the general behavioral patterns between different members of the Minimus complex; however, there is good evidence to suggest they differ from one another in a number of important ecological and epidemiological aspects (Van Bortel et al. 1999, Chareonviriyaphap et al. 2003, Potikasikorn et al. 2005, Rongnoparut et al. 2005). It is also possible that the greater response of A in both contact and noncontact trials might be the consequence of more frequent exposure to malathion or related organophosphate in Mae Nam Noi compared with Pu Teuy, although this seems unlikely. We have documented greater repellency responses in species A to DDT and pyrethroids in an area where residual chemical was routinely applied (Potikasikorn et al. 2005). Similarly, the more intense irritancy and repellency with carbaryl and cypermethrin in C could have been the result of previous exposure to these chemicals in Pu Teuy agricultural areas. The stronger escape responses seen in C may reflect a gradual adaptation toward greater sensitivity for avoiding these toxic substances (Chareonviriyaphap et al. 1997). Besides innate differences between populations, the prevailing environmental conditions (e.g., ambient temperature, relative humidity) and timing of the test could have influenced responses as well.

Both populations were considered physiologically susceptible to the three insecticides. All three test compounds demonstrated strong excito-repellency with minimal subsequent toxicity. The majority (79%) of mosquitoes of both populations that successfully escaped treated chambers presumably survived by avoiding sufficient contact with the treated surfaces The prominent repellency attribute of carbaryl (80% escaped) and cypermethrin (63% escaped) seen in species C indicates the high sensitivity of this population to avoid these, and likely, similar classes of compounds. The strong behavioral responses of *An. minimus* A and C reported previously (Potikasikorn et al. 2005) indicates that excito-repellency may at least partly be responsible for the very limited resistance seen in these mosquito populations, despite years of insecticide use.

Our finding may reflect geographical differences in populations related to land use and overall pesticide exposure, similar to observations on *An. albimanus* (Brogdon et al. 1988, Rios et al. 1988). Compared with the intensive use of chemicals in cotton growing areas of Latin America, the possibly more subtle nature of insecticide use from sites described in Thailand also might preclude any profound measurable effects on *An. minimus* populations. Extrapolation of these findings to other populations of *An. minimus* complex species in Thailand or elsewhere in Asia may not reflect accurately other localities. Further investigations are needed to examine the relationship between physiological resistance patterns and behavioral avoidance based on a combination of different levels of environmental exposure to both public health and agricultural insecticides.

Acknowledgments

We thank the Armed Forces Development Command, Sai Yok District; the Malaria Unit; and the Public Health Unit at Thong Pha-Phum District, Kanchanaburi Province, Thailand, for support in study areas. In particular, we thank Achariya Prabaripai for the statistical analysis, and Monthathip Kongmee, Vithee Meunvorn, and staff and students of the Medical Entomology Laboratory, Department of Entomology, Faculty of Agriculture, Kasetsart University, for enthusiastic assistance and cooperation that contributed to the success of this study. We also thank the International Water Management Institute (IWMI), Southeast Asia Regional Office, Pen-

ang, Malaysia, for office space and general support. This study received financial support from the Research Council of Norway, the Graduate School, Kasetsart University, and the Thailand Research Fund, Thailand.

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Received 29 December 2006; accepted 29 June 2007.

Effects of Physiological Conditioning on Behavioral Avoidance by Using a Single Age Group of *Aedes aegypti* Exposed to Deltamethrin and DDT

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J. Med. Entomol. 45(2): 251–259 (2008)

ABSTRACT The behavioral and physiological responses of 6-d-old *Aedes aegypti* (L.) adult females exposed to deltamethrin and DDT were characterized using a free-choice excito-repellency test system. Excluding varying pretest age and carbohydrate availability as possible confounders, insecticide contact (measuring irritancy) and noncontact (measuring repellency) behavioral assays were conducted on two nonbloodfed groups, either unmated or mated (nulliparous), and two blood-fed groups, either parous or newly full-engorged mosquitoes. The degree of escape response to deltamethrin and DDT varied according to the physiological conditioning. Escape rates from contact and noncontact chambers with deltamethrin were more conspicuous in nonbloodfed groups compared with mosquitoes previously bloodfed. There were no significant differences in escape responses between unmated and nulliparous test populations. With DDT, a more pronounced escape response was observed in unmated compared with other physiological conditions. More moderate escape response was seen in nulliparous mosquitoes, and the least was observed in full bloodfed test individuals, regardless of test compound. *Ae. aegypti*, regardless of pretest conditioning, was completely susceptible to deltamethrin, whereas showing high resistance to DDT. Despite profound differences in resistance, there was no significant difference in avoidance response between chemicals and mosquito conditioning. Moreover, pre- and postbloodmeals were found to influence assay outcome and thus to have relevance on the interpretation of susceptibility and excito-repellency assays.

KEY WORDS *Aedes aegypti*, behavioral responses, excito-repellency, deltamethrin, DDT

Aedes aegypti (L.), the primary vector mosquito typically resides very near or inside human dwellings preferentially feeding on humans (Christophers 1960, Polawat and Harrington 2005). Because no commercial vaccine or antiviral agents are yet available for the prevention and treatment of dengue infection, the control of this mosquito vector remains the most important method to prevent dengue virus transmission and averting dengue epidemics.

Mosquito behavior is of epidemiological importance whereby favoring or inhibiting a mosquito preferentially feeding on a human, potentially ingesting an infectious bloodmeal, or transmitting a pathogen to a susceptible host (Elliott 1972). Introduction of an exogenous element, such as residual insecticides, can disturb normal patterns of insect behavior. The avoidance of certain insecticide-treated surfaces seems to be a natural reaction of most mosquitoes; therefore, a better understanding of the impact of excito-repellency on vector control methods should enable better decisions on pesticide selection and application (Muirhead-Thomson 1960, Roberts et al. 2000).

In Thailand, deltamethrin has been regarded as an effective, relatively safe compound since introduction, and it has been widely used 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, also has been used in attempts to interrupt mosquito virus transmission in dengue active areas (MOPH 2006). The effectiveness of pyrethroids requires regular monitoring and serves as a stimulus for continued studies on the mode of action and epidemiological significance of avoidance behavior (WHO 1995). DDT has long been shown to educe strong behavioral avoidance responses by many species of mosquitoes (Kennedy 1947, Roberts and Alecrim 1991), and it remains an excellent standard by which

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0022-2585/08/0251-0259$04.00/0

comparison with other compounds can be made (Evans 1993; Chareonviriyaphap et al. 1997, 2004).

A "free choice" test system was designed that has enabled investigators to distinguish between two distinct types of behavioral responses, contact irritancy and noncontact repellency, in mosquitoes (Roberts et al. 1997). Over time, modifications and improvements have been made to the system allowing greater ease and accuracy for showing innate response of mosquitoes exposed to residual insecticides (Chareonviriyaphap et al. 2002, Tanasinchayakul et al. 2006).

The impact of insecticide on *Anopheles* species responsible for malaria transmission has been studied far more than on other mosquito genera. Relatively less work has been paid to the response of *Ae. aegypti* to insecticides (Kennedy 1947, Brown 1964, Lal et al. 1965, Moore 1977). Behavioral responses of Thai field and laboratory populations of *Ae. aegypti* to insecticides have recently been assessed under different nutritional and physiological conditions (Chareonviriyaphap et al. 2006). However, this study did not control for the confounding influence of age as a possible cause of variation to behavioral responses combined with other intrinsic physiological conditions (Hamon and Eyraud 1961, Busvine 1964, Kaschef 1970). To measure possible effects of insemination, gonotrophic status and bloodfeeding of female *Ae. aegypti* exposed to deltamethrin and DDT, we used same age mosquitoes to compare behavioral patterns more accurately.

Materials and Methods

Mosquito Population. *Ae. aegypti* was established from immature stages collected from Pu Teuy Village, Sai Yok District, Kanchanaburi Province (14° 17′ N, 99° 17′ E), ≈100 km northwest of Bangkok, between June and August 2006. Species identification and subsequent colonization was conducted at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

Mosquito Rearing. Mosquito colonization and rearing followed established methods (Kongmee et al. 2004), with only minimal modifications to meet testing requirements. All life stages were maintained under controlled conditions (25 ± 5°C and 80 ± 10% RH) in the insectary at the Department of Entomology, Kasetsart University, Bangkok, Thailand. Larval and adults were reared under a photoperiod of 12:12 (L; D) h. Upon emergence, all adults were provided cotton pads soaked with a 10% sucrose solution and withheld 12 h before testing.

Chemicals and Insecticide-Impregnated Papers. Deltamethrin [(*S*)-α-cyan-3-phenoxybenzyl(1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropanecarboxylate] (85% purity) was obtained commercially (CAS 67375-30-8, BASF Corp., Ludwigshafen, Germany). DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] (92.5% purity) was obtained commercially (CAS 59-29-3, Sigma-Aldrich, St. Louis, MO).

Based on current recommendations of the Thai national vector control program, the standard field dose of deltamethrin (0.02 g/m^2) and DDT (2 g/m^2) were used. Filter paper (Whatman no. 1, Whatman, Maidstone, United Kingdom) served as treated substrate for susceptibility tests (12 by 15 cm) and excito-repellency (ER) chambers (15 by 17.5 cm). Papers were prepared at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, according to World Health Organization specification (WHO 1996). Insecticide concentrations were prepared with acetone solvent (analytical grade). Insecticide-impregnated papers were treated at the rate of 2.75 ml of insecticide solution per 180 cm^2. Control papers were treated with a 0.67:0.33 ratio of acetone and silicone oil (a nonvolatile carrier) (WHO 2006).

Mosquito Conditioning. Six-day-old female *Ae. aegypti* representing four different test conditions were used for excito-repellency testing: 1) parous, 2) mated, 3) unmated, and 4) full bloodfed. 1) Parous females were allowed to feed on blood of live hamsters on day 2 postemergence after being held with males. Only full bloodfed females were selected and segregated into containers for oviposition. Females were provided water only up to time of testing. 2) Mated, nulliparous mosquitoes were held with males up to day 2 postemergence without access to bloodmeal. A 10% sucrose solution soaked on cotton pads was provided up to 12 h before testing and water only until testing. Dissection of spermatheca was performed on a small sample to determine proportion that had successfully mated. 3) Unmated (infertile) females were obtained by segregating individual pupae into containers until emergence, after which females were placed together and provided with 10% sucrose and water only as described for mated, nulliparous females. 4) Mated, full bloodfed mosquitoes were held with males and selected after feeding on live hamsters 3 h before testing. Dissection of spermatheca was performed on a small sample to determine proportion that had successfully mated.

Insecticide Susceptibility Tests. The susceptibility of each test population/condition was assessed by direct contact exposure to a single diagnostic dose of either deltamethrin (0.05%) or DDT (4%) on insecticide-treated test papers following standard testing procedures for *Ae. aegypti* (WHO 1998). For each test trial, five sets of WHO exposure/holding test cylinders (two control and three treatment) were used. Control cylinders contained filter paper impregnated with solvent-oil alone, and treatment cylinders provided with recommended "diagnostic" concentrations of insecticide in solvent for determination of susceptibility (WHO 1998). For each test population, 25 female mosquitoes were exposed for 1 h to deltamethrin or 30 min DDT. After test and control exposures, knockdown during that period were recorded for each chamber, and all mosquitoes were subsequently transferred to separate (each cylinder) clean holding containers and provided 10% sucrose solution. Total knockdown and mortality was recorded after 24 h postexposure. Each matched test-control series was

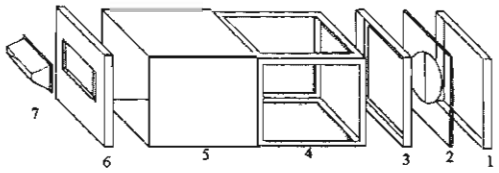

Fig. 1. Illustration of the free-choice excito-repellency test chamber for study of insecticide avoidance behavior of mosquitoes. 1, rear door cover; 2, Plexiglas panel with rubber-sealed door; 3, Plexiglas holding frame; 4, screened inner chamber; 5, outer chamber; 6, front panel; and 7, exit portal.

repeated three times to derive mean susceptibility (% mortality) per test population/condition and adjusted using Abbott's formula when appropriate (Abbott 1925).

Excito-Repellency Tests. For all tests we used an improved ER test chamber design (Tanasinchayakul et al. 2006) but without the automated system for the counting of escaping mosquitoes. Briefly, the main supporting structure is fabricated stainless steel, each side wall measuring 23 by 23 cm^2. The chamber walls have an aluminum side tongue and groove configuration on joining ends that makes it easier to set up and disassemble for transportation and storage. The frame of the inner chamber is constructed of 22.5- by 19-cm stainless steal beams, which include metal holders for securing test papers on either of two sides for the dual purpose of either providing contact or noncontact exposure designs. For noncontact tests, a thin sheet of fine mesh iron screening secured on the opposite side of the test paper provides a 1.5-cm gap that prevents mosquito tarsal contact with the test paper. A Plexiglas panel at the rear of the chamber is equipped with an 11.5-cm-diameter opening sealed with overlapping dental latex to prevent escape during handling. Last, there is a forward exit portal (13.5 by 2 cm) connected to a funnel projecting from the opposite end of the box (Fig. 1).

Each test series consisted of two chemical-treated test chambers and two paired control chambers fitted with appropriate papers. Female mosquitoes were held in 473-cm^3 (16-fl. oz.) capacity cups for ≈8–10 h before testing, and they were provided only water soaked on cotton. For each test chamber, 15 mosquitoes were carefully introduced into each of four chambers by using a mouth aspirator. Mosquitoes were allowed a 3-min adjustment period inside the test chamber before opening the escape funnel to begin the observation period. A receiving cage was connected to the exit portal for collecting exiting mosquitoes. Mosquitoes escaping were recorded at 1-min intervals for a period of 30 min. All tests were conducted between 0800 and 1600 hours and under laboratory conditions and ambient temperature (17–32°C) and relative humidity (50–75% RH). Tests were replicated four times per test population-condition.

Immediately after the 30-min exposure, the number of dead or knocked down mosquitoes remaining inside the chamber and those that had escaped into the receiving cage were recorded for each of the four test chambers. All live specimens that had escaped or remained inside the test chamber were transferred to clean holding cups and provided a 10% sucrose solution. All test mosquitoes were maintained separately in respective lots for 24-h postexposure to record mortality.

Data Analysis. In contact susceptibility tests, control mortalities exceeding 5% were corrected and adjusted before determining baseline susceptibility in each test population (Abbott 1925). Kaplan–Meier survival analysis was used to generate survival curves for behavioral avoidance data to estimate rate of mosquito escape and then compare differences in mosquito escape between the four different test populations/physiological conditions and insecticides (Kleinbaum 1996). Survival analysis provides a more robust statistical treatment of sequential excito-repellency data relative to other quantitative methods whereby minimizing the loss of valuable information while estimating temporal mosquito escape probabilities (Roberts et al. 1997). The time in minutes for 25, 50, and 75% of the test population to escape was estimated and used for "escape time" summary statistics (ET_{25}, ET_{50}, and ET_{75}). A log-rank test was used to compare patterns of escape behavior (Mantel and Haenszel 1959). This method allows detection of differences between survival curves that result when the death (defined as "escape") rate in one group is consistently different from a corresponding rate in a second group and assuming the ratio of this rate is consistent over time. With excito-repellency data, the log-rank test examined pairwise escape patterns at 1-min intervals, with the discriminating level of statistical significance set at 0.05%. All statistical testing was conducted using SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

Six-day-old *Ae. aegypti* conditioned to different physiological states were exposed to a single concentration each of deltamethrin (0.05%) and DDT (4%) to access susceptibility level. Insemination status was based on either evidence of oviposition (parous state) or a random sampling and dissection of spermatheca (nulliparous and bloodfed) for detection of stored sperm. As this species generally mates extremely well under laboratory conditions and confined spaces, 2 or more days of free mating with males and dissection results indicate insemination status was at or very near 100%. Regardless of physiological condition, all test populations were found completely susceptible to deltamethrin, whereas high levels of resistance to DDT were detected (Table 1). There was no marked difference in test mortality between replicates within each test condition (data not presented). DDT produced the highest mortality (10%) in infertile, nonbloodfed mosquitoes and only 3% mortality in the blood-engorged females.

Percentage of escape responses and total mortality were recorded for each population (four test replicates) under different conditioning when exposed to deltamethrin and DDT in contact and noncontact trials (Tables 2 and 3). Only slightly higher mortality

Table 1. Percentage of mortality of unmated, nulliparous, parous, and bloodfed 6-d-old *Ae. aegypti* after contact with deltamethrin and DDT by using standard WHO susceptibility test procedures

Insecticide[a]	Condition	No. tested	% mortality ± SE
Deltamethrin	Parous	75[b]	100
	Control-parous	75	0
	Nulliparous	75	100
	Control-nulliparous	75	0
	Unmated	75	100
	Control-unmated	75	0
	Bloodfed	75	100
	Control-bloodfed	75	0
DDT	Parous	75	6.7 ± 3.64
	Control-parous	75	0
	Nulliparous	72	9.3 ± 3.17
	Control-nulliparous	75	0
	Unmated	75	10 ± 3.61
	Control-unmated	75	0
	Bloodfed	75	3 ± 1.58
	Control-bloodfed	75	0

[a] Diagnostic dosage DDT (4% at 2.0 g/m^2) and deltamethrin (0.05% at 0.02 g/m^2).
[b] Three replicates (25 mosquitoes per replicate).

of escaped mosquitoes in contact trials exposed to deltamethrin (range, zero to four deaths) was observed compared with DDT (one to three deaths) (Table 2). Similarly, nonescaped mosquitoes from deltamethrin-treated contact chambers resulted in higher (zero to eight deaths) mortality than DDT (0–1 death). With one exception (deltamethrin-parous condition), lower mortalities for escaped and nonescaped mosquitoes were seen in all noncontact trials compared with paired contact tests (Table 2 and 3).

Contact with deltamethrin and DDT elicited stronger escape response compared with paired controls and noncontact trials, regardless of physiological condition at the time of test ($P < 0.05$) (Table 2). Significant differences in pattern of escape were seen in all noncontact trials compared with paired controls

Table 2. Mean percentage of escape and total mortality of preconditioned 6-d-old *Ae. aegypti* after contact with deltamethrin and DDT in excito-repellency tests

Insecticide[a]	Condition	No. tested	% escaped	No. dead	
				Escaped	Remain
Del	Unmated	57	78.9	1	
Del-C	Unmated	60	30.0	0	0
Del	Nulliparous	59	78.0	2	2
Del-C	Nulliparous	59	33.9	0	1
Del	Parous	95	45.3	4	7
Del-C	Parous	94	14.0	0	0
Del	Bloodfed	58	51.7	3	8
Del-C	Bloodfed	58	6.9	0	0
DDT	Unmated	60	91.7	0	0
DDT-C	Unmated	59	30.5	0	0
DDT	Nulliparous	59	74.6	1	0
DDT-C	Nulliparous	59	22.0	0	0
DDT	Parous	59	57.6	3	1
DDT-C	Parous	56	17.9	0	0
DDT	Bloodfed	60	40.0	0	0
DDT-C	Bloodfed	59	0	0	0

[a] DDT, 2 g/m^2; Del, 0.02 g/m^2; and C, control.

Table 3. Mean percentage of escape and total mortality of preconditioned 6-d-old *Ae. aegypti* after noncontact with deltamethrin and DDT in excito-repellency tests

Insecticide[a]	Condition	No. tested	% escaped	No. dead	
				Escape	Remain
Del	Unmated	59	42.4	0	0
Del-C	Unmated	58	24.1	0	0
Del	Nulliparous	58	46.5	0	0
Del-C	Nulliparous	57	21.1	0	0
Del	Parous	93	28.0	3	25
Del-C	Parous	93	10.7	0 0	
Del	Bloodfed	59	5.1	0	0
Del-C	Bloodfed	58	3.4	0	0
DDT	Unmated	58	56.9	0	1
DDT-C	Unmated	58	24.1	0	0
DDT	Nulliparous	58	48.3	0	0
DDT-C	Nulliparous	58	13.8	0	0
DDT	Parous	57	42.1	2	0
DDT-C	Parous	54	7.4	0	0
DDT	Bloodfed	59	13.6	0	0
DDT-C	Bloodfed	60	0.0	0	0

[a] DDT, 2 g/m^2; Del, 0.02 g/m^2; and C, control.

(Table 3). In contract trials, unmated/nonbloodfed mosquitoes produced the greatest escape responses, 91.7% (DDT) and 78.9% (deltamethrin), followed by nulliparous/nonbloodfed mosquitoes, 74.6% (DDT) and 78% (deltamethrin) (Table 2). Similarly, in noncontact trials, higher numbers of unmated (56.9% DDT and 42.4% deltamethrin) and nulliparous mosquitoes (48.3% DDT, 46.5% deltamethrin) escaped from chambers treated with either deltamethrin and DDT compared with parous and blood-engorged mosquitoes. Bloodfed mosquitoes had the slowest rate of escape from both contact and noncontact tests by using DDT. Deltamethrin produced a greater delay in escape in parous versus full bloodfed females.

Mean times in minutes for 25, 50 and 75% (ET_{25}, ET_{50}, and ET_{75}) of the test population to escape treated chambers within 30 min are provided in Table 4. In deltamethrin contact trials, both unmated and nulliparous females had near identical escape times with a maximum ET_{75} of 16 min. The number of parous and bloodfed individuals escaping from deltamethin were relatively low so that ET_{50} and ET_{75} values could not be obtained. For DDT, ET values for unmated mosquitoes were similar to deltamethrin, whereas ET_{50} and ET_{75} values for nulliparous were approximately 2 times greater than those of unmated females. Both parous and bloodfed mosquitoes produced far slower responses with only parous females managing at least 50% escape within 30 min.

The patterns escape over a 30-min period are indicative of escape probability between the four test populations in contact and noncontact trials by using deltamethrin (Fig. 2) and DDT (Fig. 3). No significant differences ($P < 0.05$) in escape patterns were seen between unmated and nulliparous females exposed to deltamethrin in contact and noncontact trials. However, escape responses were markedly different for unmated and nulliparous compared with parous and bloodfed mosquitoes ($P > 0.05$). Mean escape patterns were similar for parous and bloodfed test pop-

Table 4. Escape time in minutes for 25, 50, and 75% 6-d-old conditioned *Ae. aegypti* to escape insecticide-treated chambers

Chemical	Condition	Contact			Noncontact		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Deltamethrin	Parous	5	—[a]	—	20	—	—
	Nulliparous	1	3	16	2	—	—
	Unmated	1	4	16	8	—	—
	Bloodfed	5	—	—	—	—	—
DDT	Parous	3	19	—	16	—	—
	Nulliparous	2	8	28	4	—	—
	Unmated	2	5	14	8	21	—
	Bloodfed	8	—	—	—	—	—

[a] Too few mosquitoes escaped from exposure chambers so that ET values could not be estimated for a 30-min exposure period.

ulations in deltamethrin contact trials. DDT contact produced a significant greater ($P < 0.05$) rate of escape in unmated females compared with all other conditions (Fig. 3). For DDT noncontact trials, there was no significant difference in escape response between unmated, nulliparous and parous mosquitoes, whereas full bloodfed females produced the slowest overall response. Pairwise comparisons of escape responses within like conditions found no significant difference between chemicals in irritability (P = 0.148–0.539) or repellency (P = 0.108–0.606).

Discussion

Our study compared the behavioral responses of *Ae. aegypti* of like age under different physiological conditions when exposed to chemicals. Unmated and nulliparous mosquitoes showed greater irritant and repellent escape responses than recently bloodfed and parous mosquitoes, regardless of chemical. This is in contrast to a previous study that found parous *Ae. aegypti* (8–9 d old) with higher sensitivity to pyrethroids, resulting in greater spontaneous escape activity (Chareonviriyaphap et al. 2006). Although others have demonstrated copulation and oviposition-induced changes in the patterns of flight activity periodicities in *Ae. aegypti* and *Anopheles gambiae* (Jones 1981), insemination status alone did not seem to unduly influence our assay results.

The physiological state of a mosquito represents an important set of factors that can influence escape movement from chemical-treated surfaces (Roberts et

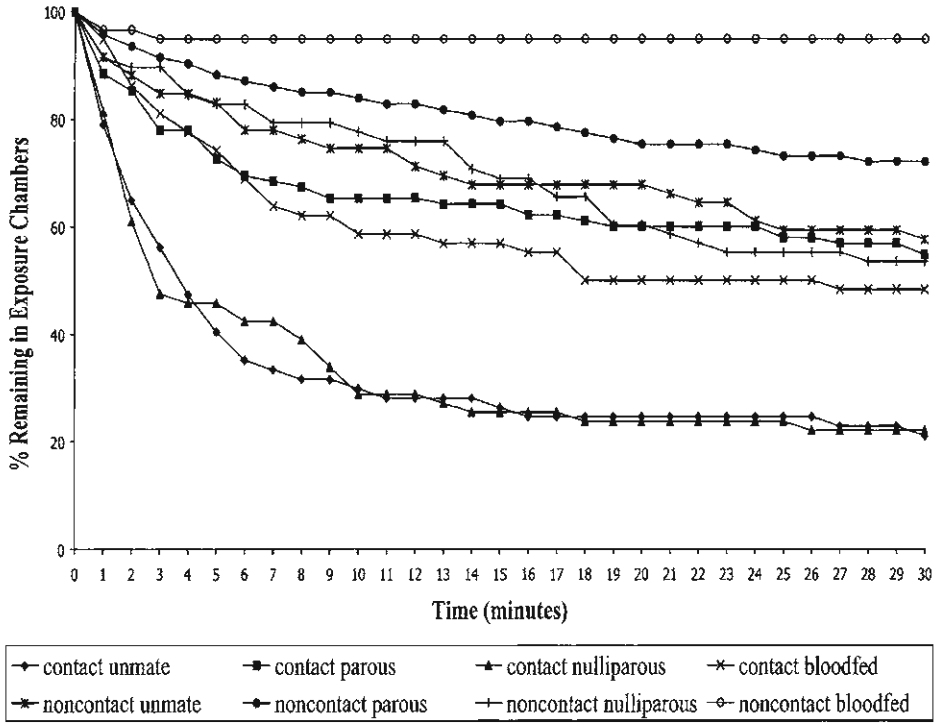

Fig. 2. Comparison of escape patterns of female *Ae. aegypti* preconditioned to one of four different physiological states exposed to 0.02 g/m^2 deltamethrin in contact and noncontact trials.

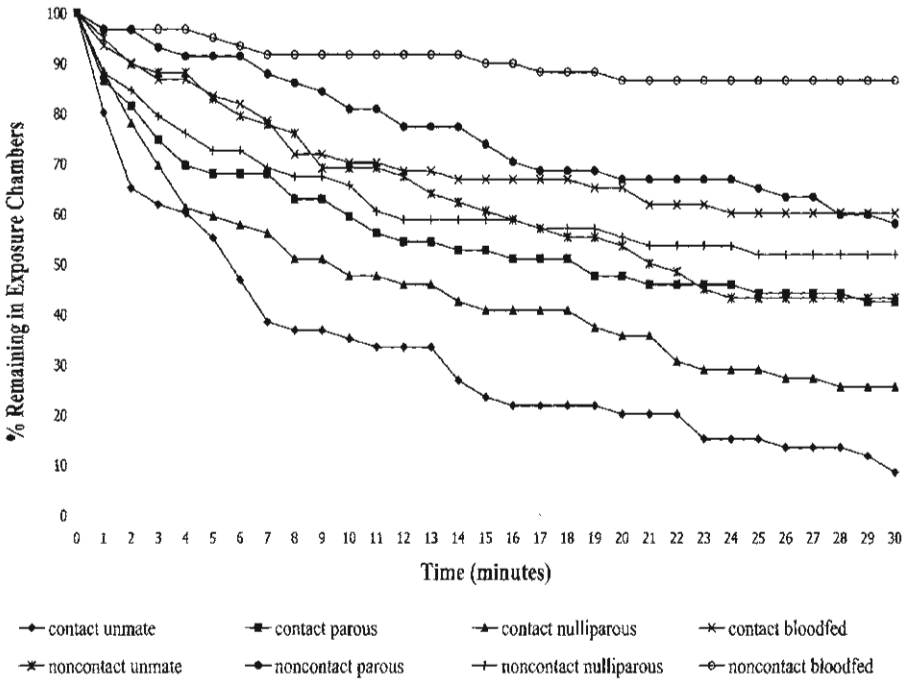

Fig. 3. Comparison of escape patterns of female *Ae. aegypti* preconditioned to one of four different physiological states exposed to 2 g/m^2 DDT in contact and noncontact trials.

al. 1984, Sungvornyothin et al. 2001, Chareonviriyaphap et al. 2006). Intrinsic factors known to influence susceptibility and behavioral response include carbohydrate (energy) reserves, age, bloodfeeding, and gonotrophic condition of female mosquitoes (Hadaway and Barlow 1956, Busvine 1964, Xue and Barnard 1999). Age can influence both susceptibility (Raffaele et al. 1958, Lines and Nassor 1991) and irritability to insecticides (Busvine 1964). David and Bracey (1946) noted a decline in DDT tolerance with advancing age in *Ae. aegypti*, and glutathione transferase (GST)-mediated DDT resistance also has shown a marked decline in activity with increased age (Hazelton and Lang 1983). Fewer investigations have been done comparing age and behavior, but generally, older mosquitoes have been found less irritable than younger mosquitoes, possibly related to lower or depleted energy reserves (Hamon and Eyraud 1961, Kaschef 1970). In our study, age and sucrose availability were controlled and environmental factors, such as temperature, humidity, and light, were maintained within a reasonably defined range so as not to unduly cause disparate responses.

Ae. aegypti test colony was collected from an area with perennial malaria transmission (MOPH 2006). For >40 yr DDT was commonly used as an indoor residual spray to control anopheline vectors in the Pu Teuy area but ceased over a decade before this study was conducted. Deltamethrin is a much more recent introduction and the insecticide of choice for nonresidual space spray during dengue outbreaks in Thailand (Kongmee et al. 2004). However, very little of this compound or any other synthetic pyrethroid has been applied in Pu Teuy, an area where dengue transmission has also been apparently absent (MOPH 2006).

Regardless of conditioning, all individuals were completely susceptible to deltamethrin, whereas the same test population was found highly resistant to DDT. Previous studies also had documented a high degree of resistance to DDT (>90%) in this mosquito population despite the long interval from last exposure (Chareonviriyaphap et al. 2006, Suwonkerd et al. 2006). Because cross-resistance was not observed, our results would indicate the knockdown resistance (*kdr*) or *kdr*-like genetic mutation is not involved. Rather, the metabolic mechanism may involve elevated levels of GSTs, resulting in increased DDT-dehydrochlorinase activity (Prapanthadara et al. 1995). Possibly in parallel with GST, detoxification could involve oxidation reactions by elevated cytochrome P450-dependent microsomal monooxygenase systems (Wilkinson 1983). Susceptibility patterns varied depending upon physiological state. Although not striking, higher mortality to DDT was seen in unmated and nulliparous test population compared with parous (previous bloodfed) and engorged test population. Because blood can serve as an additional nutritional reserve (glycogen and fat), unmated and nonbloodfed mosquitoes may have had less vigor or tolerance deltamethrin and DDT compared with other physiological states (Clements 1992).

Physiological resistance has been associated with either increasing or reducing irritability depending on the mosquito species, chemical concentration, and test conditions (Brown 1958, de Zulueta 1959, Elliott 1964, Gaaboub and Dawood 1974). In many cases, DDT-resistant *Ae. aegypti* has either been suspected or proven to be significantly less irritable than susceptible strains (Hecht et al. 1960, Cullen and de Zulueta 1962, Brown 1964, Busvine 1964). Although we did not make a similar comparison, DDT produced significant excito-repellency in the face of high levels of resistance. Moreover, the dramatic contrast in resistance profile in our population had no significant effect on escape response to either deltamethrin or DDT.

DDT produced stronger (noncontact) repellent activity than deltamethrin, presumably because of greater fumigant (vapor pressure) properties than the later. This is in agreement with previous findings on the repellency of DDT and pyrethroids (Roberts el al. 2000, Chareonviriyaphap et al. 2004). As in this study, decreased excito-repellency in blood-engorged *Ae. aegypti* is likely the result of the additional physical burden (weight) of the meal and greater reluctance to take flight (Kongmee et al. 2004, Chareonviriyaphap et al. 2006). Although the configuration of the test chambers may have played a part to inhibit escape of blood-laden mosquitoes (i.e., reduced agility), we think any impact of the testing devise is relatively small compared with increased weight itself. Increased weight also may have influenced escape behavior of parous mosquitoes, which tend to be heavier than nulliparous females. This is supported by the numerous reports of reduced irritability of mosquitoes under different conditions soon after a bloodmeal (Hecht et al. 1960, Busvine 1964, Brown and Pal 1971, Roberts et al. 1984, Sungvornyothin et al. 2001), although Brown (1964) reported very little difference in *Ae. aegypti* before and 1 h after bloodfeeding. Jones (1981) observed blood-engorged mosquitoes with greatly reduced flight activity after feeding and becoming only active again around the third day when fully gravid. Under similar test conditions, Busvine (1964) found unfed mosquitoes (mated or unmated) often demonstrated stronger irritant/repellent behavior than bloodfed.

The characteristic strong anthropophagic and endophilic behaviors of *Ae. aegypti* has presented an enormous challenge for vector control specialists to devise new or improved methods to sufficiently reduce mosquito populations and disease transmission risk (WHO 1999). Since the early 1990s, pyrethroids, including deltamethrin, have been commonly used in Thailand as space sprays for controlling household nuisance and vector mosquitoes, including *Ae. aegypti*, and they also have been used in attempts at interruption of virus transmission in communities reporting active dengue cases (Chareonviriyaphap et al. 1999). Outdoor and peridomestic space spraying alone has often failed to achieve any meaningful control of indoor adult *Ae. aegypti* populations because the chemical fails to reach the intended target resting inside homes (Reiter and Gubler 1997, Mani et al. 2005). Generally, without simultaneous attention to larval habitats and source reduction activities, adult populations often quickly rebound. However, when residual insecticides are applied indoors by using portable space spray devices (e.g., ultralow-volume units, mist blowers, and thermal foggers), more effective and longer lasting control of adult *Aedes* have been seen compared with conventional outdoor application methods (Sulaiman et al. 1993, Lee et al. 1997, Perich et al. 2001). For example, Pant et al. (1974) reported up to 7 mo effective control of indoor *Ae. aegypti* by using fenitrothion applied as an aerosol with a mist blower.

The greater tendency for nonengorged mosquitoes to escape a treated surface area alters normal resting/feeding behavioral patterns and reduces the opportunity for bloodfeeding and potential for virus transmission. This enhanced hyperactivation response in nonbloodfed compared with the more subdued reaction of bloodfed mosquitoes has lead to several interpretations of the epidemiological consequences of excito-repellency to effectively control vector populations and disease. Any residual chemical with sufficiently strong irritant and repellent properties applied to indoor surfaces has the dual potential to decrease both adult longevity and reduce vector–human contact by behavioral avoidance. Even if the majority of indoor resting *Ae. aegypti* preferentially rest on unsprayed surfaces, repellency action alone could reduce transmission risk by disrupting the normal resting and feeding patterns of a vector (Roberts et al. 2000). This is supported by evidence that individuals sleeping in rooms with pyrethroid-treated bed-nets or curtains are afforded adequate protection because of significant "deterrency" of vectors from entering the house (Miller et al. 1991). Deltamethrin, acting as a potent deterrent that would inhibit successful bloodfeeding, is deemed advantageous for enhancing personal protection. Furthermore, our findings do not support the notion that deterrence is necessarily independent of excito-repellency stimulated by the insecticide active ingredient (Lindsay et al. 1991). Although certain commercial product formulations may contain "inert ingredients" (e.g., aromatic hydrocarbon solvents) that might influence behavior, our paired treatment–control assays have shown that deterrency is the result of the irritant and repellent properties of the parent chemical and not a function of solvent or oil-based carrier.

Differences in the physiological condition of mosquitoes have considerable bearing on behavioral avoidance assays. Even when tests are carried out on apparently homogenous organisms, there can be numerous other factors responsible for unexplained variations in response (Busvine 1964). Because we carefully controlled for age in this study, extrapolation of these laboratory findings to more heterogeneous field populations or under less controlled conditions is cautioned. Any altered behavioral that might interfere with vector feeding must be considered when assessing the epidemiological effect of insecticides on disease transmission. In view that the primary measure of

successful vector control is the reduction of transmission risk and disease incidence, rather than simply the quantitative reduction of vector densities, a better understanding of impact of excito-repellency is needed. We also agree that susceptibility tests alone should not be the sole criteria or evidence for critical decisions on the usefulness of a chemical or its replacement (Davidson and Zahar 1973). The continued refinement and use of excito-repellency assays offer a better means to objectively evaluate the full attributes of an insecticide and its potential to suppress disease transmission.

Acknowledgments

We thank the Thailand Research Fund and the Kasetsart University Development Institute for financial support.

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Received 2 March 2007; accepted 24 November 2007.

Genetic structure and gene flow of *Anopheles minimus* and *Anopheles harrisoni* in Kanchanaburi Province, Thailand

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Received 19 September 2007; Accepted 20 January 2008

ABSTRACT: Isozyme frequencies were compared in seven field collections of *Anopheles minimus* complex using starch gel electrophoresis. Mosquito collections were sampled from four districts in Kanchanaburi Province where malaria is endemic. From eight enzyme systems, nine loci and seven polymorphisms were detected, indicating limited genetic differentiation among the seven collections (F_{ST} = 0.061). The highest percent polymorphic loci were observed in Bong Ti Noi (BTN) Village (55.6%), whereas the least percent polymorphism was seen in Tha Kradan (TK) Village (22.2%). Comparing villages Pra Jedee (PJ) with Pu Teuy C (PTC) and Huai Khayeng (HK) with Pra Jedee (PJ), gene flow among collections varied from 3.72 to 62.25 reproductive migrants per generation. Among the seven collections, no correlation was seen between genetic and geographical distances ($P > 0.05$). *Anopheles minimus* (former species A) and *Anopheles harrisoni* (former species C) from Pu Teuy fit most closely in the same cluster, possibly indicating relatively recent divergence between taxa. The genetic and epidemiological ramifications of these findings are discussed. ***Journal of Vector Ecology* 33 (1) 158-165. 2008.**

Keyword Index: *Anopheles minimus, Anopheles harrisoni*, isozyme, genetic, gene flow, malaria, Thailand.

INTRODUCTION

In Thailand, malaria is still one of the most important infectious diseases despite decades of organized disease control in reducing both mortality and morbidity countrywide (WHO 2004). Seventy percent of the malaria cases are documented from the relatively undeveloped borders and hill region of eastern Myanmar where *Anopheles minimus* complex mosquitoes are common and represent important malaria vectors in Thailand (Reid 1968, Ismail et al. 1975).

The *An. minimus* complex, Theobald 1901, is composed of two formally named species, *An. minimus* (=species A) and *An. harrisoni* (=species C), and the informally designated *An. minimus* E (Harbach et al. 2006, 2007, Somboon et al. 2001, 2005).

Two sibling species within this complex, *An. minimus* and *An. harrisoni*, occur in Thailand along the Thai-Myanmar border (Sucharit et al. 1988, Baimai 1989, Green et al. 1990, Kengluecha et al. 2005, Garros et al. 2006, Sungvornyothin et al. 2006a, b). *Anopheles minimus* is the predominant member of the complex in the country and recognized as an important malaria vector, whereas *An. harrisoni* has only been reported from western Thailand and appears to play a minor role in transmission based on its limited distribution and greater zoophilic feeding predilection (Rwegoshora et al. 2002, Kengluecha et al. 2005, Trung et al. 2005, Sungvornyothin et al. 2006a).

In Kanchanaburi Province, sympatric collections of *An. minimus* and *An. harrisoni* have been identified from Pu Teuy Village, in Sai Yok District (Green et al. 1990, Sungvornyothin et al. 2006a, b) and in neighboring Sri Sawat District (Kengluecha et al. 2005). Larval habitats surveyed in Kanchanaburi Province found *An. minimus* in Pu Teuy where it had been reported previously undetectable or absent (Kengluecha et al. 2005). Rwegoshora et al. (2002) found sympatric populations of *An. minimus* and *An. harrisoni* present in a 1:3 ratio in Pu Teuy, and Sungvornyothin et al. (2006b) subsequently found relatively low frequency (4% based on molecular analysis) of *An. minimus* compared to *An. harrisoni* in the same study site during a two-year collection period. Both sympatric species described in this study are difficult to accurately distinguish on morphological characters alone, thus requiring molecular methods for precise identification (Rattanarithikul and Panthusiri 1994, Harrison 1980, Garros et al. 2004, 2006, Sungvornyothin et al. 2006b).

The reasons for the predominance of *An. harrisoni* in Pu Teuy are not clear but might be related to the prevailing environmental conditions that have preferentially favored this species by providing a competitive advantage over *An. minimus*. The natural evolutionary process is influenced by numerous environmental factors that account for varying rates of species adaptation or extinction that can lead to changes in species frequency over time (Dombeck and Jaenike 2004). Human activities in the province

have gradually increased deforestation and economic development, whereas the natural environment of Pu Teuy has remained relatively intact with more limited interference, which may play a significant role in current species composition.

An understanding of the differences in biology, behavior, and genetic structure of members within a vector species complex and their true geographical distribution helps to better describe vectorial capacity and relative roles in disease transmission and becomes especially critical when dealing with cryptic species and developing targeted vector prevention and control strategies. The apparent unique species frequency seen in Pu Teuy lead to an investigation into the genetic relationship of *An. harrisoni* and *An. minimus* in Kanchanaburi Province. In this study, we analyzed morphological, geographical, and genetic relationships among seven *An. minimus* s.l. collections in four separate districts in the province to determine the genetic variations between collections. Using this approach, we analyzed the genetic relationship between the two sibling species and identified possible barriers and corridors of gene flow.

MATERIALS AND METHODS

Collection sites

Anopheles minimus s.l. collections were made in six different locations in Kanchanaburi Province, western Thailand (Figure 1). GPS coordinates and a brief description of locations is provided.

Site 1. Bong Ti Noi (BTN) Village in Sai Yok District (14°17'N, 98° 56'E.) is located in a mountainous area (elevation 320 m above sea level) surrounded by dry forest and cultivated vegetable fields. A stream runs through the village during the dry season, increasing dramatically in water volume during the wet season.

Site 2. Huai Khayeng (HK) Village in Thong Pha Phum District (14°68'N, 98° 59'E) is located south of Khao Laem near Wachiralongkon Dam (420 m asl). The environment is surrounded by evergreen trees and dry forest. There are several natural streams running from the foot of the hills to the dam.

Site 3. Na Suan (NS) Village in Si Sawat District (14°70'N, 99° 09'E) is located east of Si Nakharin Dam (280 m asl). The area is surrounded by evergreen forest, cassava, and vegetable fields. The primary stream runs from an enclosed forest to the dam.

Site 4. Tha Kradan (TK) Village in Si Sawat District (14°40'N, 99° 08'E) is located west of Si Nakharin Dam (285 m asl). This hilly area is predominately evergreen forest interspersed with corn fields.

Site 5. Pra Jedee (PJ) Village in Sangkhla Buri District (14°66'N, 98° 59'E) is located in the far western part of the province along the national border with Myanmar near the Wachiralongkon Dam (460 m asl). The area is surrounded by deep natural forest and rubber plantations. A natural stream runs through the site and provides the primary water source for villagers.

Site 6. Pu Teuy (PT) Village in Sai Yok District (14°17'N, 99° 11'E.) is located in a hilly zone mostly surrounded by primary dense forest (420 m asl). During the dry season a slow running stream with dense primary vegetation along its margins represents the nearest primary larval habitat for *An. minimus* s.l. (Baimai 1989, Chareonviriyaphap et al. 2003). *Anopheles minimus* collections from Pu Teuy are referred to as "PT" and *An. harrisoni* collections as "PTC."

Mosquito collections

Anopheles minimus s.l. were collected as immatures (larvae/pupae) and adults. Immatures were reared to adults in an environmentally-controlled insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. As it is often difficult to obtain sufficient numbers of immature *An. minimus* s.l. in the field, collections from evening human-landing collections were also performed to increase study sample size. Strict segregation of field specimens in the insectary was ensured to prevent contamination between collections. Adult mosquitoes were either tested shortly after emergence or immediately frozen (-20° C) until processed.

Mosquito identification

Anopheles minimus s.l. specimens were initially identified using morphological keys (Harrison 1980, Rattanarithikul et al. 2006). Species were separated by the presence or absence of the humeral pale spot on the costa vein of the wing. *Anopheles minimus* was identified by the absence of a humeral pale spot on the costa of both wings, whereas *An. harrisoni* typically has a humeral pale spot present on one or both wings. This wing pattern has been described as characteristic of *An. harrisoni* 88% of the time, but the reliability of morphological identification drops to only 47% for *An. minimus* (Sungvornyothin et al. 2006b).

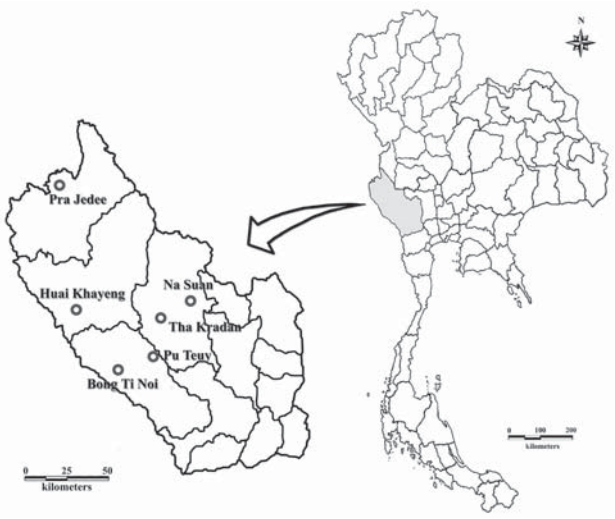

Figure 1. Collection sites in Kanchanaburi Province, Thailand. Pu Teuy, the only site with sympatric collections of *An. minimus* and *An. harrisoni*.

Starch gel electrophoresis

Anopheles minimus s.l. adults from each collection were processed and assayed using horizontal starch gel electrophoresis as described previously (Harris and Hopkinson 1976, Manguin et al. 1995, Chareonviriyaphap and Lerdthusnee 2002). Eight enzyme systems and a morpholine (Morph) buffer system were used (Pasteur et al. 1988). Starch gels were composed of 55 g potato starch (Sigma Chemical Co., St. Louis, MO), 25 g sucrose, and 550 ml gel buffer. Each mosquito was ground in 25 µl of grinding buffer and homogenate absorbed onto three 0.4 x 1.4 cm cellulose polyacetate wicks (Gelman Sciences Inc., Ann Arbor, MI). The Morph system was run for approximately 5 h at a constant power of 16 volts/cm (Manguin et al. 1995). Gel was stained and incubated at 37°C for 15-60 min and bands scored for each specimen. Two or more alleles appearing at the same locus was defined polymorphic. Any locus containing one allele was considered monomorphic. Different alleles of the same locus demonstrated different banding patterns depending upon the migration speed. The most common allele was designated as "100" (Pasteur et al. 1988).

Data analysis

Chi-square tests were performed to observe any significant differences between observed and expected allelic frequencies. Analysis of allele frequencies, heterozygosity per locus, conformity to Hardy-Weinberg expectations, and genetic distances were calculated using BIOSYS-1 (Swofford and Selander 1989). Differentiation among collections was determined using *F*-statistics (F_{ST}). The effective migration rate (N_em) and exchange of genes between collections were estimated from the F_{ST} values as $N_em = (1\text{-}F_{ST})/4\ F_{ST}$ (Nei 1978, Wright 1978). GENEPOP-3.1 was used to estimate the degree of isolation by distance between collections (Rousset 1997), i.e., the relationship between pairwise estimates of F_{ST} and logarithms of geographical distance to determine whether geographical distance among collections serves as a barrier to gene flow.

RESULTS

Using eight enzyme systems, nine putative loci were detected (Tables 1 and 2). The number of polymorphic loci by collection included NS (6), BTN and PT (5), PJ (4), PTC and HK (3), and TK (2) (Table 3). Among all collections, *Got-1* and *Pgm-1* were observed polymorphic, while *Hk-1* and *Tpi-1* were monomorphic. The allele frequencies of all polymorphic loci are given in Table 3.

Of 63 comparisons, there were four significant deviations from the Hardy-Weinberg equilibrium ($P < 0.05$), representing approximately 7% of expected deviations by chance alone (Table 2). The Bonferroni correction test was run and significance level adjusted accordingly (0.05/63), so any value where $p < 0.0008$ is significant. The seven-collection 'pooled' isozyme comparison based on the 9 loci revealed an expected mean heterozygosity (H_{exp}) from 0.036 to 0.136, an average of 0.083 $\pm$ 0.036 (Table 2). The largest H_{exp} was observed in collection BTN (0.136$\pm$0.050), whereas the least was in TK (0.036$\pm$0.024). Likewise, the percent polymorphic loci varied from 66.7 in NS to 22.2 in TK (Table 3). The observed mean heterozygosity from all collections was not significantly different from Hardy-Weinberg H_{exp} ($t_{0.025}$, df = 6, -1.353, $P > 0.05$). The mean F_{ST} from all polymorphic loci was low with a value of 0.061. The largest F_{ST} was associated with *Pgd-1* (0.090), the least *Had-1* (0.039). Four loci, *Got-2, Idh-1, Pgm-1,* and *Pgd-1*, produced small F_{ST} values ($0.050 \leq F_{ST} \leq 0.15$), whereas three loci, *Got-1, Had-1,* and *Mdh-2* showed negligible genetic differentiation.

Gene flow between collections was estimated from the calculated effective migration rate (N_em), wherein *Ne* is the effective collection size and *m* is the migration rate between collections. As *m* represents the proportion of migrants (number of migrants/*Ne*), N_em is an estimate of the number of migrants, regardless of actual collection size, that would permit a determination of degree of genetic differentiation among collections. The genetic divergence between the seven local collections ranged from N_em 3.72 (PJ vs PTC) to 62.25 (HK vs PJ) (Table 4). Pairwise analysis $F_{ST}/(1\text{-}F_{ST})$ indicated no correlation ($P > 0.05$) between gene flow and geographic

Table 1. Enzyme systems and loci used in electrophoresis on adult *An. minimus* s.l.

Enzyme system	E.C. *	No. of loci**
Got (Glutamate oxaloacetate transaminase)	2.6.1.1	2
Had (β-Hydroxyacid dehydrogenase)	1.1.1.30	1
Hk (Hexokinase)	2.7.1.1	1
Idh (Isocritate dehydrogenase)	1.1.1.42	1
Mdh (Malate dehydrogenase)	1.1.1.37	1
6-Pgd (6-Phosphogluconate dehydrogenase)	1.1.1.44	1
Pgm (Phosphoglucomutase)	2.7.5.1	1
Tpi (Triose phosphate isomerase)	5.3.1.1	1

*Enzyme commission number.
**Number of scored bands per phenotype.

Table 2. Allele frequency and sample size (*n*) of six collections of *Anopheles minimus* s.l. from Kanchanaburi Province (Thailand).

Locus / allele	*Anopheles minimus* collections						
	BTN[1]	HK[2]	NS[3]	TK[4]	PJ[5]	PT[6]	PTC[7]
Got-1							
n	30	33	11	12	18	39	12
127	0.05	0.152	0.091	0.083	0.167	0.026	0
100	0.917	0.833	0.864	0.917	0.806	0.974	1
73	0.033	0.015	0.045	0	0.028	0	0
Got-2							
n	30	18	11	12	18	27	12
-120	0.117	0	0.091	0	0	0	0
-100	0.833	0.861	0.909	1	0.889	1	1
-40	0.05	0.139	0	0	0.111	0	0
Had-1							
n	30	18	11	12	18	27	12
166	0	0	0.045	0	0	0	0
100	1	1	0.955	1	1	1	1
Hk-1							
n	30	18	11	12	18	27	12
100	1	1	1	1	1	1	1
Idh-1							
n	30	18	11	12	18	29	12
127	0	0	0.045	0	0	0.069	0.167
100	1	1	0.955	1	1	0.931	0.833
Mdh-2							
n	30	18	11	35	31	27	12
100	0.067	0	0.091	0	0	0.056	0.125
-67	0.933	1	0.909	1	1	0.944	0.875
Pgm-1							
n	30	12	11	12	18	39	12
115	0.05	0.083	0.045	0.042	0.056	0.051	0.083
100	0.75	0.917	0.955	0.917	0.944	0.949	0.917
77	0.2	0	0	0.042	0	0	0
Pgd-1							
n	30	18	11	12	18	17	12
138	0.067	0	0	0	0.056	0.176	0
100	0.867	1	1	1	0.944	0.824	1
63	0.067	0	0	0	0	0	0
Tpi-1							
n	30	15	11	7	15	11	12
100	1	1	1	1	1	1	1
H_{exp}**	0.136	0.07	0.097	0.036	0.083	0.076	0.075
	-0.05	-0.04	-0.03	-0.024	-0.039	-0.033	-0.039

[1]Bong Ti Noi, [2]Huai Khayeng, [3]Na Suan, [4]Tha Kradan, [5]Pra Jedee, [6]Pu Teuy, [7]Pu Teuy C.
*Deviation from Hardy-Weinberg equilibrium ($P < 0.05$).
**Average expected genetic heterozygosity with standard error in parenthesis.

Table 3. Genetic variability at eight loci of pooled collections of *An. minimus* s.l. from six sites in Kanchanaburi Province (Thailand).

Collection	Average alleles / locus	% polymorphic loci [1]	Mean heterozygosity	
			H_{obs}	H_{exp} [2]
Bong Ti Noi (BTN)	2.0±0.3	55.6	0.104 ±0.039	0.136±0.050
Huai Khayeng (HK)	1.4±0.2	33.3	0.073 ±0.038	0.077±0.040
Na Suan (NS)	1.8±0.2	66.7	0.101± 0.032	0.097±0.030
Tha Kradan (TK)	1.3±0.2	22.2	0.037 ±0.024	0.036±0.024
Pra Jedee (PJ)	1.6±0.2	44.4	0.068 ±0.037	0.083±0.039
Pu Teuy (PT)	1.6±0.2	55.6	0.065 ±0.029	0.076±0.033
Pu Teuy (PTC)	1.3±0.2	33.3	0.083±0.044	0.075±0.039
			Average	0.083±0.036
			$t_{0.025}$ = -1.353 [ns]	

[1]Locus considered polymorphic when frequency of the most common allele < 0.95.
[2]Unbiased estimate and standard error (Nei 1978).
[ns]Not significant.

distance among the seven *An. minimus* collections (Table 4). Coefficient of determination of isolation by distance among collections was not significant (r^2 = 0.010).

DISCUSSION

In this study, genetic variation and gene flow among seven collections of *An. minimus* s.l. collected in the Kanchanaburi Province were compared using information from isozyme allele frequencies and morphological criteria. In addition, this study allowed us to examine the genetic relationship between collections of *An. minimus* and *An. harrisoni* in the only locality where they were found in sympatry.

The average heterozygosity in all collections of *An. minimus* s.l. was much lower (Hexp=0.083) than those from the previous studies of eight collections throughout Thailand (Hexp=0.360) (Komalamisra 1989) and three collections from southern China (Hexp=0.340) (Sawabe et al. 1996). The reason for this is unclear but it could be associated with the low number of polymorphic loci detected in this study. Genetic variation among the seven collections was small (*Fst* = 0.061), indicating sufficient random mating between collections in contiguous areas (*Fis* = 0.058). For comparison, a mean F_{ST} value of 0.040 was found for *An. maculatus* populations in Thailand, suggesting that gene flow also occurs among these populations (Rongnoparut et al. 1999). It appears that all collections in Kanchanaburi Province represent a genetically closed cluster, except two collections from Pu Teuy which were slightly different from the other five locations. Paired groupings indicated high gene flow between collections from Huai Khayeng (HK) (Thong Pha Phum District) and Pra Jedee (PJ) (Sangkhla Buri District), the two villages located nearest the Thai-Myanmar border and separated by a 72 km distance. Gene flow was also high between NS and TK (both in Si Sawat District) and likely explained by the relative close geographical proximity to one another (< 4 km).

Larval habitats of *An. minimus* s.l. in Pu Teuy Village are somewhat different from the more common or typical sites described (Kengluecha et al. 2005). They are derived from limestone spring-fed water into a slow moving stream coursing zigzag through the village protected by dense native vegetation and shaded along both margins. Typical larval sites in other locations are primarily streams derived from deep forest surface waters with littoral margins generally characterized as having less vegetation and shade, and surrounded by small shrubs and trees. Various lotic, slow running water habitats can have significant environmental differences in inorganic and biologic characteristics, which in turn, can directly or indirectly influence mosquito species presence, absence, and diversity. The influence of large or subtle changes and differences in larval ecology may translate into intraspecific differences often seen between collections of the same species (Hynes 1984, Laird 1988, Williams and Feltmate 1992).

The sympatric collections of *An. harrisoni* and *An. minimus* from Pu Teuy were found to be genetically similar, but this does not necessarily infer interbreeding between the two species. No hybrids have yet been found in Thailand. However, electrophoretic data of these two sibling species from northern Vietnam showed natural hybrids at a rate of 0.88% (Van Bortel et al. 1999). In the *An. gambiae* complex from Africa, the observed frequency of hybrids between *An. gambiae* and *An. arabiensis* lies between 0.1% based on cytotaxonomy or 0.15% using rDNA-PCR method (Temu et al. 1997) to 0.76% reported from electrophoretic data (Mahon et al. 1976). This issue of hybrids between species of the *An. minimus* complex should be further investigated.

Thus, we surmise that *An. harrisoni*, showing only minor phenotyical differences, has likely descended from parental *An. minimus* relatively recently; whether the

Table 4. Pairwise *F*-statistics and effective migration rate (N_em) of all loci between seven collections of *An. minimus s.l.* in Kanchanaburi Province (Thailand).

	BTN	HK	NS	TK	PJ	PT	PTC
BTN	Infinite						
HK	0.037 (6.51)	Infinite					
NS	0.031 (7.81)	0.020 (12.25)	Infinite				
TK	0.043 (5.56)	0.027 (9.01)	0.021 (11.65)	Infinite			
PJ	0.036 (6.69)	0.004 (62.25)	0.019 (12.91)	0.025 (9.75)	Infinite		
PT	0.039 (6.16)	0.055 (4.30)	0.032 (7.56)	0.044 (5.43)	0.041 (5.85)	Infinite	
PTC	0.054 (4.38)	0.062 (3.78)	0.028 (8.68)	0.052 (4.56)	0.063 (3.72)	0.035 (6.89)	Infinite

result of sympatric or parapatric reproductive isolating mechanisms is yet to be determined. The apparent competitive displacement of a closely related, more efficient malaria vector species with a relatively less competent one is epidemiologically important and there is a real need for more careful assessment of the distribution of members of the *An. minimus* complex throughout its range for purposes of directing appropriate vector control efforts where needed. Mosquito dispersal is an important component of natural gene flow that influences the genetic structure of mosquito populations. This information is epidemiologically relevant for understanding relatively small, area-specific vector bionomics and risk of potential malaria transmission. The temporal and spatial variations with respect to expression of enzymes that confer resistance to insecticides or vector capacity for development and transmission of disease pathogens may be strongly influenced by patterns and frequency of gene flow between localities. Several studies have reported a correlation between genetic distances and variation in vector-virus competent phenotypes in *Aedes aegypti* (Ocampo and Wesson 2004, Sukonthabhirom et al. 2005). Locations with a substantial or increased rate of gene flow could also share the same characteristics that influence pathogen susceptibility and insecticide resistance patterns. Although gene flow in *Anopheles* mosquitoes was once thought to be rare, more recent studies have shown that it is not restricted in some notable cases (Lehmann et al. 1996, Rongnoparut et al. 1999). Defining the population structure associated with vector capacity and disease transmission can greatly assist predictive modeling and timely planning for allocation of vector monitoring and control methods.

We acknowledge that a limitation in this study was reliance on using morphological identification of *An. minimus* and *An. harrisoni* alone that may result in a certain degree of misidentification between the two species (Sungvornyothin et al. 2006a, 2006b). In addition, using F_{ST} statistics may result in misleading conclusions or interpretation between ongoing gene flow and shared ancestral polymorphism, especially with more recent co-ancestry. Isozymes may also be subjected to selection pressure, and thus are not clearly appropriate as the best of natural markers. In the future, we would recommend incorporating molecular assays for identification of specimens as the most reliable method for distinguishing these two species of the Minimus Complex (Garros et al. 2004).

Acknowledgments

We thank the Armed Forces Development Command, Sai Yok District, Kanchanaburi Province, Thailand, for support in all study areas. Financial support was received from the Thailand Research Fund (TRF) and the Kasetsart University Research Development Institute (KURDI), Thailand.

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Journal of Vector Ecology

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BITING PATTERNS OF *ANOPHELES MINIMUS* COMPLEX (DIPTERA: CULICIDAE) IN EXPERIMENTAL HUTS TREATED WITH DDT AND DELTAMETHRIN

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ABSTRACT. Biting patterns of natural population of *Anopheles minimus* s.l. females into experimental huts treated with DDT and deltamethrin were carried out at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, west Thailand. Two experimental huts, control and treatment, were constructed in the fashion of local Thai homes. Pre-spray (baseline) peak biting activity of *An. minimus* females occurred at 1900-2200 hr. Although weaken, post-treatment exposure continued to show greater landing activity during the first half of the evening. An overall greater proportion of *An. minimus* females entered the hut treated with deltamethrin compared to DDT. The hut fitted with DDT-treated net panels showed a 71.5% decline in attempted blood feeding, whereas exposure to deltamethrin-treated panels resulted in a 42.8% human-landing reduction. DDT exhibited significantly more pronounced ($P < 0.05$) effects in overall reduction of biting activity than deltamethrin.

Keyword index: Anopheles minimus, behavioral responses, excito-repellency, experimental hut, deltamethrin, DDT

48 INTRODUCTION

49 Malaria is known as the most serious vector borne disease in the tropical and
50 subtropical regions with transmission occurring in over 105 countries worldwide (Roll
51 Back Malaria 2006). Approximately 70% of malaria cases occur on the African
52 continent whereas 30% remain in Americas and Asia [World Health Organization
53 (WHO) 2006]. In Thailand, malaria remains a major and reemerging health problem,
54 although vector control programs have been successful in reducing morbidity and
55 mortality which often results in socioeconomic losses (Ministry of Public Health
56 (MOPH) 2006). Approximately seventy percent of the malaria cases are documented
57 from the undeveloped national borders of eastern Myanmar where a member of
58 efficient malaria vectors like *Anopheles minimus* complex, one of the most important
59 malaria vectors in Thailand, is most prevalent (MOPH 2006). Although *An. minimus*
60 populations in some areas demonstrate exophagic and zoophagic behavior, major
61 endophagic and anthropophagic behaviors remain as significant characteristics of this
62 important vector (Rattanarithikul et al. 1996, Sungvornyothin et al. 2006).

63 The *An. minimus* complex has shown different behavioral responses to
64 intradomicilary use of insecticides (Herrison 1980, Parajuli et al. 1981, Ismail et al.
65 1975). In Thailand, indoor residual spray (IRS) is routinely conducted to interrupt
66 human-vector contact and transmission (Chareonviriyaphap et al. 2001, MOPH 2006).
67 For years, DDT was the chemical of choice and was used extensively in malaria-
68 endemic areas. Because of theoretical adverse environmental impacts and general
69 negative public perceptions, DDT was removed from malaria control in Thailand in
70 2000 and replaced by synthetic pyrethroids (Chareonviriyaphap et al. 2000).

71 Pyrethroids have been widely accepted for controlling disease vectors due to
72 their low mammalian toxicity (Elliot e1 al. 1987). Deltamethrin, a commonly used

73 synthetic pyrethroid in public health programs, has been the mainstay for IRS use to
74 combat malaria transmission in Thailand (Pothikasikorn et al. 2005, MOPH 2006).
75 There have been numerous attempts to accurately measure the behavioral
76 responses of mosquitoes to insecticides (Smith 1965, Roberts et al. 1984, Rutledge et
77 al. 1999, Bangs 1999, Grieco et al. 2000, Pates and Curtis 2005). Experimental hut
78 studies provide valuable information on the behavioral responses of natural occurring
79 mosquito populations. Understanding the behavioral responses of different disease
80 vectors to test compounds can facilitate vector control operations by helping in the
81 selection of the most effective interventions possible and in targeting the primary
82 disease vectors. However, little has been documented on the house entering behavior
83 of *An. minimus* females into experimental huts treated with either DDT or
84 deltamethrin. For this reason the effects of chemicals applied to the interior of homes
85 on the behavior of this important vector warrants further study. The experimental huts
86 used in the current study have been used to evaluate the flight behavior of *Aedes*
87 *aegypti* in Thailand (Suwondkerd et al. 2006). The data presented here are the results
88 of the first comparison of the behavioral responses of *An. minimus* to DDT and
89 deltamethrin, as measured by levels of biting activity both pre- and post-spray.
90
91

MATERIALS AND METHODS

Study site: The study was conducted in Pu Teuy, a village located in Sai Yok District, Kanchanaburi Province, western Thailand ($14^0$17'N, $99^0$11E'). It has a population of 1,400 with the major occupation being agricultural practices. The rural site is located in mountainous terrain mostly surrounded by intact forest, approximately 500 m from the nearest house at Pu Tuey Village. The main water body near the collection site is a narrow, slow running stream, bordered with native vegetation (Chareonviriyaphap et al. 2003). This stream represents the main larval habitat for *An. minimus* s.l. (Baimai 1989, Kengluecha et al. 2005, Sungvornyothin et al. 2006). Indoor residual spraying (IRS) using DDT had occurred routinely in this area for approximately 60 years (1940-2000), however, DDT was replaced by deltamethrin, a synthetic pyrethroid, in 2000. IRS was discontinued in the area during the course of our investigation.

Insecticide susceptibility tests. The susceptibility of *An. minimus* s.l. to DDT (4%) and deltamethrin (0.05%) was assessed by exposing female mosquitoes to a single diagnostic dose on insecticide-treated test papers, as recommended by WHO and following standard testing procedures (WHO 1998). After a 30 min exposure for DDT and 60 min exposure for deltamethrin, test and control mosquitoes were transferred to separate clean holding containers and mortality was recorded 24 hrs post-exposure. Tests were repeated four times. Mosquito survival was used as an indicator of the degree of physiological resistance.

***Experimental hut*:** Two identical experimental huts were used for the study of the entering and feeding behaviour of *An. minimus* s.l. Huts used in the present study were previously used to evaluate the flight behaviour of *Ae. aegypti* in Thailand (Chareonviriyaphap et al. 2005; Suwonkerd et al. 2006). The huts were built using

117 locally acquired materials and consisted of sections of iron fence pipe along with
118 custom-welded galvanized pipes. Pieces of untreated wood planks, measuring 1 x
119 2.5 m were joined together into panels measuring (1 x 3 m) to serve as the side walls.
120 Floors were adjusted and aligned with cement blocks with an 'A' frame style zinc
121 roof. The apex of the roof measured 3.5 m from the ground. The eaves on all four
122 sides of the hut were sealed with 1/12-in aluminum wire mesh fastened across the
123 eave opening. All three windows, one on each of three walls, and one door remained
124 open during the collection period. The two huts were positioned 100 m apart on an
125 open plot of land surrounded by mountainous terrain and agricultural fields.
126 ***Preparation and use of nets in huts*:** In order to evaluate chemicals in the
127 treated hut without applying compound directly to the wall surfaces, a series of panels
128 were developed for holding treated netting which could be positioned around the
129 interior surface of the hut. The aluminum frame that housed the netting contained
130 holes in each corner and were held in place to the hut walls using bolts. There is a 9
131 cm gap between the aluminum panel and the wood planks to prevent the netting from
132 touching the interior walls. Wing nuts were used to facilitate the rapid placement and
133 removal of the metal panels for washing after the conclusion of the experiment.
134 The field application rate of DDT and deltamethrin were used in this investigation.
135 Netting impregnated with DDT at 2 g/m^2 and deltamethrin at 0.02 g/m^2 were prepared
136 using acetone diluents following the method of Grieco et al. (2005). The treatment
137 nets (3 m^2) were soaked with treatment solutions (18.6 ml) in metal pans and covered
138 with a heavy, smaller pan. Additional nets were treated with acetone (18.6 ml.) to
139 serve as untreated controls. All nets were allowed to air-dry for 60 min before use in
140 the experimental huts (Grieco et al. 2005). The interior of the treatment hut was lined

141 with netting material treated with either 2 g/m^2 of DDT or 0.02g/m^2 of deltamethrin.
142 The control hut was lined with netting prepared with only the solvent, acetone.
143 ***Pre spray collection*****:** Two untreated experimental huts were used during the
144 pre-spray period. Simultaneous indoor collections were performed in the two
145 untreated huts to obtain the baseline data on the normal pattern of *An. minimus* biting
146 in the experimental huts during the rainy season (August 2006). The baseline
147 collections also provided a determination of comparability of the two huts in regard to
148 *An. minimus* densities and activity patterns prior to spraying. Collectors were divided
149 into two teams of four persons each. The first team worked from 1800-2400 hr for
150 each hut with two collectors inside of each hut, followed by the second team
151 beginning from 0000 hr to 0600 hr. Human-landing collections were conducted for
152 45 min with a 15-min break at the end of each hr. On the following night, collectors
153 who worked during a particular sampling period (either the early or late sampling
154 period) were rotated to avoid collector bias. Each collector exposed their lower legs
155 and collected all landing mosquitoes by mouth aspirator. Collected mosquitoes were
156 retained in plastic holding cages, labeled by hr and hut, and provided cotton soaked
157 with a 10% sugar solution. Specimens were transferred to the field laboratory and
158 microscopically identified the following morning. Additional details on human-
159 landing collection methods are given in previous work (Sungvornyouthin et al. 2006).
160 Hourly ambient outdoor temperature and humidity were recorded during the period of
161 mosquito collection. This study received full review and approval from the Kasetsart
162 University Human Use and Ethics Committee before conducting this work. Anti-
163 malarial chemoprophylaxsis was offered to each study participant. Given that
164 chemoprophylaxsis alone will not necessarily prevent patent infections, all collectors

were afforded access to professional malaria diagnosis and immediate treatment if contracting malaria during the period of study.

***Post spray collections*:** During the post-spray collections, one hut served as a control and the other hut served as the treated structure. The same hut was used for DDT (September 2006) and later for deltamethrin (October 2006). In order to evaluate chemicals without applying compound directly to the hut walls, a series of panels were made for holding treated netting which could be positioned around all interior surfaces of the hut, excluding floor and ceiling. The aluminum frame that housed the netting contained holes in each corner and were held in place to the hut walls using bolts. A 9 cm gap between the aluminum panel and the wood planks prevents the netting from touching the interior walls. The interior of the treatment hut was lined with netting material treated with either 2g/m^2 of DDT or 0.02g/m^2 of deltamethrin, whereas the control hut was lined with netting prepared with only acetone solvent used in the preparation of the insecticide-treated netting. All three windows and one door were left open during the period of collection to allow female mosquitoes to freely enter.

***Data analysis*: Pre-sprayed:** Collection periods were grouped into four categories, early evening (1800-2100 hr), late evening (2100-2400 hr), early morning (0000-0300 hr) and before sunrise (0300-0600 hr). The mean number of collected mosquitoes from the huts prior to spraying (huts 1 and 2) was compared using an independent-sample *t*-test, one-way analysis of variance (ANOVA). The test of normality for the numbers of *An. minimus* collected in each hut was conducted using either the normal probability plot and Komogorov–Smirnov Test (K-S Test) or Shapiro–Wilk Test using SPSS (SPSS version 15.0. Inc., Chicago, IL). The accepted significance level was determined at 0.05% (*P*-value < 0.05).

190 **Post spray:** Collection periods were also grouped into four categories as listed
191 above. The mean number of mosquitoes from the sprayed hut and its matched control
192 were compared (DDT treated hut vs. untreated hut and deltamethrin treated hut vs.
193 untreated hut) using a paired–sample *t*-test and ANOVA in SPSS (SPSS version 15.0.
194 Inc., Chicago, IL).

RESULTS

The pattern of biting activity for natural populations of *An. minimus* in experimental huts was observed during the rainy season (Figure 1). From a total of twenty all-night collections, 260 and 248 *An. minimus* females were captured from huts 1 and 2, respectively. One prominent peak of biting activity was observed during 1900-2200 hr whereas a second weak peak was observed at 0300-0600 hr. When collection times were tabulated into four categories, the lowest proportion of *An. minimus* females entering the two huts was found to occur during early morning hours (0000-0300 hr) [33 for hut 1 (12.69%) and 39 for hut 2 (15.72%)] (Table 1). The greatest proportion of biting occurred during the first half of the night. Broken down by hut, there was 69.61% biting before midnight in the first hut and 66.53% in the second hut. Furthermore, greater numbers of *An. minimus* females were biting in the early evening [(119 hut 1 (45.76%) and 104 hut 2 (41.93%)] compared with the other periods. Ratio of numbers biting in the two huts was 1:0.95. The Levene's test for equality or homogeneity of variances demonstrated that the two experimental huts had equal variances without any significant differences in numbers biting (Student's t-test, $t = -0.268$, $df = 38$, $p > 0.05$).

After the DDT treated nettings were placed in the hut, an additional ten nights of human-landing collections were performed to assess biting pattern of *An. minimus* females in treatment and control huts. The pattern of biting activity of *An. minimus* females in the control hut was similar to what was observed under pre-spray conditions. A significant reduction in the number of *An. minimus* females biting in the DDT treated hut was observed throughout the night with a major pronounced reduction in the number of mosquitoes collected during the first half of the night (1800-0000 hr) ($P < 0.05$). Two hundred and ninety two females were collected

220 from the unsprayed hut whereas 110 females were collected from the DDT treated
221 hut. During the before sunrise period (0300-0600 hr), female mosquitoes almost
222 disappeared from the hut treated with DDT. Only five were collected from the DDT
223 treated hut compared to 58 from the control hut. Overall, 627 *An. minimus* females
224 (77.8%) were caught from the untreated hut (control) whereas only 179 (22.2%) were
225 captured from the DDT treated hut (Table 2).

226 The effects of deltamethrin on biting activity of *An. minimus* in treated huts
227 were investigated. The two huts, deltamethrin treated hut and control hut, were
228 prepared in the same manner as previously described for DDT. The pattern of *An.*
229 *minimus* biting activity in the control hut was found to be similar to that observed
230 under pre-spray conditions. In the deltamethrin treated hut, there was a significant
231 reduction in the number of mosquitoes collected compared to the control hut ($P <$
232 0.05). A decrease in the numbers biting was observed in the deltamethrin treated hut
233 in the early evening (1800-2100 hr) and low levels of biting persisted through the
234 remainder of the night. Overall, 509 *An. minimus* females (63.6%) were collected
235 from the untreated hut whereas 291 (36.4%) females were collected from the
236 deltamethrin treated hut (Table 2).

237 A comparison of mean number of *An. minimus* between the hut treated with
238 DDT (17.9) and its matched unsprayed control hut (62.7) also showed the huts to be
239 significantly different (t value = -2.179: $p < 0.05$). Moreover, a comparison of mean
240 number of *An. minimus* between the deltamethrin treated hut (29.1) and its matched
241 unsprayed control hut (50.9) were also significantly different (t value = -5.313: $p <$
242 0.05).

243 Comparatively large numbers of *An. minimus* females were collected from the
244 unsprayed hut compared to the treated hut. There was a 71.5% reduction in the

245 number of *An. minimus* caught in the DDT treated hut as compared to the control hut
246 (Table 3 and Fig. 3) and a 42.8% reduction in the deltamethrin treated hut compared
247 to the control hut (Table 2 and Fig. 3).

DISCUSION

Like DDT, most pyrethroids are known to elicit behavioral responses in insects (Roberts and Andre 1994, Roberts et al. 1997, Chareonviriyaphap et al. 1997). In Thailand, vector control using deltamethrin for IRS was first launched in 1994 (Chareonviriyaphap et al. 1999). The extensive use of pyrethroids since that time should be a major stimulus for extensive testing and field evaluation of this class of chemistry on the behavioral responses of malaria vectors.

Behavioral responses to DDT and deltamethrin by several malaria vectors have previously been reported from Thailand (Ismail et al. 1975; Prasittisuk et al. 1996; Suwonkerd et al. 1997, Chareonviriyaphap et al. 2001, 2004, Sungvornyothin et al. 2001, Pothikasikorn et al. 2005). In spite of these reports, the true impact of DDT and the pyrethroids on behavioral responses of mosquitoes and the chemical actions that interrupt disease transmission remains unclear and poorly understood. Most work on the behavioral responses of vectors to insecticides was conducted in the laboratory and relied on the excito-repellency test system (Chareonviriyaphap et al. 2001, 2004, Sungvornyothin et al. 2001, Pothikasikorn et al. 2005). Very few field trials to quantitatively evaluate the responses of *Anopheles* mosquitoes to insecticides have been performed using experimental huts in Thailand (Ismail et al. 1975, Prasittisuk et al. 1996, Suwonkerd et al. 1997).

The present study suggests that the excito-repellency effect must be accurately assessed under field conditions for a clear understanding of how these chemicals function. Result demonstrated that DDT strongly reduced *An. minimus* biting inside of treated huts. There was a 71.5% reduction of *An. minimus* females collected in the hut treated with DDT compared with the matched control. This indicates a strong excito-repellent action of DDT. Hut studies with Anopheline vectors from Belize

273 resulted in a similar conclusion that DDT produced both an irritant and repellent
274 action (Bangs 1999, Grieco et al. 2000). In Thailand, *Anopheles minimus* females
275 showed strong avoidance behavior by not entering experimentally treated huts with
276 DDT (Ismail et al. 1975, 1976, Suthus et al. 1986). Similar work of Roberts et al.
277 (1991) observed that *Anopheles darlingi* females from Brazil completely disappeared
278 after experimental huts were sprayed with DDT.

279 In addition to DDT, deltamethrin also reduced *An. minimus* populations from
280 inside the experimental hut. However, the pattern of *An. minimus* behavior elicited by
281 deltamethrin was quite different from behavior elicited by DDT. *Anopheles minimus*
282 females almost disappeared from the DDT treated hut during the second half of the
283 night (0000-0600 hr) whereas they continued to bite in the deltamethrin treated hut
284 throughout the night. This difference in behavioral responses is consistent with
285 observations on house entering behavior of *An. vestitipennis* after huts were sprayed
286 with deltamethrin and DDT (Bangs 1999, Grieco et al. 2000). In the *An. vestitipennis*
287 studies there were higher numbers of mosquitoes entering the hut treated with
288 deltamethrin than with DDT, indicating the powerful spatial repellency of DDT
289 compared to deltamethrin. Specifically, there was a 97% reduction of mosquitoes
290 entering a hut treated with DDT whereas there was only a 66% reduction of *An.*
291 *vestitipennis* in the deltamethrin treated hut (Grieco et al. 2000).

292 The current strategy of using human landing collections was adopted due to
293 the low numbers of *An. minimus* at the study site. The use of traps would have further
294 reduced the numbers collected, a result that would have reduced statistical power of
295 the data. Reduced biting produced by both of these compounds is probably a result of
296 the combined effects of spatial repellency and contact irritancy. Mosquitoes may
297 have entered the treated huts, rested on the insecticide treated surface, become

298 irritated and left without biting, thus giving the perception of repellency. While the
299 data presented here cannot clearly define the nature of the response (i.e., contact
300 irritancy or spatial repellency), it does clearly demonstrate the effectiveness of DDT
301 in preventing indoor biting by *An. minimus*. While deltamethrin did not have as
302 dramatic a reduction on the biting population as DDT, it too significantly reduced *An.*
303 *minimus* biting inside of huts. The differences in the patterns of response to the two
304 chemicals indicate that the two compounds may be eliciting different actions. This
305 will have to be studied further using entrance and exit traps to quantify the differential
306 contributions of spatial repellent and contact irritant actions in the reduction of indoor
307 biting and disease transmission.

308 In conclusion, without a better understanding of the relationship between
309 insecticide residues and mosquito behavior, vector control strategies will continue to
310 be hampered by not knowing which of several chemical actions are actually serving to
311 prevent disease transmission inside homes. Studies on the avoidance behavior of *An.*
312 *minimus* using insecticide treated huts provides significant baseline data and critical
313 information on how female mosquitoes respond to chemicals in a natural setting.
314 Such information will facilitate the national vector control program by providing the
315 detailed field entomological knowledge on how insecticides are functioning to control
316 malaria and other vector-borne diseases. Additional work on the behavioral
317 responses of mosquitoes to insecticides is needed. Such studies must be performed
318 using experimental huts fitted with entrance and exit traps in order to define the
319 entrance and exit behaviors of important disease vectors in presence of behaviorally
320 active insecticides.

ACKNOWLEDGEMENT

We would like to thank Dr. D.R. Roberts, Emeritus Professor of the Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences for critical review. Special thanks go to Drs. J.P Grieco and N.L. Achee of the Uniformed Services University of the Health Sciences for their technical advice during field study. We are grateful the Armed Forced Development Command, Sai Yok District, Kanchanaburi Province, Thailand for permission to use the study area. Funding for this research came partly from the National Institutes of Health, U.S.A. (Grant # 5U01AI054777-02), the Thailand Research Fund, the Center for Agricultural Biotechnology, and the Kasetsart University Development Institute, Thailand.

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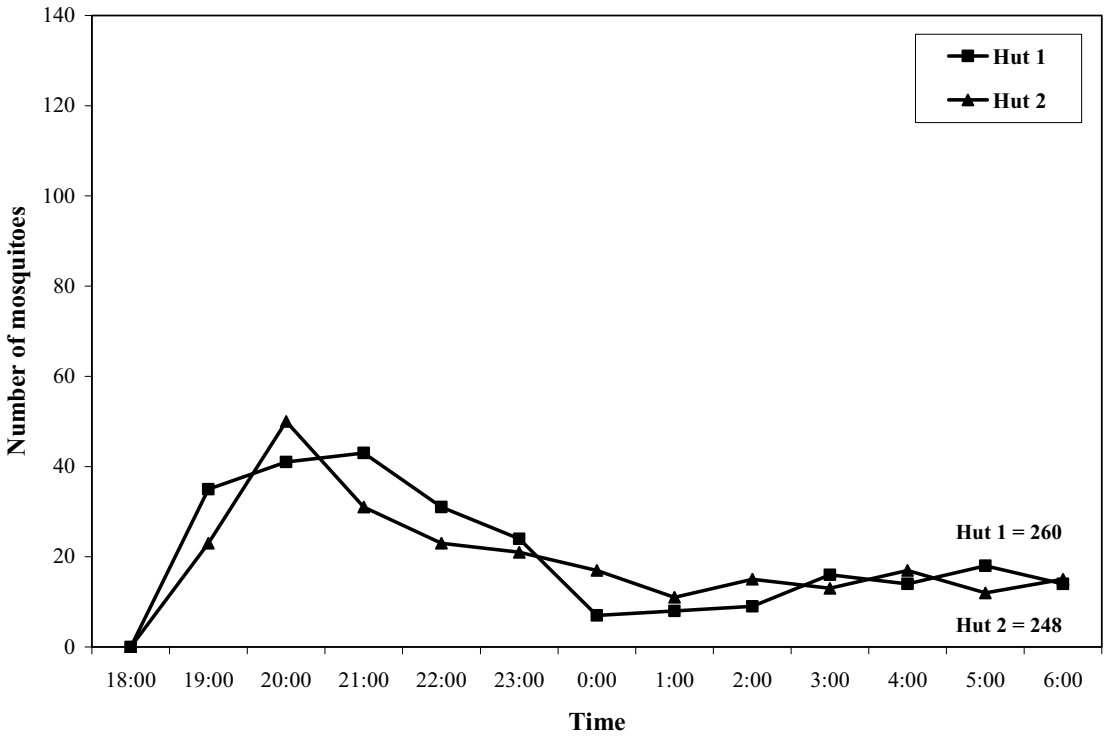
Table 1. Number of *Anpheles minimus* s.l. collected from human-landing collections conducted for twenty nights in untreated huts (huts 1 and 2)

Huts	Number of *An. minimus* (N)					
	1800-2100	**2200-2400**	**0000-0300**	**0400-0600**	**Total (N)**	**Ratio**
Hut 1	119	62	33	46	**260**	**1**
Hut 2	104	61	39	44	**248**	**0.95**
Total	223	123	72	90	**508**	

Table 2. Number of *Anpheles minimus* s.l. collected during four time periods from human-landing collections during 10 collection nights in huts treated with DDT and deltamethrin along with their matched control. Collection totals are separated into four sample periods to correspond to evening (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr), and dawn (0400-0600 hr)

Huts / **Times**	**Number of *An. minimus* (N)**					
	1800-2100	**2200-2400**	**0000-0300**	**0400-0600**	**Total (N)**	**% Reduction**
DDT (Hut 1)	110	46	18	5	179 (22.2%)	71.5%
Unsprayed (Hut 2)	292	186	91	58	627 (77.8%)	
Deltamethrin (Hut 1)	86	77	82	46	291 (36.4%)	42.8%
Unsprayed (Hut 2)	172	115	130	92	509 (63.6%)	

Fig. 1


Hut 1
Hut 2
Number of mosquitoes
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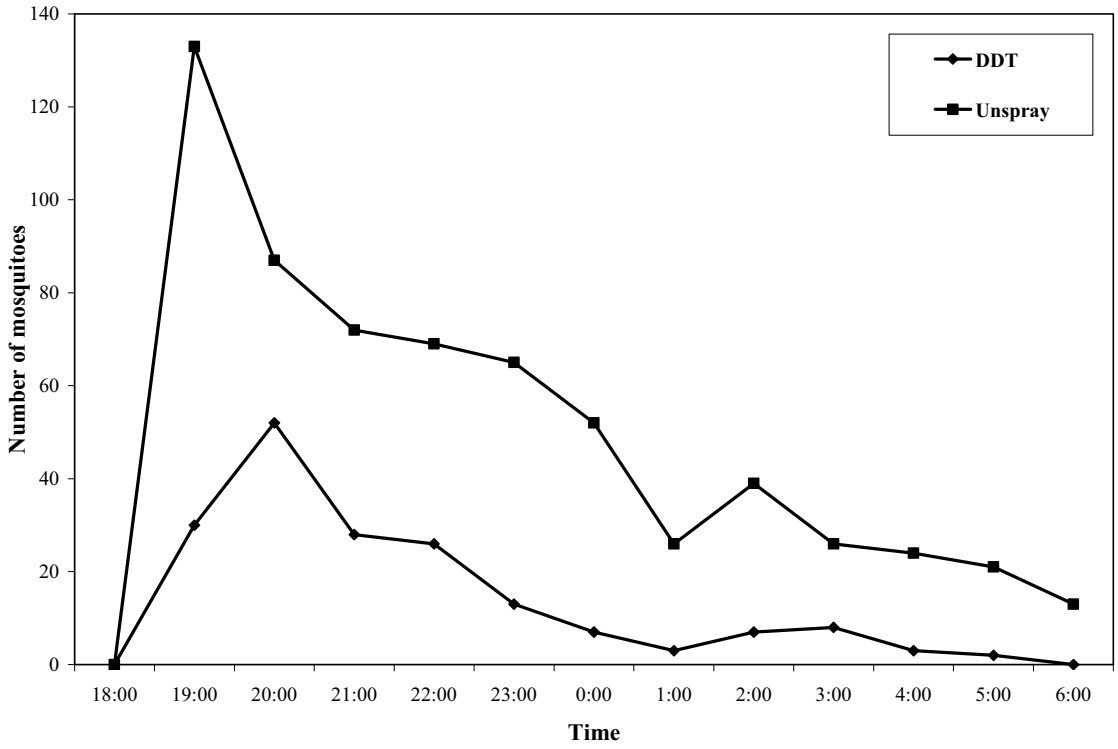
120
100
80
60
40
20
0
18:00
19:00
20:00
21:00
22:00
23:00
0:00
1:00
2:00
3:00
4:00
5:00
6:00
Time
Hut 1 = 260
Hut 2 = 248

Fig. 2


140
120
100
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60
40
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0
Number of mosquitoes
18:00
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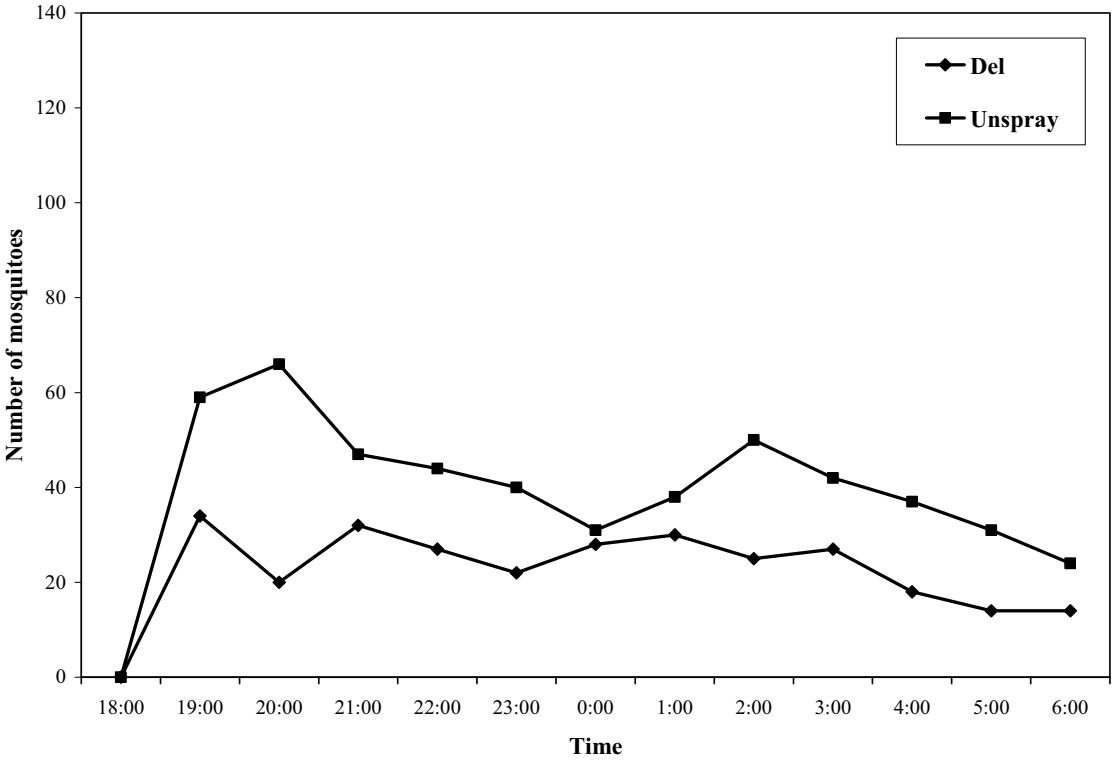
1:00
2:00
3:00
4:00
5:00
6:00
Time
DDT
Unspray

Fig. 3


Del
Unspray
Number of mosquitoes
140
120
100
80
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40
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22:00
23:00
0:00
1:00
2:00
3:00
4:00
5:00
6:00
Time

Figures legend

Fig. 1 Number of *Anopheles minimus* s.l. collected from human landing collections during 20 nights at Pu Teuy Village, Sai Yok, Kanchanaburi Province, Thailand, a comparison between untreated huts 1 and 2 during the pre-spray period.

Fig. 2 Number of *Anopheles minimus* s.l. collected from human landing collections during 10 collection nights at Pu Teuy Village, Sai Yok, Kanchanaburi Province, Thailand, a comparison between untreated hut and DDT treated hut.

Fig. 3 Number of *Anopheles minimus* s.l. collected from human landing collections during 10 collection nights at Pu Teuy Village, Sai Yok, Kanchanaburi Province, Thailand, a comparison between untreated hut and deltamethrin treated hut.

Journal of Pesticide Science

Biochemical detection of insecticide resistance mechanisms in *Aedes aegypti* (Linnaeus)

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RUNNING TITLE: Biochemical resistance of *Aedes aegypti*

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INTRODUCTION

Dengue haemorrhagic fever is one of the most serious viral diseases transmitted by a day biting mosquito, *Aedes aegypti*. The mosquito resides in and around human houses and seeks a blood source primarily on human (Gubler 1997). Approximately, 50-100 million people around the world are at risk of dengue infection (WHO 2006). Prevention and control of the disease are almost dependent on vector surveillance and vector control methods. Most vector surveillance relied exclusively on indicators that have been designed to detect the presence or absence of mosquito larvae or pupae. In addition, elimination through the source reductions (larval habitats) has been proposed but this approach is somewhat expensive, needs full community participation and is invariably unsuccessful (Kongmee et al. 2004). Furthermore, adult control using synthetic insecticides are commonly used in homes and this could be an important cause of insecticide resistance in the house-haunting mosquito like *Ae. aegypti*.

Development of insecticide resistance in *Ae. aegypti* to synthetic pyrethroids, a commonly used insecticide in dengue control program, has been extensively reported in Thailand (Chareonviriyaphap,1999, Paeporn et al. 2003, Paeporn, 2004, Ponlawat et al, 2005, Jirakanjanakit et al. 2007). Common insecticide resistance mechanisms in insect pests against synthetic pyrethroids include P-450 mediated monoxygenases, elevated non-specific esterases, and reduced sensitivity of sodium ion channels along nerve axons (Oppenoorth 1985, Roberts and Andre 1994, Chareonviriyaphap et al. 2003). In addition, increased levels of glutathione S-transferases (GSTs) have been associated with conferring pyrethroid inhibition in several insects, including *Ae. aegypti* (Grant and Matsumura 1988). Kostaropoulos et al. (2001) reported that elevated GSTs were found to

bind to molecules of several synthetic pyrethroids, compromising effectiveness and toxicity by a sequestering mechanism.

The conventional method for measuring resistance is based mainly on the World Health Organization (WHO) susceptibility test (WHO 1998) which requires a comparatively high number of mosquitoes for testing. This susceptibility test can be complemented by biochemical assays that give additional information on the underlying mechanisms of insecticide resistance. The microplate assay is often used to evaluate enzyme levels in laboratory and field populations (WHO 1991). This test is based on reactions that produce visual color differences. In this study, series of biochemical enzyme assays for detection of resistance and to define the underlined mechanisms involved in pyrethroid resistance in *Ae. aegypti.*

MATERIALS AND METHODS

Mosquito strains

Seven strains of *Ae. aegypti* were used in the study. All mosquitoes used in this study were reared at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bamgkok, Thailand until testing.

1.Chiang Mai strain. This strain was originally collected from the water containers inside homes at Ban Pang Mai Deang Village in Mae Teang District, Chiang Mai Province (19 ^{o}14′ N, 98 ^{o} 82′ E, 600 m above the sea level). This strain was resistance to DDT (Thanispong et al. unpublished data).

2. Kanchanaburi strain. This strain was collected from the water containers inside the homes at Pu Teuy Village in Sai Yok District, Kanchanaburi Province (14^{o} 20′ N, 98 ^{o} 59′ E, 292 m above the sea level) This strain was resistance to permethrin and DDT (Thanispong et al. unpublished data).

3. Khonkaen strain. This strain was obtained from originally from the water storages outside homes in Muang District, Khonkaen Province (16^{o} 25’ N, 102 ^{o} 50′ E 48m above the sea level). This strain was found resistance to permethrin and DDT (Thanispong et al. unpublished data).

4. Nonthaburi strain. This strain was obtained from cement jars outside homes at Muang District, Nonthaburi Province (13^{o} 53′ N, 100 ^{o} 29′ E above the sea level). This strain was resistance to permethrin and DDT (Thanispong et al. unpublished data).

5. Songkhla strain. This strain was originally collected from the water containers inside homes in Muang District, Songkhla Province (7^{o} 11′ N, 100 ^{o} 35′ E, 7 m above the sea level). This strain was resistance to permethrin and DDT (Thanispong et al. unpublished data).

6. Satun strain. This strain was originally collected from the water jars inside the house in Muang District, Satun Province (6^{o} 37′ N, 100^{o} 03′ E, 8m above the sea level). This strain was resistance to permethrin and DDT (Thanispong et al. unpublished data).

7. Bora Bora strain. The Bora Bora strain was obtained from laboratoire de Lutte contre les Insectes Nuisbles, Montpellier, France in October 2005 and was subsequently colonized at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. This strain was found completely susceptible to all insecticides (Thanispong et al. unpublishes data).

Mosquito rearing

Standard procedure for colonizing and rearing *Aedes aegypti* followed established methods (Kongmee et al. 2004). All life stages were maintained under environmental controlled conditions (80%RH, 27 ± 2^{O}C). Larval and adults were reared under a 12:12 h light: dark photophase regime. Upon emergence, all adults were provided cotton pads soaked with a 10% sucrose solution until 12 h before testing. Only F2 generations were used for enzyme assays.

Protein assay

The total protein content of individual *Ae. aegypti* mosquitoes was determined using a BioRad protein assay system (Hercules, California). Individual mosquitoes were homogenized in 0.5 ml of phosphate buffer (0.2 mol, pH 7.0) using a plastic microcentrifuge tube and pestle. Duplicate volumes of 10 µl of each homogenate were transferred to a microplate. A volume of 290 µl of Coomassie Plus Protein Assay Reagent (CPPAR) in distilled water (dH_2O) at a ratio of 1:1 (15 ml CPPAR plus with 15

ml dH_2O) were then added to each well. The plates were incubated at 25 ^oC for 5 min and read at 590 nm end point. The estimated protein content in each mosquito was measured by the method Bradfor (1976).

Monoxygenases assay

The assay for monooxygenase activity was performed according to Vulule et al (1999) with slight modification. Two volumes of 20 µl of each homogenate were transferred to a microplate. Eighty µl of a 0.0625 M Potassium Phosphate ($KHPO_4$) was added with buffer at pH 7.2 in each well. A volume of 0.01 g of 3, 3, 5', 5'-Tetramethly Benzidine (TMBZ) in 5 ml methanol was prepared and a 0.25 M Sodium Acetone ($NaCzH_3Oz$) Buffer(pH 5.0) was added. Then, a 200 µl volume of TMBZ solution was added into each well followed by 25 µl of 3% hydrogen peroxide. The microplate was incubated for 30 min at room temperature (with a cover) and then enzyme levels determined using density values recorded at 630 nm wavelengths. Enzyme activity was determined from cytochrome c standard curve.

Esterase enzyme assay

The reaction was undertaken in Phosphate saline Buffer (PBS) (pH 6.5), containing 90 µl of 1% Triton following the method of Hemingway *et al*. (1998). A volume of 500 µl of 0.3M alpha-naphthyl acetate (or beta-naphthyl acetate) in 2.5 ml 1% triton PBS (pH 6.5) in 7ml distilled water was prepared. A 100 µl volume of this solution was added into each well. The microplate was incubated for 30 min at 25 ^oC. After 30 min the reaction was stopped by adding 100 µl of Fast Garnett solution (0.008 g of fast Garnett salt (PGBC) in 10 ml distilled water). The microplate was read immediately after 10 min at 550 nm wavelength. Absorbance values were converted to nmol naphthol

produced/min/mg protein by using naphthol standard curves and protein values was calculated from analysis of the insect homogenate as above.

Glutathione-S-Transferase assay

Glutathione-S-Transferase activity was assayed following the procedure of Hemingway *et al*. (1998). Duplicates of 10 µl volumes of each homogenate were transferred to a microplate well. A volume of 200µl of glutathione solution [0.06 g of glutathione (GSH) reduced form in 20 ml of 0.1 M Sodium Phosphate Buffer (pH 6.5) plus with 0.013 g of CDNB (1-chloro-2,4-Dinitrobenzene) in 1 ml methanol] was added into each well. The microplate was read at 340 nm wavelengths for 5 min and converted to activity using the published extinction coefficient for this reaction and the protein values calculated from analysis of the insect homogenate as above.

RESULT

Seven strains of *Ae. aegypti* mosquitoes were measured independently for susceptibility to DDT and permethrin using contact bioassay (Table 1). Strong resistance to DDT (>97%) was detected in all strains of *Ae. aegypti*, except those from the reference standard strain (Bora Bora) (Table 1). With permethrin, strong resistance was found in Kanchanaburi (91%) and Nontaburi (95%). Resistance to permethrin in Chaing Mai, Khonkaen, Songkhla, and Satun was 39%, 62%, 28%, and 35%, respectively (Table 1).

An ANOVA found no statistical differences in the total protein content among the six field strains (Table 1). All enzyme activities were measures from 1 mg protein levels (Table1). Approximately 30 specimens were used to perform the three enzyme activities, monooxygenase, GST and non-specific esterases. Significant increase in monooxygenase activity was found in Chiang Mai, Songkhla and Satun strains compared to the Bora Bora standard susceptible strain ($P<0.05$). The greatest activity of monooxygenase was found in Songkhla strain whereas the lowest was observed from Nontaburi strain. In brief, no monooxygenase activity was observed in the three strains from Kanchanaburi, Khonkean and Nontaburi ($P>0.05$). With GSTs activity, Kanchanaburi strain was found significantly increase compared to the other four field strains ($P<0.05$). No such activity was performed in Chiang Mai strain due to the shortages of test specimens (Table 1).

Alpha and beta-esterase activities are quite fluctuate among field strains. Alpha-esterase activities in two strains from Khonkaen and Satun were significantly elevated above the reference susceptible strain ($P<0.05$). There were no significant differences in alpha-esterase activities observed between reference susceptible strain and the three field

strains from Chiang Mai, Kanchanaburi and Nontaburi ($P>0.05$) (Table 2). Beta-esterase activity was found dramatically elevated in Khonkaen strain compared to the standard susceptible strain ($P<0.05$). There was no significant differences in beta-esterase level between standard susceptible strain and the other four field strains, Chiang Mai, Kanchanaburi, Nontaburi, and Satun) ($P>0.05$). Both alpha and beta-esterase activities were found significantly reduced in Songkhla strain compared to the reference susceptible strain ($P<0.05$) (Table 2).

DISCUSSION

Vector control in Thailand has relied mainly on the reduction of human-vector contact by using chemical compounds. Several insecticides have been used in dengue control program in Thailand. DDT was first used for dengue control as an indoor residual spray in Bangkok metropolitan area in (REF XXXX). The following 40 years of intensive use of DDT to control mosquitoes has led to the extensive selection of DDT resistance in *Ae. aegypti* (Chareonviriyaphap et al. 1999, Yaicharoen et al. 2005, Jirakanchanakit et. al. 2007). DDT was withdrawn for public health use in 2000 with the replacement of organophosphates and synthetic pyrethroids (Chareonviriyaphap et al. 1999). Several synthetic pyrethroids are available in the market for controlling household nuisance and vector mosquitoes, i.e. *Ae. aegypti* (Kongmee et al. 2004). These household products (aerosols, mosquito coils, mats, and liquid forms) containing various synthetic pyrethroids such as permethrin, deltamethrin, bifenthrin, *d*-tetramethrin, esbiothrin and allethrin have been widely used in most Thai homes (Paeporn 1996, Jirakanjanakit et al. 2007, Thanispong et al. 2008). Heavy use of synthetic pyrethroids has resulted in insecticide resistance in mosquito population (Chareonviriyaphap et al. 1999).

Mosquito populations may survive the toxic effect of insecticides by four different physiological/biochemical mechanisms, including increased production of monooxygenases, non-specific esterases, GSTs, and reduced sensitivity of sodium ion channels on the nerve membrane ('*kdr*' knockdown resistance), the target site for DDT and pyrethroids (Oppenoorth 1985, Brooke et al. 1999, Chareonviriyaphap et al. 2003). The first three mechanisms of insecticide resistance have been implicated in promoting

detoxification of pyrethroids in resistance insects (Brogdon and McAllister 1998, Vulule et al. 1999). As the whole, quantitative increases in these enzymes, associated with gene amplification or over-expression of target genes, can result in protein overproduction in insects under selection pressure, thus conferring insecticide resistance (Mouches et al. 1990).

Our results indicated that monooxygenase activity increased in all *Ae. aegypti* permethrin resistant strains compared to the susceptible standard strain (Bora Bora). Activity of monooxygenase was associated well with the permethrin resistant levels, i.e. Songkhla strain (73% resistance to peremthrin). Monooxygenases have been reported to associate with pyrethroid resistance in several mosquitoes (Ocampo et al. 2000, Hemingway and Ranson, 2000, Brooke et al. 2001, Chareonviriyaphap et al. 2003). Monooxygenases are a chain of enzymes, with the rate limiting enzyme usually being cytochrome P450 (Nelson et al. 1990). Alterations in this rate-limiting enzyme can dictate levels of resistance to pyrethroids, organophosphates and carbamates using this metabolic mechanism. In our study, there is a 2.2-fold increase in monooxygenase activity in Songkhla strain (73% resistance to permethrin) compared to the control strain (Bora Bora). Significant increases in specific monooxygenase activity were also detected in two strains from Chaing Mai (61% resistance to permethrin) and Satun (65% resistance to permethrin) when compared to those from the reference susceptible strain (0% resistance to permethrin). It seems that elevated monooxygenase activity in *Ae. aegypti* strains accompanied decreased toxicity changes based on permethrin susceptibility results. Although all strains of *Ae. aegypti* demonstrates a strong resistance

to DDT (>97%), level of resistance to permethrin was found fluctuating among *Ae. aegypti* strains. This indicated that cross resistance may not be elevated

Three strains of *Ae. aegypti* from Chaing Mai, Songkhla and Satun were collected from inside homes where household products from permethrin are strongly used. In addition, physiological factors may vary among the strains and this may contribute to differences in insecticide resistance. Based on our results, it appears that monooxygenase is the major contributor to permethrin resistance in Ae. aegypti.

Over-production of alpha and beta-esterase is a common mechanism in those insects resistant to organophosphate and carbamate insecticides (Oppenoorth 1985). In Thailand, resistance to organophosphates and carbamates in *Ae. aegypti* is very common (Chareonviriyaphap et al. 1999, Polawat et al. 2005, Jirakanjanakit et al. 2007). In this study, elevated esterase activities in all strains may associate with organophosphate and carbamate resistance in *Ae. aegypti* mosquitoes. Greatest activity of both alpha and beta esterases in Khonkaen strain may be associated with malathion resistance (Tanispong et al. 2008).

The presence of resistance to DDT in permethrin resistance strains are not elevated GST activity, except the Kanchanaburi strain. In addition, Lumjuan et al. 2005 found GST-2 (Epsilon class GST) in *Ae aegypti* is over expressed in the DDT and permethrin resistance strains from South America, but no found the evidence for increased levels of this GST protein in DDT/pyrethroid-resistant population form Thailand(Lumjuan et al, 2007). All test strains showed resistance to DDT, may be GSTs enzyme catalyze the metabolism of DDT to non-toxic DDE in a dehydrochlorination

reaction which dose not involve a GSH conjugate intermediate (Clark and Shamaan, 1984).

Acknowledgement

Authors would like to thank the Armed Forces Development Command, Sai Yok District, Kanchanaburi Province for supporting activites in the study areas. This project was jointly supported by the Thailand Research Fund (TRF) and the Kasetsart University Research and Development Institute (KURDI), Thailand.

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Table 1. Percent resistance of *Aedes aegypti* strains after exposure to diagnostic concentration of DDT (4%) and permethrin (0.05%) (Thanispong et al. in press)

Strain	No.tested	Percent mortality	
		DDT	Permethrin
Chiang Mai	100	97	39
Kanchanaburi	100	98	91
Khonkaen	100	97	62
Nonthaburi	100	100	95
Songkhla	100	100	27
Satun	100	100	35
Bora Bora	100	0	0

Table 2. Mean values and standard deviation of activities of monooxygenase and gulththione-S-transferase in *Aedes aegypti* strains compared with Bora, the susceptible strain.

Strains	Total protein Mean ± SD mg protein/ml per mosquito(n)	Monooxygenase Mean ± SD nmole product/min/mg protein(n)	GST Mean ± SD nmole DNB/min/mg protein(n)
Chiang Mai	0.0038 ± 0.0012(39)	0.0765 ± 0.0213(39) *	NA
Kanchanaburi	0.0058 ± 0.0004(40)	0.0603 ± 0.0040(40)	0.0857 ± 0.0396(39)
Khonkaen	0.0061 ± 0.0006(40)	0.0568 ± 0.0053(40)	0.0521 ± 0.0217(39) *
Nonthaburi	0.0060 ± 0.0004(40)	0.0543 ± 0.0028(40)	0.0543 ± 0.0238(39) *
Songkhla	0.0022 ± 0.0006(39)	0.1241 ± 0.0351(39) *	0.0397 ± 0.0198(28) *
Satun	0.0054 ± 0.0007(40)	0.0701 ± 0.0061(40) *	0.0348 ± 0.0221(37) *
Bora	0.0067 ± 0.0003(40)	0.0538 ± 0.0034(40)	0.0795 ± 0.0360(36)

* Significant increase in mean differences compared to the Bora Frence Polynesia susceptible strain ($p<0.005$, Fishher's least significant difference test)

NA: Not Applicable.

Table 3. Mean values and standard deviation of activities of non-specific esterase (α and β esterases)in *Aedes aegypti* strains compared with the Bora, the susceptible strain.

Strains	α Esterase mean ± SD nmole α naphthol/min/mg protein(n)	β Esterase Mean ± SD nmole β naphthol/min/mg protein(n)
Chiang Mai	0.0845 ± 0.0276(39)	0.0836 ± 0.0189(39)
Kanchanaburi	0.1040 ± 0.0109(40)	0.0805 ± 0.0055(40)
Khonkaen	0.2892 ± 0.1173(40) *	0.2171 ± 0.0994(40) *
Nonthaburi	0.1058 ± 0.0108(40)	0.0733 ± 0.0045(40)
SongKhla	0.0561 ± 0.0146(39) *	0.0282 ± 0.0070(39) *
Satun	0.1126 ± 0.0143(40) *	0.0860+0.0066(40)
Bora	0.0895+0.0098(40)	0.0752+0.0066(40)

* Significant increase in mean differences compared to the Bora Frence Polynesia susceptible strain($p<0.005$, Fishher's least significant difference test)

Journal of the American Mosquito Control Association

BEHAVIORAL RESPONSES OF CATNIP (*NEPETA CATARIA* L.) BY TWO SPECIES OF MOSQUITOES, *AEDES AEGYPTI* (L.) AND *ANOPHELES HARRISONI* HARBACH AND MANGUIN, IN THAILAND

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ABSTARCT. An investigation of the biological effect of catnip oil (*Nepeta cataria* L.) on the behavioral response of field collected *Ae. aegypti* and *An. harrisoni* was conducted using an automated excito-repellency test system. *Aedes aegypti* showed significantly higher escape rates from the contact chamber at 5% catnip oil compared to other concentrations ($P < 0.05$). With *Anopheles harrisoni*, a high escape response was seen at 2.5% catnip oil from the contact chamber, while in the noncontact chamber, a higher escape response was observed at a concentration of 5%. In summary, the behavioral action of catnip oil was evaluated on two field caught mosquito species. Results showed that this compound exhibits both irritant and repellent actions.

KEY WORDS Behavioral responses, irritancy, repellency, *Aedes aegypti*, *Anopheles harrisoni,* Catnip, *Nepeta cataria* L.

INTRODUCTION

Many areas of the world are at risk for a wide variety of arthropod-borne diseases with millions of cases each year (World Health Organization (WHO) 2007). A significant growth in human population, demographic movement from rural to more crowded urban areas and an increase in tourism-based facilities contributing to an increasing trend in disease transmission. Prevention of these diseases remains almost entirely dependent on various methods of vector control. Control of the vector by insecticides remains the most important means of reducing disease transmission and protection from mosquito bites (Reiter and Gubler 1997, Roberts et al. 1997, WHO 1999).

Chemicals protect humans from the bite of mosquitoes through three different actions: irritation after making contact, repelling prior to contact, or by killing the insects (toxicity) (Grieco *et al*., 2007). Most research has focused on the toxic function of chemicals whereas comparatively few have concentrated on non-toxic chemical actions. Non-toxic action can be categorized into two distinct mechanisms, contact irritancy and noncontact repellency. Irritant responses result from physical contact with chemical-treated surfaces, whereas repellency is an avoidance response devoid of making actual contact with the chemical (Chareonviriyaphap *et al*., 1997; Roberts *et al*., 1997). Much of the early research on behavioral responses was concentrated on the synthetic chemicals (Evans, 1993, Chareonviriyaphap *et al*., 2001; Kongmee *et al*., 2004; Grieco *et al*., 2005, 2007; Pothikasikorn *et al*., 2005, 2007). In Thailand, synthetic compounds, including organophosphates, carbamates, and pyrethroids have been used with varying degrees of success in national public health vector control programs (Reiter and Gubler, 1997). Since 1994, the Ministry of Public Health (MOPH) in Thailand has recommended the use of deltamethrin in

79 public health to control malaria and dengue haemorhagic fever. Recent studies have
80 reported the spread of deltamethrin resistance in several field *Culex quinquefastiatus*
81 and *Ae. aegypti* populations from Thailand (Somboon *et al*., 2003; Jirakanjanakit *et*
82 *al*., 2007; Sathantriphop *et al*., 2006). Alternative compounds or new methods of
83 controlling mosquito vectors are needed. One source of alternatives lies in botanical
84 compounds which are commonly used as "insect repellents". These compounds are
85 effective, safe and increasingly available for domestic use against indoor and outdoor
86 biting mosquitoes and arthropod pests.
87 One option for preventing the transmission of a vector-borne pathogen to a
88 host is the use of a tropical insect repellents. N, N-diethyl-3-methylbenzamide
89 (DEET), one of the most common insect repellents, is effective at protecting humans
90 from mosquito bites (Qiu *et al*., 1998). Recently, several botanical extracts, such as
91 eucalyptus (*Eucalyptus citriodora* Hook), citronella grass (*Cymbogon nardus* Rendle),
92 thyme (*Thymus vulgaris* L.), clove (*Syzygium aromaticum* L.), and catnip (*Nepeta*
93 *cataria* (L.)) were tested as alternative tropical mosquito repellents (Barnard, 1999;
94 Tawatsin *et al*., 2001; Zhu *et al*., 2006). Among these, the essential oil from catnip
95 proved to be a safe and promising insect repellent. This oil contains two stereoisomer
96 forms of nepetalactone (E,Z and Z,E isomer). The two stereoisomers have been
97 reported to function as insect repellents against 13 families of insects (Eisner, 1964).
98 The E,Z-nepetalactone form showed to be a stronger repellent against German
99 cockroaches than the Z,E-nepetalactone one (Peterson *et al*., 2002). Catnip oil was
100 also reported to be a good repellent compound for short term action against house
101 flies and American cockroaches (Schultz *et al*., 2004). Additionally, catnip oil was
102 found to be a good spatial repellent compound in protecting humans from mosquito
103 bites for at least six hours past treatment (Bernier *et al*., 2005; Zhu *et al*., 2006).

However, no investigation has been performed to identify the two distinct categories of behavioral responses, irritancy and repellency, of mosquitoes to catnip oil. We investigated the active properties of catnip oil using two species of mosquitoes, *Aedes aegypti*, a vector of dengue and *Anopheles harrisoni*, a vector of malaria in Thailand. Irritant and repellent responses were quantitatively assessed using an automated excito repellency (ER) test system (Tanasinchayakul *et al*., 2006).

MATERIALS AND METHODS

Mosquito populations: Populations of *Ae. aegypti* and *An. harrisoni* were used in this study. *Aedes aegypti* was established from immature stages whereas *An. harrisoni* were collected by cow bait from 1800-2400 hours between April-September 2006. For cow baited collections, one cow was placed in a net trap and mosquitoes were collected from inside the net for 15 min/hour. The captured mosquitoes were kept in mosquito cups and provided with 10% sugar solution. *Anopheles harrisoni* mosquitoes were identified using the morphological keys of Rattanarithikul *et al*., (2006) the following morning.

Mosquito conditioning: Unfed three to five day- old female *Ae. aegypti* mosquitoes were used in this study. All female mosquitoes were deprived of sucrose solution and water 12 h before testing. With *Anopheles harrisoni*, only field collected mosquitoes were used for to testing and they were not starved since they were active and host – seeking at time of capture.

Insecticide impregnated papers: Different concentrations (1%, 2.5%, 5% and 10%) of essential oil from catnip were impregnated onto test papers measuring 12 by 15 cm for susceptibility tests and 15 by 17.5 cm for excitio-repellency test, following the standard WHO procedure (WHO 1998). Catnip oil was received from the Chemicals Affecting Insect Behavior Lab (CAIBL), United States Department of Agriculture, Beltsville, Maryland. Nepetalactones (E,Z ~ 48% and Z,E ~ 40% isomers) and β-caryophyllene (~9%) are the major constituents in catnip oil. *E,Z* and *Z,E* nepetalactone isomers were 99% chemically pure and 95-98% stereo-chemically pure according to capillary gas-liquid chromatography (Chauhan and Zhang 2004). The structures of nepetalactone isomers were confirmed by GC-mass spectroscopy

(GC-MS) and nuclear magnetic resonance spectral analysis (Eisenbraun et al. 1980). Racemic nepetalactone was formulated by mixing 1:1 ratio of *E,Z* and *Z,E*-nepetalactones, and homogeneity was confirmed by GC.

Dose response assay: The standard WHO tarsal contact test (WHO, 1996) was used in this study. For each test, five cylinders (two for controls and three for treatments) were used. Control cylinders contained filter paper impregnated with solvent (acetone) whereas, treatments contained filter paper impregnated with the different concentrations of catnip oil in solvent. For each test population, 25 female mosquitoes were exposed for 1 h to catnip oil. Following test and control exposures, knockdown was recorded and all mosquitoes transferred to separate clean holding containers and provided with 10% sucrose solution. Total knockdown and mortality was recorded after 24 h post-exposure. Each matched test-control series was repeated 4 times per test population

Excito-repellency tests: In this study, I used an automated field excito-repellency test system as described in a recent publication (Tanasinchayakul *et al*., 2006). The main supporting structure was fabricated using stainless steel, each side wall measuring 23x23 cm^2. The chamber walls were constructed with an aluminum side tongue and groove configuration on adjoining ends which made the assay easier and faster to set up and disassemble for transportation and storage. The frame of the inner chamber was constructed of 22.5x19 cm stainless steal beams. The frame included metal holders for securing test papers on either of two sides for the dual purpose of providing a contact or a noncontact exposure configuration. For noncontact tests, a thin sheet of fine mesh iron screening secured on the opposite side of the test paper allows for a 1.5 cm gap that prevented mosquitoes from makings tarsal contact with the test paper. A Plexiglas^{TM} panel at the rear of the chamber was

166 equipped with a 11.5 cm diameter hole sealed with overlapping dental dam to prevent
167 escape during handling. Each assay chamber contained a forward exit portal
168 (13.5x2cm) connected to a funnel projecting from the box (Figure 11).
169 The photoelectric sensor (FX-301, SUNX Limited, Aichi, Japan) detects and
170 counts escaping mosquitoes (Fig. 1, #2). The sensor has two operational mode
171 switches (#3), a jog switch, and a MODE key require for operating the system. To
172 record data during testing, the DATA Logger CL 123 (#5) is connected to the
173 photoelectric sensor and records values at three signal channels, one analog and two
174 digital. The DATA Logger CL123 is a small, battery-operated device (#4) with
175 software to record and transfer data in tabular and graphic form to the computer
176 system (#6) (Tanasinchayakul *et al*., 2006).
177
178 Each test series consisted of two chemically-treated test chambers and two
179 paired control chambers fitted with appropriate papers. Female mosquitoes were held
180 in 473 cm^3 (16 fl. oz.) capacity cups for approximately 8-10 h prior to testing and
181 were provided with only water soaked cotton pads. For each test chamber, 15
182 mosquitoes were carefully introduced into each of the four chambers using a mouth
183 aspirator. Mosquitoes were allowed a 3 min adjustment period inside the test
184 chamber prior to opening the escape funnel to begin counting. A receiving cage was
185 connected to the exit portal for collecting exiting mosquitoes. Escaping mosquitoes
186 were recorded at 1 min intervals for a period of 30 min. All tests were conducted
187 between 0800-1600 hours and replicated 4 times per test population.
188
189 Immediately following the 30-min exposure, the number of dead or
190 knockdown specimens remaining inside the chamber and those that had escaped into

191 the receiving cage were recorded for each of the four chambers. Also, all live
192 specimens that had escaped or remained inside the test chamber were transferred to
193 clean holding cups and provided with a 10% sucrose solution. All test mosquitoes
194 were maintained separately in lots for 24 h post-exposure at which time mortality was
195 recorded.

196 *Data analysis:* In contact susceptibility tests, control mortalities exceeding
197 5% were corrected and adjusted for determining baseline susceptibility in each test
198 population (Abbott, 1925). For excito-repellency data, a life table survival analysis
199 approach was used to estimate mosquito escape rates and compared differences in
200 mosquito escape rates between test populations and insecticides (Roberts *et al*., 1997).
201 Survival analysis provides a robust statistical treatment of sequential excito-
202 repellency data, and relative to other quantitative methods describing behavioral
203 avoidance, survival curves minimize loss of valuable information while estimating
204 temporal mosquito escape probability (Roberts *et al*., 1997). The time in minutes for
205 25, 50 and 75% of the test population to escape was estimated using life table analysis
206 and these estimates were used as the "escape time" summary statistics (ET_{25}, ET_{50},
207 and ET_{75}).

208 A log-rank method is used to compare patterns of escape behavior. This test is
209 designed to detect differences between survival curves that result when the death (or
210 escape) rate in one group is consistently higher than the corresponding rate in the 2nd
211 group and the ratio is consistent over time. With excito-repellency data, the basic idea
212 underlying the log-rank test involves examining escape observations by 1-min
213 intervals. The log-rank method was proposed by Mentel and Haenzel (1959). The
214 discriminating level for statistical significance was set at 0.05%.
215

RESULTS

Dose response assay: Bioassays were conducted to obtain the dose response mortality on test populations of two mosquito species (*Ae. aegypti* and *An. harrisoni*), collected from Kanchaburi Province, western Thailand, using the WHO susceptible test for adult mosquitoes (WHO 1998). From preliminary screening, three concentrations of catnip oil (1%, 5% and 10% for *Ae. aegypti* and 1%, 2.5% and 5% for *An. harrisoni*) were selected for the bioassay and behavioral assay. Catnip oil exhibited low toxicity for the two test populations (Table 1). Percent mortality of two test populations was comparatively low, regardless of test concentrations. Mortality varied between 0-3% for *Ae. aegypti* and 0-7% for *An. harrisoni* (Table 1). With *Ae. aegypti*, 94% percent knockdown at 1 hour was observed from 5% catnip oil and a 43% knockdown at 10% catnip oil whereas a 55% percent knockdown of *An. harrisoni* was observed from 5% catnip oil.

Excito-repellency test: Percent escape responses of the two test populations exposed to different concentrations of catnip oil were recorded in contact and noncontact trials (Tables 2 and 3). With *Ae. aegypti* in contact trials, the greatest escape responses were observed from 5% catnip oil (80%) whereas the lowest escape responses were observed from 1% catnip oil (35%). At the highest concentration (10%), a high percentage of knockdown specimens was observed from those that had escaped (21.21%) and those that remained in the test chamber (40%). In noncontact trials, the highest escape responses were observed from 10% catnip oil (53.57%) and the lowest was seen in 1% catnip oil (31.03%). Percent knock down was not as high as that observed from the contact trials. The highest knockdown rate was seen from those nonescaped specimens at 10% catnip oil (34.61%) whereas the percent of

knockdown was comparatively low for those females that had escaped, ranging from 0-6.67%. With *An. harrisoni* in contact trials, a marked escape response was observed at 2.5% catnip oil (71.19%), compared to 5% catnip (58.62%) and 1% catnip (16.95%). In noncontact trials, escape responses were comparatively high at 2.5% catnip oil (63.16%) and 5% catnip oil (67.87%) compared to 1% catnip oil (15%). In general, high percent knockdown was observed at the higher concentrations of catnip oil. Contact trials produced higher numbers of knock down specimens than those from noncontact trials. The greatest percent of knockdown was observed from females failing to escape at 5% catnip oil in contact trials (62.50%).

Twenty four hour mortalities of *Ae. aegypti* and *An. harrisoni* females after exposure in contact and noncontact trials with catnip oil are given in Tables 6 and 7. Lower mortality rates were recorded for *Ae. aegypti* as compared to *An. harrisoni* when tested against different concentrations of catnip oil. With *Ae. aegypti* in contact trials, percent mortalities of escape and nonescape females varied from 0-8%. No mortality was observed from non-contact trials for all test concentrations (Table 2). With *An. harrisoni* in contact trials, the percent mortality of nonescaping females was high (2.04-20.83%) compared to escaping females (9.52-14.70%). Similarly, high mortality rates were observed from noncontact trials in both escaping and nonescaping females, ranging from 2.78 to 10.53% for escaping and 1.96-16.67% for nonescaping females (Table 3).

Escape times (ET) from chambers treated with different concentrations of catnip oil, measured in 1-min intervals, are designated based on the percentage of the test population escaping, 25% (ET25), 50% (ET50) and 75% (ET75), the treated chamber within 30 min (Table 4). For 1% catnip oil, the *Ae. aegypti* test population had an ET25 value of 15 min in contact trials and of 18 min in noncontact trials

266 whereas an ET25 value could not be calculated for *An. harrisoni* in both contact and
267 noncontact trials due to the lack of mosquito movement. At 2.5% catnip oil, ET25 and
268 ET50 for *An. harrisoni* values were 4 and 9 min, respectively, for contact trials and 3
269 and 11 min, respectively, for noncontact trials. At 5% catnip oil, the ET25 value was
270 2 min for *Ae. aegypti* and 4 min for *An. harrisoni* in contact trials whereas the ET25
271 values in noncontact trials were 8 and 6 min for *Ae. aegypti* and *An. harrisoni*,
272 respectively. The ET50 value was also low (4 min) for *Ae.aegypti* whereas it was
273 comparatively high for *An. harrisoni* in contact (14 min) and noncontact trials (12
274 min) (Table 4). At 10% catnip oil, *Ae. aegypti* had a low ET25 values of 2 min in
275 contact trials and 3 min in noncontact trials whereas ET 50 values of 16 and 20 min in
276 contact and noncontact trials, respectively. ET75 values for both contact and
277 noncontact trials at different concentrations of catnip oil could not be estimated
278 because too few specimens departed the exposure chamber (Table 4).
279 Contact vs. noncontact escape responses of *Ae. aegypti* to 1%, 5% and 10%
280 catnip oil were compared (Table 5). Escape probabilities in contact and noncontact
281 trials were significantly higher than in controls for all cases *($P < 0.05$),* except for 1%
282 catnip oil when the contact trials were not significantly different from the control.
283 Significant differences in escape responses were observed in 5% catnip oil between
284 contact and noncontact trials *($P < 0.05$).* Likewise, the contact vs. noncontact escape
285 response of *An. harrisoni* to 1%, 2.5% and 5% catnip oil were compared. No
286 significant differences in escape response were observed in all pairs when contact
287 trials was compared to noncontact trial, regardless of test concentration *($P > 0.05$).*
288 Statistically significant differences in escape responses were observed at 2.5% and 5%
289 catnip oil when control was compared to contact and noncontact trials.

290 Statistical comparisons between 2 doses of catnip oil (1%, 5% and 10% for
291 *Ae. aegypti* and 1%, 2.5% and 5% for *An. harrisoni*) in contact and noncontact trials
292 demonstrated that there were significant differences between all pairs ($P < 0.05$),
293 except when catnip oil at 2.5% was compared to 5% in *An. harrisoni* test population
294 ($P > 0.05$) (Table 6).
295 Fig. 2 and 3 show the proportions of mosquitoes remaining in the exposure
296 chambers at different test concentrations. These proportions are used to show patterns
297 of escape rates. The patterns are used to compare escape probabilities between
298 contact and noncontact trials for *Ae. aegypti* (Fig. 2) and *An. harrisoni* (Fig.3). A
299 higher escape response of *Ae. aegypti* was observed when exposed to 5% catnip oil
300 in contact trials compared to non-contact trials. Significantly lower escape responses
301 were found at 1% and 10% catnip oil in both contact and non-contact trials when
302 tested against *Ae. aegypti* (Fig. 2). The patterns of escaped females of *An. harrisoni*
303 were significantly greater at 2.5% and 5% catnip oil than at 1% catnip oil (Fig. 3).
304
305

DISCUSSION

Understanding the behavioral responses of mosquito vectors, especially avoidance behavior to test compounds, is of paramount importance to any mosquito control program. There have been numerous attempts to accurately measure the behavioral responses of mosquitoes to insecticides using several types of excito-repellency test system (Roberts *et al*., 1984; Chareonviriyaphap *et al*., 1997; Rutledge *et al*., 1999; Sungvornyothin *et al*., 2001). Due to the inherent complexities of accurately measuring excito-repellency in mosquitoes, no test method had been adequate and fully accepted. No test recommended by the WHO will discriminate between the two types of behavioral responses, contact irritancy and noncontact repellency (Roberts *et al*., 1984). However, an experimental test system described by Roberts *et al*. (1997) addresses a number of deficiencies attributed to behavioral test systems. This test system was first used to test the avoidance behavior of *An. abimanus* from Belize, Central America (Chareonviriyaphap *et al*., 1997). This prototype test system has been modified further into the collapsible chamber designed for the greater ease of use (Chareonviriyaphap *et al*., 2002) and has proved valuable in the evaluation of behavioral responses in several laboratory and field populations of mosquitoes in Thailand and Indonesia (Chareonviriyaphap *et al*., 2004; Kongmee *et al*., 2004; Pothikasikorn *et al*., 2005, 2007; Muenworn *et al*., 2006; Chareonviriyaphap *et al*., 2006). However, this system was still cumbersome and required a minimum of two investigators to observe and record data during the 30-min testing period.

Recently, an assay for evaluating the three types of chemical actions, contact irritancy, spatial repellency and toxicity, in adult mosquitoes was developed (Grieco

331 *et al*., 2007), but this system was not designed as a field-adaptable assay. To
332 overcome these technical problems when conducting field studies, a more compatible
333 design was developed and is referred to as an "automated, field-compatible device for
334 testing excito-repellency behavior (Tanasinchayakul *et al*., 2006). This system
335 consists of two major modifications from the previous model: a substantial reduction
336 in the size of the test box and the use of an electronic sensor for automated counting
337 of mosquitoes as they departed the test chamber through the opened gate into the
338 external holding box. This device has been successfully used to measure the
339 behavioral responses of *Ae. aegypti* from Bangkok, Thailand to deltamethrin
340 (Tanasinchayakul *et al*., 2006). Moreover, an automated excito repellency test
341 system provides the advantage it makes it easier for automatically counting escaping
342 mosquitoes from the chamber and recording data by computer system. This system
343 can eliminate error from confounding factors by human such as human odor, body
344 heat, and carbon dioxide. An additional advantage is the system requires only one
345 investigator to observe and collect escaped mosquitoes from the receiving cage.
346 In this study, we observed the behavioral responses of two field collected mosquito
347 species, *Ae. aegypti* and *An. harrisoni* collected from Kanchanaburi, western
348 Thailand, to catnip oil, a promising plant derived compound from catnip (Peterson
349 and Coat, 2001).

350 Chemicals protect human from the bite of mosquitoes in three different ways,
351 irritate, repel or kill the mosquitoes (Grieco *et al*., 2007). In this study, *Ae. aegypti*
352 demonstrated clear behavioral escape responses to catnip oil in both contact and
353 noncontact trials compared to the control trials. Greater contact irritancy escape
354 responses from 5% catnip oil were documented in *Ae. aegypti*, compared with 1% and
355 10% catnip oil. All tests showed mosquitoes successfully departed treated surfaces

356 and chambers before receiving a lethal dose of test compound. Higher knockdown
357 rates were observed at the higher doses, regardless of test condition, indicating a
358 strong vapor from the test chemical affected the test specimens. However, a high
359 percent of recovery (>92%) was observed, indicating no toxic action of catnip oil.
360 Recently, there were several studies to examine the repellency effect of catnip oil in
361 mosquito species and other insects (Bernier *et al*., 2005; Chauhan *et al*., 2005;
362 Peterson and Coat, 2001; Schultz *et al*., 2004; Webb and Russell, 2007; Zhu *et al*.,
363 2006). With *An. harrisoni*, contact irritancy and noncontact repellency were quite
364 high, especially at 2.5% catnip. Knockdown rates were somewhat greater at the higher
365 doses with greater percent mortality of both contact and noncontact mosquitoes,
366 suggesting *An. harrisoni* were more sensitive to the toxic action of catnip oil.
367 The protection time of catnip oil has been reported elsewhere. Catnip oil was
368 shown to be an effective repellent up to 6 hours against *Ae. albopictus* (Zhu *et al*.,
369 2006). In Australia, catnip oil demonstrated mean protection times, ranging from 0
370 min for *Ae. aegypti* up to 240 ± 60 min for *Cx. quinquefasciatus* (Webb and Russell,
371 2007). In contrast, catnip oil showed a long protection time to *Ae.vigilax, Cx.*
372 *annulirostris* and *Cx. quinquefasciatus* compared to other potential natural plant
373 extracts (Webb and Russell, 2007). In this study, the protection time of catnip oil on
374 mosquito populations was not evaluated. However, we found that catnip oil has
375 strong irritant and repellent actions on mosquito test populations as indicated by the
376 comparatively low escape time (ET).
377 In summary, several studies have investigated mosquitoes repellents derived
378 from plant extracts (Tawatsin *et al*., 2001; Suwonkerd and Tantrarongroj, 1994), but
379 none have described contact irritant and non contact repellent actions. With the
380 existence of a field-automated excito-repellency test system, the two behavioral

381 actions of catnip oil on two field collected mosquito species were quantitified. The
382 resulting data will help better understand how catnip oils act against mosquitoes and
383 how they might be used in the future.
384

ACKNOWLEDGEMENTS

We thank the Thailand Research Fund (TRF), the Kasetsart University Development Institute (KURDI), and Deployed War Fighter Protection (DWFP) Program for financial support. Special thanks to Mr. Pisit Poolprasert for statistical analysis.

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WHO [World Health Organization]. 2007. Health topic [Internet]. [accessed November 20, 2007]. http://www.who.int/topics/en/.

549 Table 1. Percent mortality of *Aedes aegypti* and *Anopheles harrisoni* populations from
550 Kanchanaburi expose to different doses of catnip oil using standard WHO
551 susceptibility test procedures.

552

553 Mosquito	Dosage	Number Tested	%KD	% Mortality ± SE
554				
555				
556 *Ae. aegypti*				
557	1%	100	0	0
558	5%	100	4	0
559	10%	100	43	3 ± 0.75
560 *An. harrisoni*				
561	1%	100	0	0
562	2.5%	100	3	3 ± 0.48
563	5%	100	55	7 ± 0.63

564

Table 2. Escape response and percent mortality of female *Aedes aegypti* from Kanchanaburi after contact and non-contact with catnip oil in excito-repellency tests.

Conditions	Dosage	Treatment Chamber				Control Chamber		% Mortality			
								Treatment		Control	
		No. Tested	% Esc	% KD Esc	% KD Not Esc	No. Tested	% Esc	Esc[1]	Not[2] Esc	Esc	Not Esc
Contact											
	1%	60	35.00	0	0	56	21.43	0	0	0	0
	5%	55	80.00	6.81	18.18	58	13.79	2.27	0	0	0
	10%	58	56.90	21.21	40.00	58	18.97	3.03	8.00	0	0
Non-contact											
	1%	58	31.03	0	0	57	14.04	0	0	0	0
	5%	55	40.00	0	9.09	59	10.17	0	0	0	0
	10%	56	53.57	6.67	34.61	59	11.86	0	0	0	0

[1] Esc = Escaped mosquitoes

[2] Not Esc = Not Escaped mosquitoes

Table 3. Escape response and percent mortality of female *Anopheles harrisoni* from Kanchanaburi after contact and non-contact with catnip oil in excito-repellency tests.

Condition	Dosage	Treatment Chamber				Control Chamber		% Mortality			
								Treatment		Control	
		No. Tested	% Esc	% KD Esc	% KD Not Esc	No. Tested	% Esc	Esc[1]	Not[2] Esc	Esc	Not Esc
Contact											
	1%	59	16.95	0	0	56	1.79	0	2.04	0	0
	2.5%	59	71.19	11.36	18.18	59	8.47	9.52	17.64	0	0
	5%	58	58.62	35.29	62.50	58	8.62	14.70	20.83	0	0
Non-contact											
	1%	60	15.00	0	0	55	1.82	0	1.96	0	0
	2.5%	57	63.16	0	9.09	58	10.34	2.78	14.04	0	0
	5%	56	67.86	5.26	38.89	54	5.56	10.53	16.67	0	0

[1] Esc = Escaped mosquitoes

[2] Not Esc = Not Escaped mosquitoes

Table 4. Escape time (ET) in minutes for 25%, 50% and 75% of 2 species of field Mosquito to escape treated chambers with catnip oil (*Nepeta cataria*)

Mosquitoes	Dosage	Contact			Non-contact		
		ET 25	ET 50	ET 75	ET 25	ET 50	ET 75
Ae. aegypti							
	1%	15	-[1]	-	18	-	-
	5%	1	4	16	8	-	-
	10%	2	16	-	3	20	-
An. minimus							
	1%	-	-	-	-	-	-
	2.5%	4	9	-	3	11	-
	5%	4	14	-	6	12	-

[1] Very few mosquitoes escaped from exposure chambers so that the ET values could not be estimated for a 30-min exposure period.

Table 5. Comparison of escape response between paired control and non-contact trials, control and non-contact trials, and paired contact and non-contact trials for 2 species of field mosquito with catnip oil (*Nepeta cataria*) in excito-repellency tests.

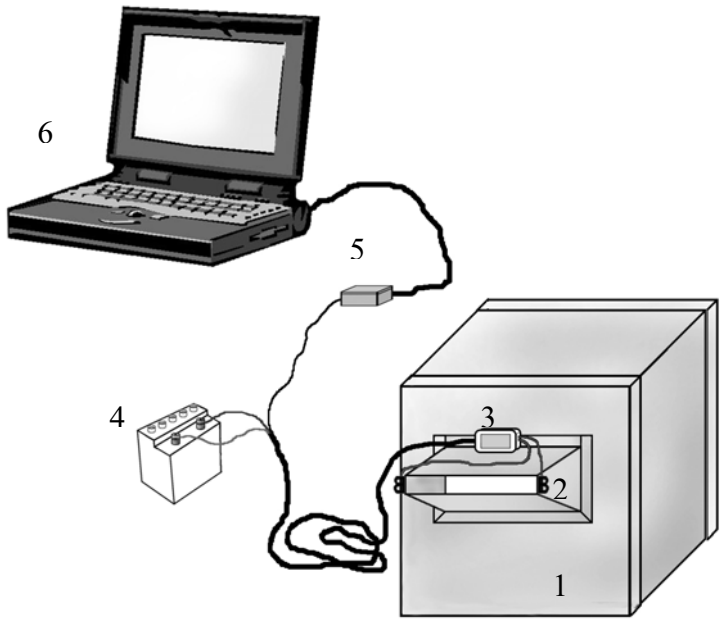
Mosquitoes	Dosage	Control[1] vs. Non-contact (*P*)	Control vs. Contact (*P*)	Contact[1] vs. Non-contact (*P*)
Ae. aegypti	1%	0.040*	0.131	0.558
	5%	0.000*	0.000*	0.000*
	10%	0.000*	0.000*	0.593
An. harrisoni	1%	0.169	1.000	0.998
	2.5%	0.000*	0.000*	0.335
	5%	0.000*	0.000*	0.568

[1] The * identifies results of log-rank tests with statistically significant (0.05 level of probability) diffences in escape response between paired of trials.

Table 6. Comparison of escape response between dasage for 2 species of field mosquito with catnip oil (*Nepeta cataria*) after contact and non-contact in excito-repellency tests.

Mosquitoes	Dosage	Contact trial (*P*)	Non-contact trial (*P*)
Ae. aegypti			
	1% vs. 5%	0.000*	0.008*
	1% vs. 10%	0.012*	0.000*
	5% vs. 10%	0.030*	0.009*
An. minimus			
	1% vs. 2.5%	0.000*	0.000*
	1% vs. 5%	0.000*	0.000*
	2.5% vs. 5%	0.128	0.858

Fig. 1



708 Fig. 2

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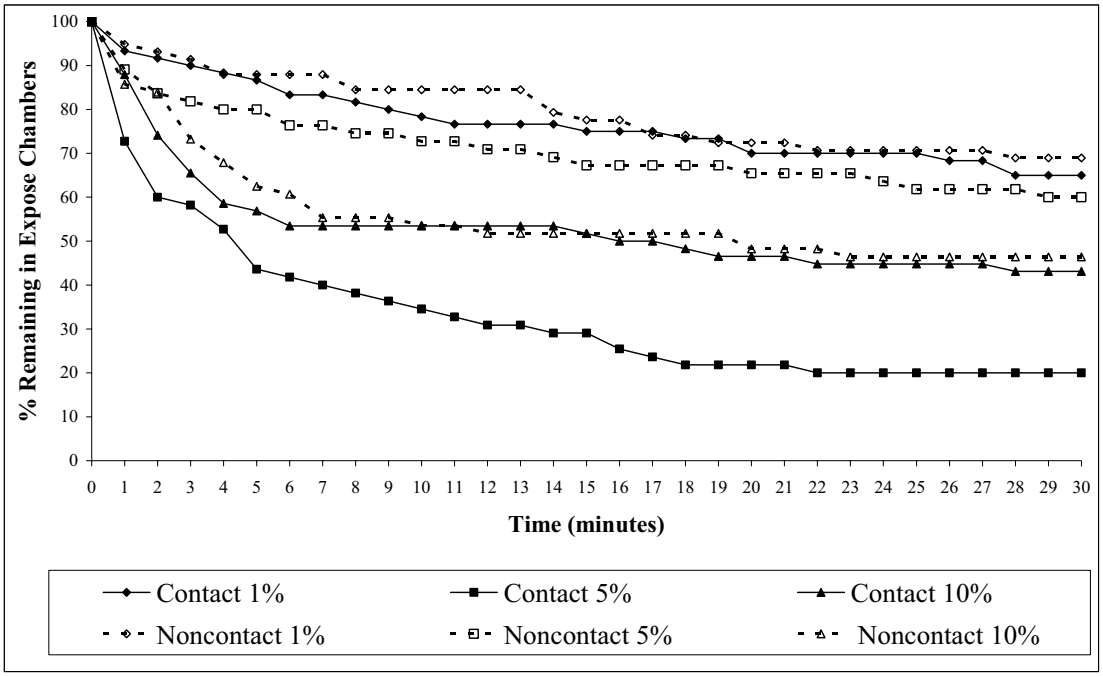

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725 Fig. 3

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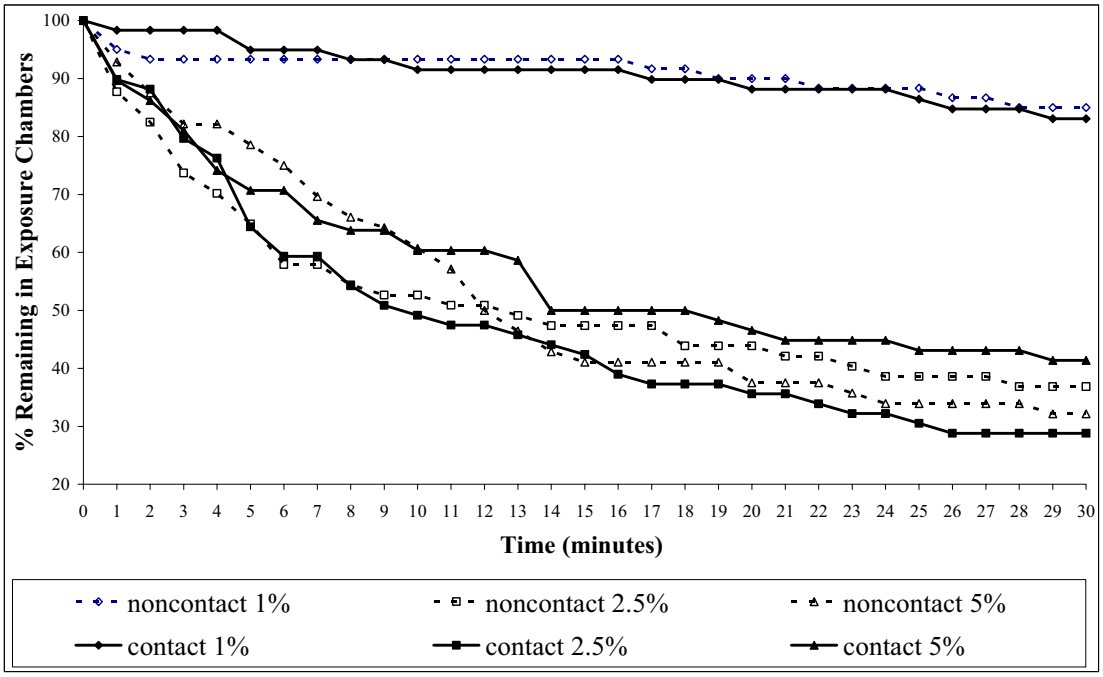

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FIGURES LEGEND

Fig. 1 An automated excito-repellency test system.1 = excito-repellency chamber, 2 = photoelectric sensor, 3 = operation mode, 4 = battery, 5 = DATA Logger CL 123, 6 = Computer system

Fig. 2 Comparison of escape pattern of female *Aedes aegypti* from Kanchanaburi in contact and non-contact trials exposed to different dosage of catnip oil (*Nepeta cataria*).

Fig. 3 Comparison of escape pattern of female *Anopheles harrisoni* from Kanchanaburi in contact and non-contact trials exposed to different dosage of catnip oil (*Nepeta cataria*).

GIS Tool and Molecular Identification for the Study of the Minimus Complex, a Vector of Malaria in Thailand

Introduction

Geographical information system (GIS) and remote sensing technology are useful tools in the study of arthropod borne diseases such as malaria. These technologies, in combination with field data, allow researchers the ability to identify potential vector breeding sites and target control intervention methods. In Thailand, species within the *Anopheles minimus* complex plays a major role in malaria transmission. Previous research characterizing the habitats of *An. minimus* and *An. harrisoni* have shown differences in larval habitat distribution based on land cover, however, the satellite imagery used did not include multi-temporal data. In addition, *An. minimus* has recently been reported at the proposed study site where previously it was not collected. This indicates potential changes in the surrounding environment that will re-examine the correlation between land use and the distribution of *An. minimus* and *An. harrisoni* larval habitats for positive and negative locations. This will require establishing a database of habitat and mosquito attributes as well as the acquisition and processing of satellite imagery.

Objectives

The objectives were to characterize the specific environmental variables that found in breeding site of *An. harrisoni*, but not found in breeding site of *An. minimus*, to observe the larval density of *Anopheles minimus* and *Anopheles harrisoni* during different seasons and to identify the two species of the Minimus complex, *Anopheles minimus* and *Anopheles harrisoni* collected from two different ecological breeding habitats by morphological and molecular identification

Materials and Methods

Study area

The study area was in a malaria-endemic area of western Thailand near the Myanmar border and covers the 2 villages of Ban Pu Teuy and Ban Bong Ti Noy in Sai Yok District,

Kanchanaburi Province. Ban Pu Teuy was the potential site for *An. harrisoni* and Ban Bong ti Noy was the potential site for *An. minimus*.

Larvae collection

Larvae were collected from breeding habitats in Ban Pu Teuy and Ban Bong Ti Noy in Sai Yok District, Kanchanaburi Province. Breeding habitat was separated to the main stream and others (stream pools, swamps, pits, etc.) in both study area. Larvae were reared to adults for species identification by morphological and molecular techniques. Geographical and ecological data were recorded for each of the collections. The coordinates for each larval habitat were recorded using a Global Positioning System unit.

Larvae were collected during December 2006- November 2008, every month at Ban Pu Teuy and every two month at Ban Bong Ti Noy. Distance between sampling point about 30 m. The collection teams have two persons per team. Ten dips per person per point, 20 dips were taken in each point (3 m long from point). The number of larvae life stage was recorded for each point.

Environmental variables

The data concerning geography and ecology were recorded from each point such as the depth, width, temperature, flow rate of water current, transparency (using secchi disc to determine transparency of water) and water analysis , these value of water from each point were measured by EZDO 7021 meter. Classified vegetation was coverage, emergent and debris. The density of vegetation variables at sample point were score as 0-3. For coverage vegetation, mean light intensity on each side of stream margin is criteria and emergent vegetation and debris score 0 to 3, 0 (none), 1 (1 to 20% density of vegetation in 1 m^2), 2 (21-40%) and 3 (> 40%).

Morphological identification

Adult mosquitoes were identified using the morphological keys of Harrison (1980), Peyton and Scanion (1966), Rattanarithikul and Panthusiri (1994) and Rattanarithikul et al. (2006) Specimens identified as *An. minimus* if present the presector pale spot (PSP) and as *An. harrisoni* if present the humeral pale spot (HP)

Molecular identification

Multiplex PCR assay of Garros et al. (2004) was performed to confirm the identification of *An. minimus* species complex

Data analysis

These information were combined with spatial data using GIS tools.

Results

The total of sampling points (Figure 1) at Ban Pu Teuy were 73 points, the elevation of this area approximate 300 m above sea level and at Ban Bong Ti Noy were 58 points and 2.2 km long, the elevation of this area approximate 100 m above sea level.

Figure 1 Sample point coordinates of larvae collection and house at Ban Pu Teuy (left) and Ban Bong Ti Noy (right), Sai Yok District, Kanchanaburi Province

At Pu teuy, the coverage vegetation has mostly middle value and high value of emergent vegetation, there were few point. Debris at the sample point of mainline of the stream has the high value. The physical data of the stream of both study area as shown is table 1.

Table 1 environmental variables data of the stream in study sites, Pu Teuy and Bong Ti Noy, Sai Yok District, Kanchanaburi Province from Dec 06 – May 08

Geographical and ecological	**Pu Teuy**	**Bong Ti Noy**
Height of stream bank	<1 m	>1 m
Water depth	Max = 0.60 m Min = 0.04 m	Vary in each season
Flow rate of water current	Vary in each point	- Vary in each point - Constant flow rate in rainy season ~ 2 s/m
Transparency of water	Clear water	0.35 – 0.50 m (rainy season)
Temperature	24-27 ^{o}C	24-30 ^{o}C
Water analysis		
- pH	- 6.84 – 7.04	- 7.00 – 8.04
- Conductivity	- 749 – 770 μs/cm	- 250 – 355 μs/cm
- TDS	- 495 – 512 ppm	- 160 – 235 ppm
- Salinity	- 357 – 369 ppm	- 100 – 169 ppm

Table 2 shows Anopheles larvae were the high number in summer and cold season. January 2007 and 2008 had lower number of larvae than the others in the same season. Because of in January that have clearing of a vegetation along the stream for drainage purpose

Table 2 Number of Anopheles immature stages collected from the stream, a potential habitat for *An. harrisoni* at **Pu Teuy**, Sai Yok District, Kanchanaburi Province from Dec 06 – May 08

Month	Number of Anopheles immature stages					
	# L1	# L2	# L3	# L4	# Pupa	Total
Dec-06	582	561	322	149	5	1,619
Jan-07	160	229	158	142	6	695
Feb-07	561	671	354	171	26	1,783
Mar-07	439	515	383	221	14	1,572
Apr-07	729	594	353	276	10	1,962
May-07	861	693	390	267	14	2,225
Jun-07	601	411	143	119	6	1,280
Jul-07	691	368	196	151	3	1,409
Aug-07	417	284	122	65	5	893
Sep-07	789	398	210	163	7	1,567
Oct-07	799	398	194	162	12	1,565
Nov-07	1,292	444	211	154	19	2,120
Dec-07	655	490	281	170	11	1,607
Jan-08	263	308	231	157	1	960
Feb-08	778	432	352	247	6	1,815
Mar-08	320	271	167	126	3	887
Apr-08	1,307	766	319	215	5	2,612
May-08	816	425	254	151	4	1,650

The main line (yellow circle) of the stream were found anopheles larvae more than the branch line of the stream (Figure 3)

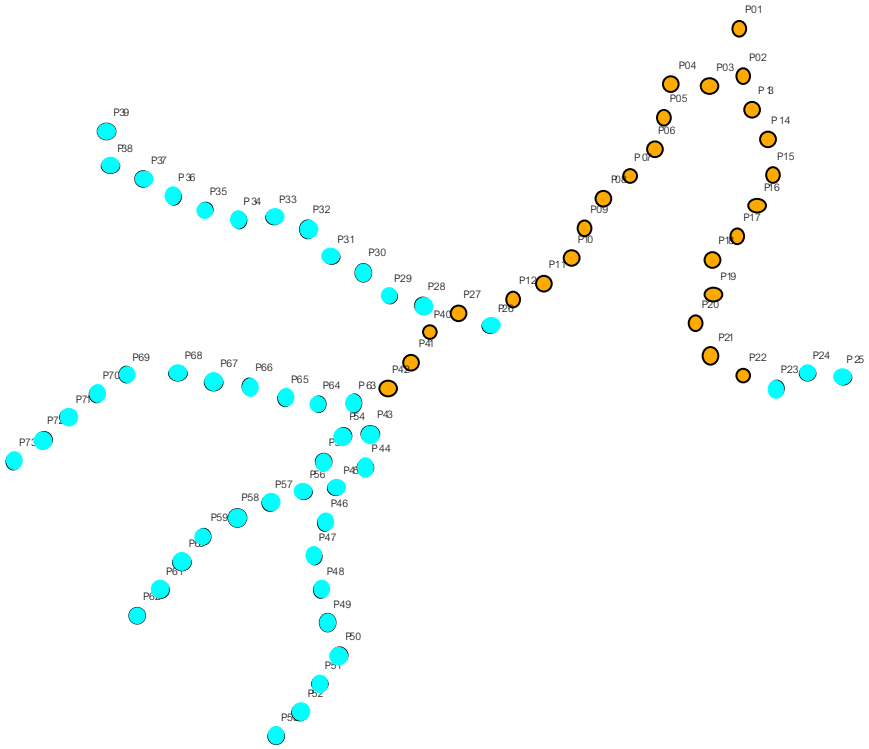

Figure 3 Sample points (light blue circle) of the stream at Pu Teuy that have the mean number of anopheles larvae was collected less than 20 in each month

The number of Anopheles immature stages collected from the stream at BTN was decrease in the rainy season, may fast flowing stream be change nature of stream margin. After that, the number is increasing (Table 3)

Table 3 Number of Anopheles immature stages collected from the stream, a potential habitat for *An. harrisoni* at **Bong Ti Noy**, Sai Yok District, Kanchanaburi Province from Dec 06 – May 08

Month	**Number of Anopheles immature stages**					
	# L1	**# L2**	**# L3**	**# L4**	**# Pupa**	**Total**
Jan-07	435	419	260	222	29	1,365
Mar-07	356	317	218	155	17	1,063
May-07	374	65	22	16	1	478
Jul-07	2	0	1	1	0	4
Sep-07	1	1	0	0	0	2
Nov-07	97	56	26	37	5	221
Jan-08	543	399	381	369	87	1,779
Mar-08	536	557	246	221	43	1,603
May-08	328	129	101	99	9	666

Morphological identification results of mosquitoes collected between Dec 2006 and May 2008 from Pu Teuy (Table 4) found *An.minimus, An. harrisoni,* Maculatus gr., *An. varuna, An. jamesii, An. aconitus,* Aitkenii gr. and Barbirostris gr. The number of *An. harrisoni* was highest

Table 4 Number of Anopheles mosquitoes collected from the stream at **Pu Teuy**, Sai Yok District, Kanchanaburi Province and identified by using morphological characters

Month	*An. minimus*			*An. harrisoni*			*Maculatus gr.*			*An. varuna*			*An. jamesii*			*An. aconitus*			*Aitkenii gr.*			*Barbirostris gr.*		
	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F
Dec-06	22	5	**27**	92	130	**222**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Jan-07	4	1	**5**	68	67	**135**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Feb-07	12	2	**14**	199	202	**401**	0	1	**1**	1	1	**2**	0	2	**2**	0	0	**0**	0	0	**0**	0	0	**0**
Mar-07	10	5	**15**	226	245	**471**	0	0	**0**	1	0	**1**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Apr-07	21	1	**22**	202	209	**411**	1	3	**4**	0	0	**0**	0	0	**0**	0	1	**1**	0	0	**0**	0	0	**0**
May-07	6	7	**13**	77	117	**194**	5	6	**11**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Jun-07	21	5	**26**	152	184	**336**	4	8	**12**	0	0	**0**	0	0	**0**	0	0	**0**	1	2	**3**	0	0	**0**
Jul-07	18	4	**22**	103	123	**226**	3	1	**4**	0	0	**0**	0	0	**0**	0	1	**1**	0	2	**2**	1	3	**4**
Aug-07	5	0	**5**	54	71	**125**	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	1	0	**1**	0	0	**0**
Sep-07	18	1	**19**	82	122	**204**	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	1	1	**2**	1	0	**1**
Oct-07	18	3	**21**	116	132	**248**	2	0	**2**	0	0	**0**	0	0	**0**	0	0	**0**	0	1	**1**	0	0	**0**
Nov-07	20	8	**28**	137	160	**297**	3	2	**5**	0	0	**0**	0	0	**0**	0	0	**0**	1	0	**1**	0	0	**0**
Dec-07	22	20	**42**	129	185	**314**	2	3	**5**	0	0	**0**	0	1	**1**	0	0	**0**	0	0	**0**	1	1	**2**
Jan-08	7	7	**14**	82	97	**179**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Feb-08	21	8	**29**	212	228	**440**	6	7	**13**	0	0	**0**	1	0	**1**	0	0	**0**	0	0	**0**	0	0	**0**
Mar-08	10	7	**17**	143	160	**303**	2	6	**8**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Apr-08	27	6	**33**	225	242	**467**	5	4	**9**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
May-08	9	8	**17**	171	186	**357**	9	4	**13**	0	0	**0**	0	0	**0**	0	0	**0**	4	3	**7**	3	2	**5**

Table 5 shows the species of mosquitoes larvae were collected from BTN such as *An.minimus, An. harrisoni,* Maculatus gr., *An. varuna, An. culicifacies, An. aconitus, An. philippinensis,* Barbirostris gr. and *An. vagus.* The number of *An.minimus* was highest, follow by Maculatus gr. mosquitoes, which mostly are *An. sawadwongporni*

Table 5 Number of Anopheles mosquitoes collected from the stream at **Bong Ti Noy**, Sai Yok District, Kanchanaburi Province and identified by using morphological characters

Month	***An. minimus***			***An. harrisoni***			***Maculatus gr.***			***An. varuna***			***An. culicifacies***			***An. aconitus***			***An. philippinensis***			***Barbirostris gr.***			***An. vagus***		
	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F
Jan-07	137	146	**283**	0	0	**0**	25	36	**61**	1	1	**2**	0	5	**5**	2	6	**8**	0	0	**0**	2	2	**4**	0	0	**0**
Mar-07	86	101	**187**	0	2	**2**	59	65	**124**	1	2	**3**	18	26	**44**	0	1	**1**	3	3	**6**	2	0	**2**	0	2	**2**
May-07	8	6	**14**	1	0	**1**	43	42	**85**	0	2	**2**	15	7	**22**	2	0	**2**	0	0	**0**	0	0	**0**	0	0	**0**
Jul-07	0	0	**0**	0	0	**0**	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Sep-07	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Nov-07	12	5	**17**	0	1	**1**	6	1	**7**	0	0	**0**	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**
Jan-08	223	240	**463**	0	6	**6**	36	26	**62**	0	4	**4**	2	2	**4**	0	2	**2**	0	0	**0**	2	3	**5**	0	0	**0**
Mar-08	150	177	**327**	6	13	**19**	114	96	**210**	0	3	**3**	9	7	**16**	0	1	**1**	0	0	**0**	2	1	**3**	0	0	**0**
May-08	108	100	**208**	2	4	**6**	25	22	**47**	0	3	**3**	6	5	**11**	0	0	**0**	0	0	**0**	0	0	**0**	0	1	**1**

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Journal of the American Mosquito Control Association

BITTING PATTERNS OF THE MALARIAL MOSQUITO, *ANOPHELES DIRUS* INTO EXPERIMENTAL HUTS TREATED WITH DDT AND DELTAMETHRIN

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ABSTRACT Movement patterns of natural population of *Anopheles dirus* females into experimental huts treated with DDT and deltamethrin were carried out during the wet season of 2006 and 2007 at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, west Thailand. Two experimental huts, control and treatment, were constructed in the fashion of local Thai homes. Under unsprayed conditions, biting activity of *An. dirus* females demonstrated one prominent peak at 1900-2000 hr. After hut was sprayed with DDT, a significant reduction in number of *An. dirus* females entering the treated hut was observed compared with the control ($P < 0.05$). In addition, female mosquitoes almost disappeared from DDT treated hut during the dawn period (0300-0600 hr). Apart from DDT, we also observed the movement pattern of *An. dirus* females into the hut treated with delatamethrin. Results revealed low number of female mosquitoes entered the deltamethrin treated hut compared with the control ($P < 0.05$). However, *An. dirus* females continued to enter the deltamethrin treated hut and maintained significantly high levels biting after 2200 hr and through the remainder of the night ($P < 0.05$). Overall, a greater proportion of *An. dirus* females entered the hut treated with deltamethrin than the hut treated with DDT. We conclude that DDT exhibited a stronger excito-repellent effect than deltamethrin on the natural population of *An. dirus*, a vector of malaria in Thailand.

KEY WORDS: *Anopheles dirus,* behavioral responses, excito-repellency, experimental hut, deltamethrin, DDT

INTRODUCTION

Malaria is known as the most serious vector borne disease in tropical and subtropical regions with transmission occurring in over 105 countries worldwide (Roll Back Malaria, 2006). Approximately 70% of malaria cases occur on the African continent whereas the remaining 30% occur in the Americas and Asia [World Health Organization (WHO), 2006]. In Thailand, malaria remains a major and reemerging health problem, although vector control programs have been successful in reducing morbidity and mortality which often results in socioeconomic losses (Ministry of Public Health (MOPH), 2006). Approximately seventy percent of the malaria cases are documented from the undeveloped national borders of eastern Myanmar where a member of efficient malaria vectors like *Anopheles dirus* occur (Scanlon & Sandninan, 1965; Kitthawee *et al*., 1990; MOPH, 2006). This species belongs to the Leucosphyrus group and is a forest and forest-fringe inhabiting mosquito that is considered highly endophagic and anthropophilic with high infectivity rates (0.3-13%) (Rosenburg, 1982; Baimai *et al*., 1984). The most favored breeding habitats are animal footprints, wheel-tracks and temporary ground pools. In addition, larvae are occasionally found breeding in water jars, cut tree stumps, and root holes (Rattanarithikul *et al*., 2006).

Anopheles dirus has shown different behavioral responses to intradomicilary use of insecticides (Ismail *et al*., 1974, 1975; Suwonkerd *et al*., 1990). In Thailand, indoor residual spray (IRS) is routinely conducted to interrupt human-vector contact and transmission (Chareonviriyaphap *et al*., 2001; MOPH, 2006). For years, DDT was the chemical of choice and was used extensively in malaria-endemic areas. Because of reported adverse environment impacts and negative public health issues,

DDT was removed for use for malaria control in Thailand in 2000 and replaced with synthetic pyrethroids (Chareonviriyaphap *et al*., 2000).

Pyrethroids have been widely accepted for controlling disease vectors due to their low mammalian toxicity (Elliot, 1976). Deltamethrin, a commonly used synthetic pyrethroid in public health programs, has been the mainstay for IRS use to combat malaria transmission in Thailand (Pothikasikorn *et al*., 2005; MOPH, 2006). There have been numerous attempts to accurately measure the behavioral responses of mosquitoes to insecticides using experimental huts (Smith, 1965; Roberts *et al*., 1984; Roberts *et al*., 1987; Rozendaal *et al*., 1989; Rutledge *et al*., 1999; Bangs, 1999; Grieco *et al*., 2000; Pates & Curtis, 2005). Experimental hut studies provide valuable details on the behavioral responses of natural occurring mosquito populations. Understanding the behavioral responses of different disease vectors to chemical compounds can facilitate vector control personnel by selecting and implementing the most effective intervention possible. However, little has been documented on the house entering behavior of *An. dirus* females into experimental huts treated with either DDT or deltamethrin. For this reason the effects of chemicals applied to the interior of homes on vector behavior for the reduction of man-vector contact needs to be studied. The experimental huts used in the current study have been used to evaluate the flight behavior of *Aedes aegypti* in Thailand (Suwondkerd *et al*., 2006). The data presented here are the results of the first comparison of the behavioral responses of *An. dirus* to DDT and deltamethrin to house entry both pre and post spray.

MATERIALS AND METHODS

Study site: The study was conducted at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14°17'N, 99° 11'E) (Fig. 1). The study site is mountainous and surrounded by deep forest, approximately 500 m from the nearest house at Pu Tuey Village. During the wet season (August to October), there are a variety of potential breeding sites for *An. dirus* such as temporarily animal hoof prints and small, shaded fresh water pools. The main occupation of people is logging, plant and animal hunting, and forest reservation labors.

Insecticide susceptibility tests. The susceptibility of *An. dirus* to DDT (4%) and deltamethrin (0.05%) was assessed by exposing female mosquitoes to a single diagnostic dose on insecticide-treated test papers, as recommended by WHO and following standard testing procedures (WHO, 2006). After a 60 min exposure, test and control mosquitoes were transferred to separate clean holding containers and mortality was recorded 24 hrs post-exposure. Tests were repeated four times. Based on the percentage of mortality in each population, mosquito survival was used as an indicator of the degree of physiological resistance.

Experimental hut: Two identical experimental huts were used for the study of the entering behavior of *An. dirus.* Huts used in the present study were previously used to evaluate the flight behavior of *Aedes aegypti* in Thailand (Chareonviriyaphap *et al*., 2005; Suwondkerd *et al*.,2006). Huts were built using locally acquired materials. The infrastructure of the huts consisted of sections of iron fence pipe along with custom-welded galvanized pipes. Pieces of untreated wood planks, measuring 1 m x 2.5 m were joined together into panels measuring (1m x 3 m) to serve as the 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. All three windows, one on each of three walls, and one door remained open during the entire of collection. The two huts were positioned 100 m apart on an open plot of land surrounded by mountainous terrain and agricultural fields.

Netting preparation: The field application rate of DDT and deltamethrin were used in this investigation. Netting impregnated with DDT at 2 g/m^2 and deltamethrin at 0.02 g/m2 were prepared using acetone diluents following the method of Grieco *et al*. (2005). The treatment net (30,000 cm^2) were soaked with treatment solutions (18.6 ml) in metal pans and cover with a heavily smaller pan. Additional nets were treated with acetone (18.6 ml.) to serve as untreated controls. All nets were allowed to air-dry for 60 min before use in the experimental huts (Grieco *et al*., 2005).

Pre spray collection: Two untreated experimental huts were used during the pre-spray period. Simultaneous indoor collections were performed on the two untreated huts to obtain the baseline data on the normal entering pattern of *An. dirus* into the experimental huts. The baseline collections also allowed for the determination if the two huts were comparable in regard to *An. dirus* densities and patterns prior to spraying. Collectors were divided into two teams of four persons each. The first team worked from 1800-2400 h for each hut with two collectors inside of each hut, followed by the second team beginning from 0000 h to 0600 h. Human-landing collection were conducted for 45 min with a 15-min break each hr. On the following night, collectors who worked during a particular sampling period (either the early or late sampling period) were rotated to avoid collector bias. Each collector exposed their lower legs and collected all landing mosquitoes by mouth aspirator. Collected mosquitoes were retained in plastic holding cages labeled by hr and hut of collection and were proved a cotton soaked with a 10% sugar solution. Specimens were

transferred to the field laboratory and morphological identified the following morning. Additional details on human-landing collection methods are given in previous work (Sungvornyouthin *et al*., 2006). Hourly ambient outdoor temperature and humidity were recorded during the period of mosquito collection.

Post spray collection: During the post-spray collections, one hut served as a control and the other hut was prepared as a treatment. In order to evaluate chemicals in the treated hut without applying compound directly to the wall surfaces, a series of panels were developed for holding treated netting which could be positioned around the interior surface of the hut. The aluminum frame that housed the netting contained holes in each corner and were held in place to the hut walls using bolts. There is a 9 cm gap between the aluminum panel and the wood planks to prevent the netting from touching the interior walls. Wing nuts were used to facilitate the rapid placement and removal of the metal panels for washing after the conclusion of the experiment. The interior of the treatment hut was lined with netting material treated with either $2g/m^2$ of DDT or $0.02g/m^2$ of deltamethrin whereas the control hut was lined with netting prepared with only the solvent, acetone. All three windows and one door were left open during the period of collection to allow female mosquitoes to enter.

Data analysis

Pre-sprayed: Collection periods were group into four categories, evening (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr). The mean number of collected mosquitoes from the huts prior to spraying (huts 1 and 2) was compared using an independent-sample *T*-Test, one-way analysis of variance (ANOVA). The test of normality for the numbers of *An. dirus* collected in each hut was conducted using either the normal probability plot and Komogorov–Smirnov Test

(K-S Test) or Shapiro–Wilk Test using SPSS(SPSS version 15.0. Inc., Chicago, IL). The accepted significance level was determined at 0.05% (*P*-value < 0.05).

Post spray: Collection periods were also group into four categories, evening period (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr). The mean number of mosquitoes from the sprayed hut and its matched control were compared (DDT treated hut vs. untreated hut and deltamethrin treated hut vs. untreated hut) using a paired–sample *T*-Test and ANOVA in SPSS (SPSS version 15.0. Inc., Chicago, IL).

RESULTS

Field collected *Anopheles dirus* females were exposed to a single diagnostic dose of either DDT (4%) or deltamethrin (0.05%) treated papers to assess susceptibility level to the compounds. *Anopheles dirus* was found to be completely susceptible to both compounds as indicated by 100% mortality after 24-h postexposure to the diagnositic dose (Table 1).

The movement pattern for natural populations of *An. dirus* into the experimental huts was observed during the rainy season (August 2006) (Figure 1). From a total of twenty night collections, 415 and 384 *An. dirus* females were captured from huts 1 and 2, respectively. One prominent peak was obtained during 1900-2000 hr whereas a very week peak was observed at 0100-0200 hr. When collection times were tabulated into four categories, evening (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr), the lowest proportion of *An. dirus* females, entering the two huts was found to occur during the dawn period [49 for hut 1 (11.81%) and 13 for hut 2 (3.38%)] (Table 2). The vast majority of entering populations were caught during the first half of the night with 74.93% in the first hut and 90.88% in the second hut. Specifically, greater numbers of *An. dirus* females entered the hut during the early evening [(212 hut 1 (51.08%) and 233 hut 2 (60.67%)] compared with the other periods. Ratio of females entering the two huts was 1:0.92. The Levene's test for equality or homogeneity of variances demonstrated that the two experimental huts had equal variances without any significant differences in entering behaviors of *An. dirus* mosquitoes (Student's t-test, $t = 1.128$, $df=38$, $p>0.05$).

After the DDT treated nettings were placed in the hut, an additional ten nights of human-landing collections (September 2006) were performed to assess the

movement pattern of *An. dirus* females into the experimental hut. The pattern of entering activity of *An. dirus* females into the control hut was similar to what was observed under pre spray conditions. A significant reduction in the number of *An. dirus* females collected in the DDT treated hut was observed throughout the night with a major pronounced reduction in the number of mosquitoes collected during the first half of the night (1800-0000 hr). One hundred and thirty three females were collected from the unsprayed hut whereas 51 were collected from the DDT treated hut. During the dawn period (0300-0600 hr), female mosquitoes almost disappeared from the hut treated with DDT in which 2 were collected from the DDT treated hut and 30 were collected in the control hut. Overall, 175 *An. dirus* females (73.8%) were caught from the untreated hut (control) whereas 62 (26.16%) were captured from the DDT treated hut (Table 3). Reduction rate……..

The effects of deltamethrin on the movement patterns of *An. dirus* into treated huts was investigated in October of 2006. The two huts, deltamethrin treated hut and control hut, were prepared in the same manner as previously described for DDT. The movement pattern of *An. dirus* females into the control hut was found to be similar to that observed under pre sprayed conditions. In the deltamethrin treated hut, there was a significant reduction in the number of mosquitoes collected as compared with the control hut. A decrease in the numbers collected was observed in the deltamethrin treated hut in the early evening low levels of mosquitoes being collected over the remainder for the night. Overall, 329 *An. dirus* females (68.82%) were collected from the untreated hut whereas 149 (31.17%) were collected from the deltamethrin treated hut (Table 2).

A comparison of the hut treated with DDT (6.3) with its matched unsprayed control hut (17.50) also showed the huts to be significantly different (t value = -4.652:

223 $p<0.05$). Moreover, a comparison of mean number of *An. dirus* between the
224 deltamethrin treated hut (10.50) and its matched unsprayed control hut (32.90) were
225 also significantly different (t value = -2.650: $p<0.05$).
226 In brief, high numbers of *An. dirus* females were collected from the unsprayed
227 hut compared to the treated hut. There was a 65% reduction in the number of *An.*
228 *dirus* caught in the DDT treated hut as compared to the control hut (Table 3 and Fig
229 3) and a 55% reduction in the number of *An. dirus* collected from the deltamethrin
230 treated hut as compared to the control hut (Table 3 and Fig 4).

DISCUSION

Millions of people in the tropical and subtropical world suffer from malaria, a disease transmitted by *Anopheles* mosquitoes (Bruce Chwatt, 1970). Each year, 300 to 500 million cases of malaria are reported worldwide (WHO, 2006). Malaria remains an important vector borne disease in Thailand, despite decades of successful vector control efforts and a significant reduction in malaria mortality and morbidity. Today, cases are found mostly along the international borders of eastern Myanmar, western Cambodia and northern Malaysia. Prevention of this disease remains focused on the use of vector control methods which has proven to be the most practical means of reducing malaria transmission in all endemic areas (MOPH, 2006).

The use of indoor residual sprays (IRS) with insecticide is widely accepted for combating malaria transmission. IRS with DDT was the major reason for the widespread success of malaria control in the 1950s and 1960s (WHO, 1995). Until the year 2000, DDT had been the frontline insecticide used in controlling malaria in Thailand. Because of the changing public perception of DDT and its perceived adverse long term impact on the environment, the use of DDT for IRS was eventually replaced by deltamethrin, a promising synthetic pyrethroid.

Like DDT, most pyrethroids are known to elicit behavioral responses in insects (Threlkeld, 1985). In Thailand, vector control using deltamethrin for IRS was lunched in 1994 (Chareonviriyaphap *et al*., 1999). The extensive use of pyrethroids since that time should be a major stimulus for extensive testing and field evaluation of this class of chemistry on the behavioral responses of malaria vectors. Although there have been years of DDT and deltamethrin use for malaria control, the true impact of these compounds on the behavioral responses of mosquito vectors and their potential for breaking disease transmission, remains unclear and poorly understood.

Behavioral responses to DDT and deltamethrin by several malaria vectors have previously been reported from Thailand (Pothikasikorn *et al*., 2005; Sungvornyothin *et al*., 2001; Chareonviriyaphap *et al*., 2001, 2004; Prasittisuk *et al*., 1996; Ismail *et al*., 1975; Suwonkerd *et al*., 1997). Most work on the behavioral responses of vectors to insecticides was conducted in the laboratory and relied on the excito-repellency test system (Pothikasikorn *et al*., 2005; Sungvornyothin *et al*., 2001; Chareonviriyaphap *et al*., 2001, 2004). Very few field trials to evaluate the responses of *Anopheles* mosquitoes to insecticides have been performed using experimental huts in Thailand (Prasittisuk *et al*., 1996; Ismail *et al*., 1975; Suwonkerd *et al*., 1997). The last published paper of experimental hut studies for malaria vectors in Thailand was in 1996 (Prasittisuk *et al*., 1996). In 2000, the mathematical model for understanding the repellent, irritant and toxic actions of insecticides on mosquitoes and how they function to control malaria by breaking man vector contact was developed (Roberts *et al*., 2000). This model has proven useful for guiding the testing of insecticides for preventing disease transmission. This study suggests that the excito-repellency effect must be accurately assessed under field conditions for a clear understanding of how these chemicals function. The current study aims to document the behavioral effects of DDT and deltamethrin on *An. dirus* in experimental huts. Result demonstrated that DDT strongly reduced *An. dirus* populations inside of treated huts. There was a 65% reduction of *An. dirus* females collected in the hut treated with DDT compared with the matched control. This indicates a strong excito-repellent action of DDT. Hut studies with Anopheline vectors from Belize resulted in a similar conclusion that DDT produced both an irritant and repellent action (Bangs 1999; Grieco *et al*., 2000). In Thailand, *Anopheles dirus* females showed strong avoidance behavior by not entering experimentally treated huts with DDT

281 (Suwonkerd *et al*., 1990). Similar work of Roberts *et al*. (1991) observed that
282 *Anopheles darlingi* females from Brazil completely disappeared after experimental
283 huts were sprayed with DDT.

284 In addition to DDT, deltamethrin also reduced *An. dirus* populations from
285 inside the experimental hut. However, the pattern of behavior elicited by *An. dirus*
286 females was quite different from that of DDT. *Anopheles dirus* females almost
287 disappear from DDT treated hut during the dawn period (0300-0600 hr) whereas they
288 continue to bite in the deltamethrin treated hut throughout the night. Similar results
289 were seen in the house entering behavior of *An. vestitipennis* after huts were sprayed
290 with deltamethrin and DDT (Bangs, 1999; Grieco *et al*.,2000). They found that
291 higher proportion of female mosquitoes entered the hut treated with deltamethrin than
292 hut treated with DDT, indicating the powerful repellency of DDT compared to
293 deltamethrin. Specifically, there was a 97% reduction of mosquitoes entering a hut
294 treated with DDT whereas there was only a 66% reduction of *An. vestitipennis* in the
295 deltamethrin treated hut.

296 The current strategy of using human landing collections was adopted due to
297 the low numbers of *An. dirus* at the study site. The use of traps would have further
298 reduced the numbers collected resulting in reduced power from the sample size. The
299 reduction produced by both of these compounds could be a result of the combined
300 effects of repellency and contact irritancy. Mosquitoes may have entered the treated
301 huts, rested on the insecticide treated surface, become irritated and left without biting
302 giving the perception of repellency. While the data presented here can not clearly
303 define the nature of the response (ie. contact irritancy or repellency) it does clearly
304 demonstrate the effectiveness of DDT to prevent indoor biting by *An. dirus*. While
305 deltamethrin did not have as dramatic a reduction on the biting population as DDT it

306 also significantly reduced *An. dirus* inside of huts. The difference in the patterns of
307 response to the two chemicals indicates that the two compounds may be eliciting
308 different actions. This will have to be studied further using entrance and exit traps to
309 determine whether it is a repellent response or a contact irritant response is the result
310 of the indoor biting response.

311 In conclusion, without a better understanding of the relationship between
312 insecticide residues and mosquito behavior, vector control strategies have never been
313 completely sucessful. Studies on the avoidance behavior of *An. dirus* using
314 insecticide treated huts provides significant baseline data and critical information on
315 how female mosquitoes respond to chemicals in a natural setting. Such information
316 will facilitate the national vector control program by providing the detailed field
317 entomological knowledge on how insecticides are functioning to control vector born
318 disease. Additional work on the behavioral responses of mosquitoes to insecticides
319 must continue to better understand how they break man vector contact. Addtitional
320 studies will be performed using experimental huts fitted with entrance and exit traps
321 to further define the entrance and exit response to these compounds.

322 ACKNOWELEAGEMENT

323 We would like to thank the Armed Forced Development Command, Sai Yok District,
324 Kanchanaburi Province, Thailand for permission to use the study area. Funding for
325 this research came partly from the National Institutes of Health, U.S.A. (Grant #
326 5U01AI054777-02) and the Thailand Research Fund (Grant RMU 4880032),
327 Thailand.

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Table 1 Susceptibility of *Anopheles dirus* from Pu Teuy Village, Saiyok District, Kanchanaburi Province to diagnostic doses of DDT and deltamethrin.

Insecticide	Dose (%)	Number of test	Number Dead (%Mortality)
DDT	4	100	100 (100)
Deltamethrin	0.05	100	100 (100)

500 Table 2. Number of *An. dirus* collected from human-landing collections conducted
501 for 20 nights in untreated huts (huts 1 and 2).
502

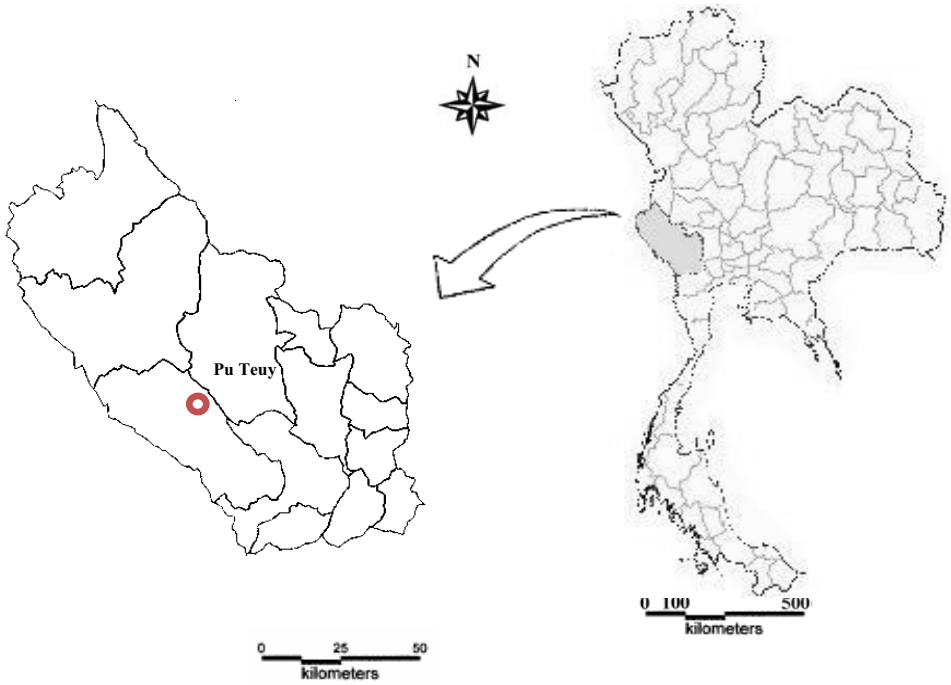
Hut / Time	Number of *An. dirus* (N)					
	1800-2100	2200-2400	0000-0300	0400-0600	Total (N)	Ratio
Hut 1	212	99	55	49	415	1
Hut 2	233	116	22	13	384	0.92
Total	445	215	77	62	799	

503
504

Table 3 Number of *Anpheles dirus* collected during four time periods from human-landing collections during 10 collection nights in huts treated with DDTand deltamethrin along with their matched untreated control. Collection totals are separated into four sample periods to correspond to evening (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr).

Treated hut / Time	Number of *An. dirus* (N)					
	1800-2100	2200-2400	0100-0300	0400-0600	Total (%)	%Reduction
DDT (Hut 1)	28	23	11	1	62 (26.16%)	65%
Unsprayed (Hut 2)	69	64	32	10	175 (73.8%)	
Deltamethrin (Hut 1)	64	26	26	29	149 (31.17%)	55%
Unsprayed (Hut 2)	123	90	69	47	329 (68.82%)	

Fig. 1



533 **Fig. 2**

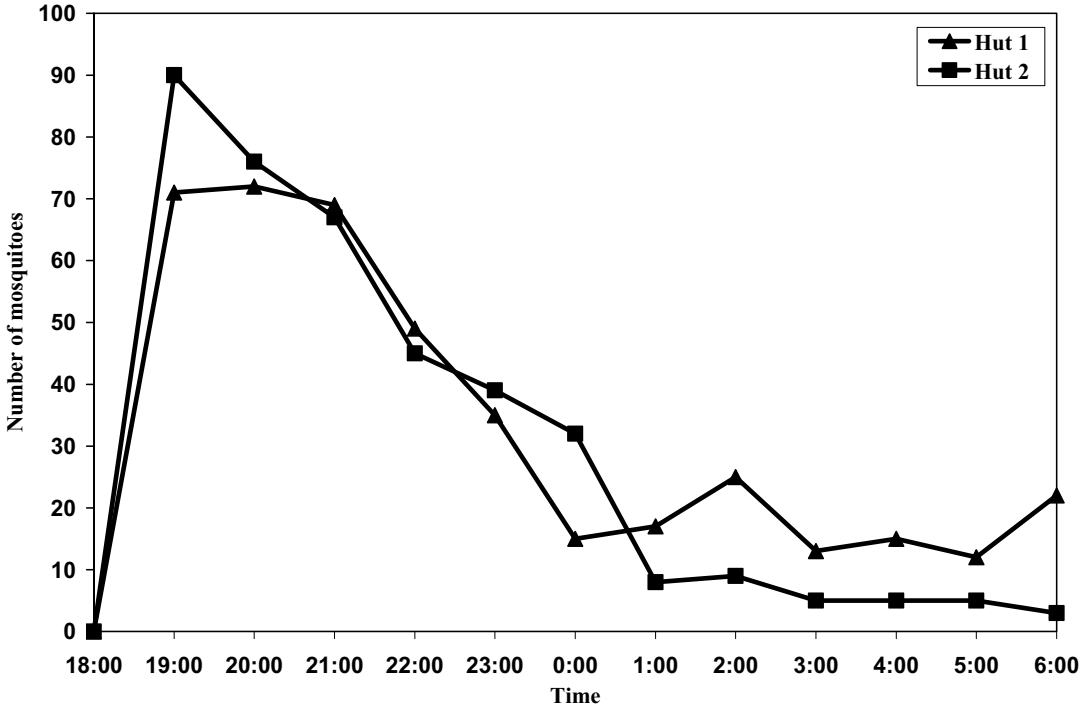


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Fig.3

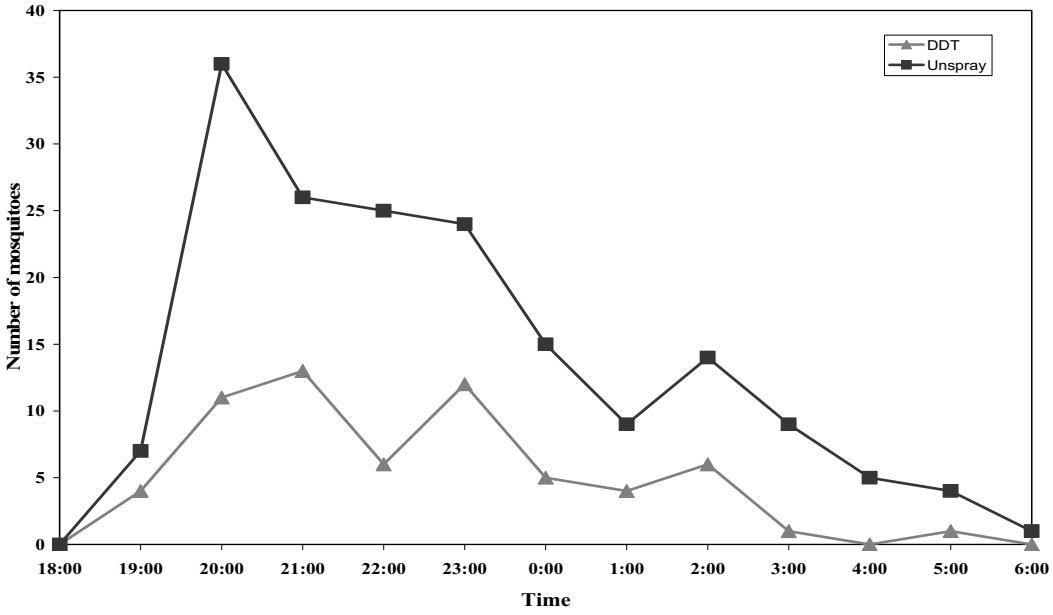
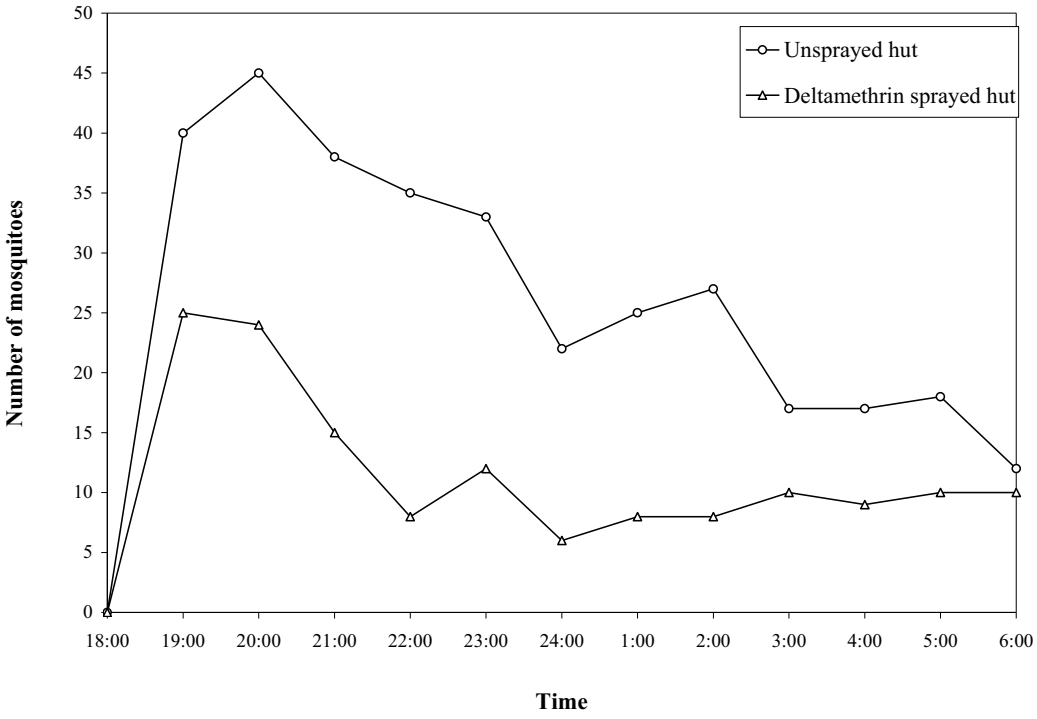

DDT
Unspray
Number of mosquitoes
0
5
10
15
20
25
30
35
40
18:00
19:00
20:00
21:00
22:00
23:00
0:00
1:00
2:00
3:00
4:00
5:00
6:00
Time

Fig 4.


Unsprayed hut
Deltamethrin sprayed hut
Number of mosquitoes
Time
0
5
10
15
20
25
30
35
40
45
50
18:00
19:00
20:00
21:00
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24:00
1:00
2:00
3:00
4:00
5:00
6:00

583 **Figure legend**

584 **Fig. 1** Map of Kanchanaburi Province and study site

585

586 **Fig. 2** Number of *Anopheles dirus* collected from human landing collections under
587 pre-spray conditions for 20 nights.

588

589 **Fig. 3** Number of *Anopheles dirus* collected from human-landing collections during
590 20 collection nights at Pu Teuy Village, Sai Yok, Kanchanaburi inside a DDT
591 treated hut and its matched unsprayed control hut.

592

593 **Fig. 4** Number of *Anopheles minimus* collected from human-landing collections
594 during 10 collection nights at Pu Teuy Village, Sai Yok, Kanchanaburi inside
595 a deltamethrin treated hut and its matched unsprayed control hut.

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