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THESIS

BIONOMICS OF NATURAL POPULATIONS OF *Anopheles minimus* AND *Anopheles harrisoni* (DIPTERA: CULICIDAE) AND BEHAVIORAL RESPONSES TO BIFENTHRIN AND DEET

RUNGARUN TISGRATOG

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Anopheline adults were surveyed at Ban Tum Sua, Mae Sot District, Tak Province, western Thailand between November 2008-September 2009. A total of 5,392 *Anopheles* was collected with the most commonly prevalent being *Anopheles minimus* (71.57%), followed by *An. maculatus* complex (27.97%) and *Anopheles dirus* complex (0.46%). The trophic behavior and host preference of *Anopheles minimus* was observed throughout the night long. Indoor and outdoor human landing activities by *An. minimus* were observed between 2400 and 0100 hours with a slight predilection to feed outdoor compared to indoor. Significantly greater number of *An. minimus* was collected from human rather than that of cow ($P <$ 0.0001). *An. minimus* was more abundant during the wet season compared with the dry and hot seasons. A better knowledge of mosquito behavior related to host and feeding predilection will facilitate the efficiency of vector control operation.

Behavioral responses of two species in the Minimus Complex exposed to an operational field dose of bifenthrin or DEET were described using an excito-repellency test system. Two test populations of *An. minimus*, one from Tak Province, western Thailand, the other from a long-established laboratory colony, and *Anopheles harrisoni* collected from Kanchanaburi Province, western Thailand, were used. Results showed that all test populations rapidly escaped after direct contact with surfaces treated with either bifenthrin or DEET compared to match-paired untreated controls. Greater escape response by exposed females to bifenthrin and DEET were observed in the *An. minimus* colony compared to the two field populations. Field collected *An. minimus* demonstrated a more rapid escape response to DEET than to bifenthrin, whereas *An. harrisoni* showed a converse response. Fewer females escaped from test chambers without direct contact with treated surfaces compared to contact tests, however, the spatial repellency response was significantly pronounced in all test populations compared to match-paired controls ($P < 0.05$). DEET was found to perform as both a contact stimulant and moderate spatial repellent.

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LIST OF ABBREVIATIONS

An.	=	Anopheles
h	=	Hour
KD	=	Knockdown
MOPH	=	Ministry of Public Health
P	=	Probability Value
SE	=	Standard Error
WHO	=	World Health Organization

BIONOMICS OF NATURAL POPULATIONS OF *Anopheles minimus* AND *Anopheles harrisoni* (DIPTERA: CULICIDAE) AND BEHAVIORAL RESPONSES TO BIFENTHRIN AND DEET

INTRODUCTION

Malaria is still one of the most important vector-borne diseases in topic and subtropical regions. Approximately 2.5 million cases of malaria are reported annually, and it is estimated that as many as 100 million cases may occur in the Southeast Asia region each year (World Health Organization (WHO), 2007a). Southeast Asia accounts for 30% of the global malaria morbidity and about 8% of the global mortality, with approximately 26,000 deaths reported per year (WHO, 2007b). Four species of the *Plasmodium* parasites commonly infect humans in which the most serious forms of the disease are caused by *Plasmodium falciparum*. *Plasmodium vivax, Plasmodium ovale and Plasmodium malariae* causes milder disease in humans that is not generally fatal. A fifth species, *Plasmodium knowlesi*, a malaria parasite of Asian macaques, can also infect humans as well as (rearly) some other primate malarias. In Thailand, the number of confirmed malaria cases has fallen from an average of 40,382 cases a year between 2000-2005 to 23,327 in 2009, a decline of 42% (WHO, 2010).

Malaria is transmitted by the bites of infective mosquito vectors in the genus Anopheles. Three main malaria vector groups are recognized in Southeast Asia; namely *An. dirus* sensu lato (Dirus Complex) occurring primarily in forested areas, *An. minimus* s.1. (Minimus Complex), widespread in the hilly forested regions and *An. sundaicus* s.1. (Sundaicus Complex), normally a brackish water mosquito present along coastal areas (Garros *et al*., 2006). In Thailand, situation is particular in which 3 anopheline species complexes, *An. dirus*, *An. maculatus* and *An. minimus* are considered to be primary malaria vectors (Chareonviriyaphap *et al*., 2000). Among three, *An. minimus* is considered to be the most important malaria vector in Thailand (MOPH 2010).

The *An. minimus* complex contains important vectors of malaria (Green *et al*., 1990). This Complex belongs to the Myzomyia series in the subgenus *Cellia,* and represents one of the major vectors of malaria in Southeast Asia. The *Anopheles minimus* complex, Theobald 1901, is composed of at least three sibling species, *An. minimus* (former species A), *An. minimus* species C and species E. A neotype for *An. minimus* (species A) has recently been designated (Harbach *et al.,* 2006) therefore this species is now recognized as *An. minimus*. *Anopheles minimus* is the most common that is widespread throughout Thailand (Baimai, 1989). Species C is restricted in two districts of Kanchanaburi Province, western Thailand and occurs in sympatry with *An. minimus* (Kengluecha *et al.,* 2005). Species C was previously collected from Mae Sot in Tak Province and Mae Rim in Chiangmai Province, north Thailand but no clear confirmation was made at the time (Rattanarithikul *et al.,* 2006). The third species, *An. minimus* species E, is restricted to the Ishigaki Island in the Ryukyu Archipelago, Japan (Somboon *et al.,* 2001, 2005). Additionally, *An. minimus* species D has been reported in Thailand, but sufficient information is lacking to support the proposed sibling species status (Baimai, 1989).

All species within this complex are impossible to identify based on morphology alone (Jaichapor *et al*., 2005; Sungvornyothin *et al*., 2006a) and clear separation between closely related sympatric species is in questions due to overlapping characters and variability of *An. minimus* (Jaichapor *et al*., 2005). Despite the existence in the literature of wing characteristics that could separate *An. minimus* from *An. harrisoni*, recent studies shown that morphological identification of the two sibling species of the Minimus Complex is not reliable and can lead to nearly 40% of misidentifications (Jaichapor *et al*., 2005; Sungvornyothin *et al*., 2006a). Isoenzymes have served as the gold standard to separate the two sympatric species of the complex (Green, 1990), however, this technique requires fresh or frozen specimens and the complete destruction of the specimen makes impossible further studies such as sporozoite detection. Recently, molecular methods were recently developed for distinguishing different species of the group. Two polymerase chain-reaction (PCR)-based techniques, an allele-specific amplification (ASA) and a single-strand conformation polymorphism (SSCP) were developed for distinguishing *An. minimus*

from *An. harrisoni* and both *An. minimus* and *An. harrisoni* along with *An. aconitus* and *An. varuna*.

A PCR-restriction fragment length polymorphism (RFLP) method was also designed for the identification of these four species, as well as for *An. pampanai*, *An. culicifacies* B, *An. jeyporiensis,* and naturally occurring hybrids between *An. minimus* and *An. harrisoni*(Van Bortel *et al*., 2000). Recently, a single multiplex PCR assay, using sequence characterized amplified region (SCAR) markers derived from individual random amplified polymorphic DNA markers, was developed as an easy and reliable identification of *An. minimus* and *An. harrisoni* (and their natural hybrids) as well as three closely related species within the larger *An. minimus* group (Kengne *et al*., 2001; Manguin *et al*., 2001)

Previous observation on behavioral differences between *An. minimus* and *An. harrisoni* in Vietnam have demonstrated that zoophilic behavior was pronounced for both species but *An. harrisoni* showed greater exophagic and exophilic than *An. minimus* in sympatry area (Trung *et al*., 2005). In non-sympatric, various behaviors were observed for *An. minimus* leading to the conclusion that this species may exhibit high behavioral heterogeneities. In Thailand, *An. minimus* and *An. harrisoni* occur in sympatry in some focal areas but few investigations have been conducted on each sibling species regarding feeding activity, resting behaviors, host preference. Sungvornyothin *et al*., (2006b) reported biting activity of *An. harrisoni* by take advantage of the PCR technology in relation to seasonal climatic variations. However, none has been observed the trophic behavior, biting activity and seasonal abundance of *An. minimus* describe by using a molecular identification assay. In addition, behavioral responses of the two species to bifenthrin and *N,N*- diethyl-3-methylbenzamide (DEET) within the Minimus complex were characterized.

OBJECTIVES

1. To describe the human-landing patterns and seasonal abundance of *An. minimus.*

2. To characterize the behavioral responses of *An. minimus* (wild and colony population) and *An. harrisoni* (wild population) to bifenthrin and DEET.

LITERATURE REVIEW

1. Malaria situation in Thailand

Malaria is one of the most important and potentially deadly, mosquito-borne diseases in Thailand. Although the incidence of malaria in Thailand has been significantly reduced during the past 50 years, malaria remains unacceptably high in some locations, especially in the scrub and forested hills areas along the more undeveloped areas of the international borders with eastern Myanmar, western Cambodia and northern Malaysia (Chareonviriyaphap *et al*., 2000; MOPH 2009). In particular, over seventy percent of recent malaria cases have been documented from the international border of Thai-Myanmar, especially in Tak and Kanchanaburi provinces where transmission is year-round (MOPH 2009; Manguin *et al*., 2010). This situation has been primarily associated with agricultural activities and exacerbated by uncontrolled population movements and political unrests in these locations (MOPH 2009).

In Thailand, confirmed malaria cases have declined approximately 61% from 81,692 in 2000 to 31,771 cases in 2009 (Table 1). Likewise, there has been a dramatic decrease in malaria associated mortality; deaths declining from 625 to 70 (nearly 89% reduction). Approximately half (32 million) of the total population of Thailand remain at risk for malaria infection, either residing in malaria active areas or receptive transmission zones. Malaria is caused by intracellular parasites called *Plasmodium*, which is transmitted by the bites of infective anopheline mosquitoes. The female *Anopheles* mosquito is an obligatory part of the parasite's life cycle, requiring development and multiplication following ingestion of from gametocytes acquired from infected blood of the human host to sporozoites, the infective stage that is transmitted back to a susceptible human. In Thailand, among the important malaria vectors are members of the Minimus Complex together with *An. dirus* complex and *An. maculatus* group mosquitoes (Rattanarithikul *et al*., 1996).

Table 1 Malaria cases from 2000-2009.

Year	Cases	cases of Pf.[1]
2000	81,692	35,440
2001	63,528	29,061
2002	44,555	20,389
2003	37,355	19,024
2004	26,690	13,371
2005	29,782	14,793
2006	30,293	14,124
2007	33,178	16,557
2008	26,150	12,108
2009	31,771	9,486

[1] Pf = *Plasmodium falciparum*

2. Malaria vector in Thailand: *Anopheles minimus* Complex

Several important vectors of malaria are widely distributed in Thailand, including *Anopheles dirus* occurring in natural and cultivated forests and forest fringes, *Anopheles maculatus* associated with hill and mountainous areas and certain member of *Anopheles minimus* complex. *An. minimus* is one of the most important malaria vectors in forested rural areas of Thailand (Baimai, 1989; Chareonviriyaphap *et al*., 2000; Kengluecha *et al*., 2005; Rattanaritthikul *et al*., 1994, 1996, 2006; Sungvornyothin *et al*., 2006b; MOPH 2009). The Minimus Complex belongs within the Minimus Subgroup, Myzomyia Series, Funestus Group of mosquitoes which include species found in Africa. *Anopheles minimus* Theobald is composed of at least two different species in Thailand, *An. minimus* and *An. harrisoni.* A third allopatric species designated *An. minimus* E occurs on Ishigaki Island of the Ryukyu Archipelago, Japan (Harbach, 2004; Somboom *et al*., 2005; Garros *et al*., 2006; Sungvornyothin *et al*., 2006b). *Anopheles minimus* is distributed over a wild

geographical areas from northern India eastward through Vietnam and northward across southern China, including Taiwan (Chen *et al*., 2002; Garros *et al*., 2005; Phuc *et al*., 2003). In Vietnam, Laos, Thailand, Myanmar and southern China *An. harrisoni* is also found (Chen *et al*., 2002; Garros *et al*., 2005; Kengne *et al*., 2001; Phuc *et al*., 2003; Sharpe *et al*., 2000; Singh *et al*., 2006; Trung *et al*., 2004). They have no specimens of *An. harrisoni* reported from Cambodia (Coosemans *et al*., 2006) *Anopheles minimus* is the predominant species found throughout most of Thailand, whereas *An. harrisoni* appears restricted to the western Thai-Myanmar border, primarily in Kanchanaburi Province (Kengluecha *et al*., 2005). Although the geographical distribution of *An. minimus* and *An. harrisoni* has been well defined in Thailand, the bionomical aspects of each species remains poorly understood, for example feeding behavior, host preference, vector capacity and competence and responses to insecticides (Chareonviriyaphap *et al*., 2004; Sungvornyothin *et al*., 2006b). In order to study these two species it is crucial that precise species identification using molecular identification tools be used to clearly differentiate the vector capacities of these two sibling species (Van Bortel *et al*., 1999; Theophil *et al*., 2002; Manguin *et al*,. 2010).

One method of controlling vector populations is through the use of chemicals, primarily insecticidal compounds. Understanding the behavioral responses of a species to chemicals facilitates vector control activities by selecting and implementing the most sustainable and successful interventions possible (Kongmee *et al*., 2004; Sungvornyothin *et al*., 2006b). Behavioral responses can be divided into two distinct forms; contact irritancy (excitation) and noncontact repellency (Roberts *et al*., 1997). Contact irritancy results from physical contact with a chemical-treated surface, whereas noncontact repellency results from a distance by detecting a chemical spatially and that result in an insect avoiding making physical contact with a treated surface. Although behavioral responses have been recorded with various Anopheles species and chemicals of in Thailand using the excito-repellency test system (Chareonviriyaphap *et al*., 1997, 2001; Muenworn *et al*., 2006; Pothikasikorn *et al*., 2005; Polsomboon *et al*., 2008), none have been so far attempted observing the behavioral responses to bifenthrin, a synthetic pyrethroid and DEET within the

Minimus Complex. In this study, observation on the behavioral responses of two wild-caught populations, *An. minimus* and *An. harrisoni*, and a laboratory-colonized population of *An. minimus*, against a recommended field concentration of bifenthrin using the excito-repellency test system. A five percent concentration of DEET (*N*,*N*-diethyl-meta-toluamide) was used as a comparison compound for repellency.

3. Importance of vector behavior on malaria transmission and impact of insecticide that modify behavior to reduce disease risk

Many study about mosquito behavior. *An. minimus* were found as endophagic and endophilic behavior in Southeast Asia. *An. harrisoni* has been shown highly anthropophilic, exophilic and feeding outdoor in combination with early feeding behavior (Harrison, 1980; Manguin *et al*., 2008). Environmental changes is evaluated to influence the behavior, hence that role of the different species in malaria transmission (Trung *et al*., 2005). Host preference of mosquitoes feeding is an important factor in evaluation the malaria vector status of *Anopheles* mosquitoes. A few chance of human-vector contact in outdoor feeding (Elliott *et al*., 1968). Behavior of vectors is factors determining the appropriateness of the most commonly used vector control measures. Understanding of vector behavior and the influence of alternative hosts and environment on behavior have direct practical implications for malaria control.

Behavioral responses of mosquito can be separated into two responses, contact irritancy and noncontact repellency (Roberts *et al*., 1997). Irritancy behavior were defined in as insect is stimulated to move away from an insecticide after direct physical contact with the chemical residue and repellency behavior were define in as insect detects to move away from an insecticide without making physical contact with the chemical (Roberts *et al*., 1997; Pothikasikorn *et al*., 2005, 2007; Tanasinchayakul *et al*., 2006).

4. Molecular identifications

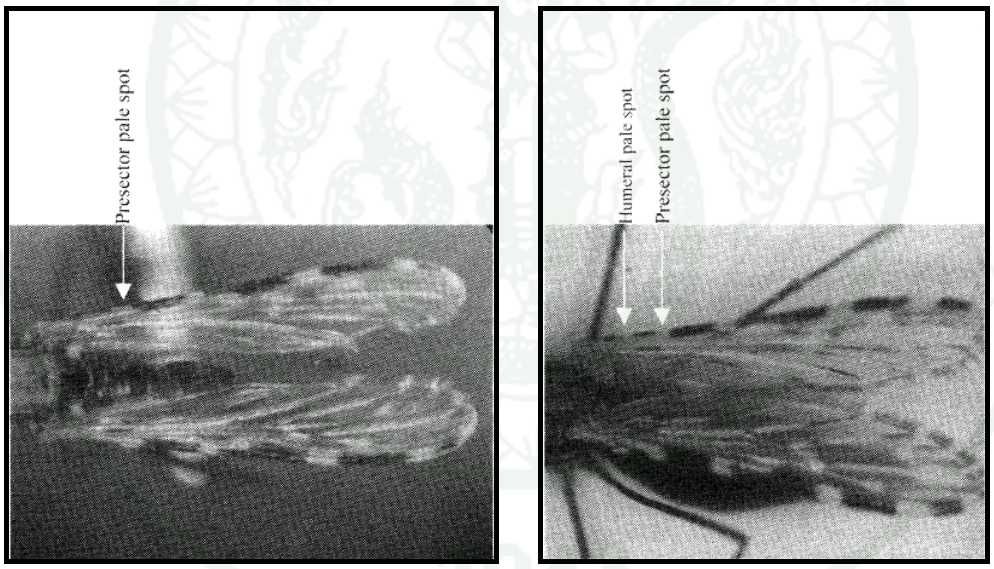
An. minimus is the one of primary vector in Southeast Asia. The 2 sibling species of Minimus Complex (*An. minimus* and *An. harrisoni*) can be sympatric. Both of 2 species were presented character that could differentiate the two species. *An. minimus* may present a presector pale spot (PSP) on costal wing vein, whereas *An. harrisoni* may present both presector pale and humeral pale spots (HP) (Figure 1). Morphology could not clearly identify 2 species (Sucharit *et al*., 1988).

Several previous studies included morphological, cytogenetic and other method for identifications such as isozymes markers (Green *et al*., 1990; Garros *et al*., 2006). Only morphological identification, the Minimus Complex cannot be properly identified and a high probability of misidentification has been consistently reported (Jaichapor *et al*., 2005; Sungvornyothin *et al*., 2006a). Several molecular approaches have been recently established for separating different species within this group. Two PCR based techniques, an allele-specific amplification (ASA) and an SSCP were developed for distinguishing *An. minimus* from *An. harrisoni* (Sharpe *et al*., 1999). A PCR-restriction fragment length polymorphism (RFLP) method was developed to distinguish the five species within the Minimus Complex and other related species, *An. minimus*, *An. harrisoni*, *An. aconitus*, *An. pampanai* and *An. varuna* (Garros *et al*., 2004; Phuc *et al*., 2003; Manguin *et al*., 2008). Currently, a single multiplex PCR assay, using sequence characterized amplified region (SCAR) markers derived from individual random amplified polymorphic DNA markers, was developed for an ease and reliability to identify the *An. minimus* and *An. harrisoni*, and their hybrids, as well as the three closely related species within the Minimus Subgroup (Kengne *et al*., 2001; Manguin *et al*., 2001). An allele specific method was used for studying of anopheline specie complexs (Paskewitz *et al*., 1993; Walton *et al*., 1999) and AS-PCR were developed for identification member of the *An. minimus* group (Garros *et al*., 2006).

The strategy for Internal Transcribed Spacer (ITS2) allele-specific amplification followed the approach of Scott *et al*., (1993) to distinguish members of

the *An. gambiae* complex. Garros *et al*., (2004a) design 6 primers for the five species of *An minimus* group, named the primers specific for the *An. minimus* group ACO, MIA, MIC, PAM, and VAR for *An. aconitus*, *An. minimus*, *An. minimus* species C(*An. harrisoni*), *An. pampanai*, and *An. varuna*., respectively.

The universal forward primer (ITS2A) is located in the conserved 5.8S gene, whereas the species-specific reverse primers are within the ITS2 spacer region. Member of *An. minimus* group can be identified by the combination of 6 primers in multiplex PCR. One of the 6 primers is the forward primer (ITS2A). Lengths of amplified species-specific products were 90 bp for *An. pampanai*, 180 bp for *An. harrisoni*, 200 bp for *An. aconitus*, 260 bp for *An. varuna*, and 310 bp for *An. minimus.*



PSP phenotype, *An. minimus* HP and PSP phenotype, *An. harrisoni*

Figure 1 Different morphological characterization of *An. minimus* and *An. harrisoni*, *An. minimus* is shown a presector pale spot (PSP) on costal wing vein, whereas *An. harrisoni* present both presector pale and humeral pale spots (HP) (Sungvornyothin *et al*., 2006a)

5. Chemicals used to control vectors and malaria transmission

Bifenthrin

Bifenthrin (IUPAC 2-methylbiphenyl-3-ylmethyl (*Z*)-(1*RS*,3*RS*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate]- 2,2-dimethylcyclopropane-1-carboxylate.) is a non-alpha cyano pyrethroid insecticide and acaricide. It has a low vapor pressure (1.81 x 10-7 mm Hg), low water solubility (less than 1 μg/l) and good stability with hydrolysis and photolysis. Bifenthrin is less toxic by the dermal route (LD 50 > 2000mg/kg body weight rabbit) than the oral route (LD50 > 56mg/kg body weight rat). It is a mild irritant to the rabbit eye but present no irritation after dermal application on abraded and intact skin. It is not a skin sensitizer in the guinea pig. In rat, dermal absorption is low. Because of these properties, bifenthrin is potentially a good candidate insecticide for treatment of mosquito nets.

Bifenthrin was used to control *An. minimus* in Maetaeung District, Chiangmai Province, northern Thailand (Prajakwong *et al*., 1998). The treatment of military tenting material with bifenthrin provided a marked reduction of mosquito entry. This chemical used against a range of agricultural pests and as an insecticide treatment for mosquito nets (Hougard *et al*., 2002) The chemical has relatively low irritant and knockdown effects.

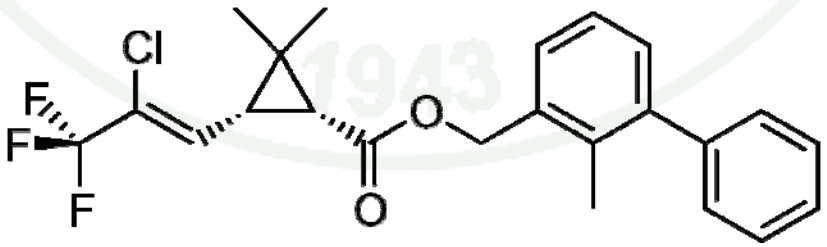


Figure 2 Bifenthrin chemical structure

DEET

DEET is the common name for *N*,*N*-diethyl-meta-toluamide (or *N*,*N*- diethyl-3-methylbenzamide), a slightly yellow oil in pure forms. It is the most common active ingredient in commercially available insect repellents. DEET was originally tested as a pesticide in agriculture and entered military use in 1946 and was registered for general public (USA) use in 1957. DEET has been shown to protect from a wide range of insect and other arthropod bites such as mosquitoes and ticks. Insect repellents containing DEET have been used for more than 60 years by millions of people worldwide to prevent bites from mosquitoes, tick, fleas, biting flies and chigger mites.

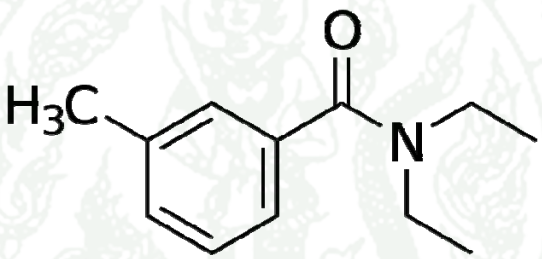


Figure 3 DEET **(***N*,*N*-diethyl-meta-toluamide**)** structure

MATERIALS AND METHODS

1. Seasonal abundance and biting activity of *Anopheles minimus* (Diptera: Culicidae) in western Thailand

1.1 Study Site

Anopheles minimus populations were collected from the Ban Tum Sua (16^{o} 41’ N 98^{o} 41$^{'}$ E), Mae Sot District, Tak Province, western Thailand. Tak is located in Thailand province, large mountainous, and covering an area of 16,406 square kilometres. The provincial capital Mae Sot is 426 kilometres north of Bangkok. To the west, Tak Province borders touches on Myanmar demarcated by mountain range and the Moei River (Figure 4.)

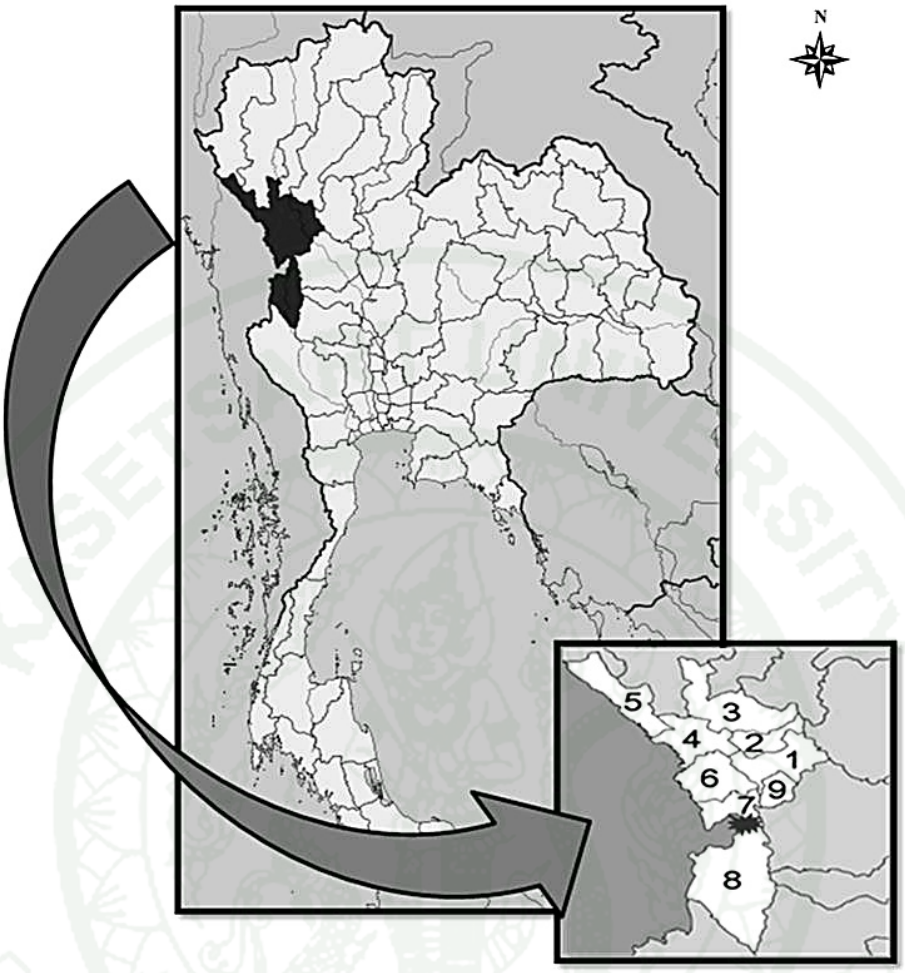


Figure 4 Collection site of Ban Tum Sua, Mae Sot District, Tak Province, western Thailand

1.2 Collection Methods

Adult mosquitoes were conducted collections every two months for two consecutive nights beginning from 06:00 PM to 06:00 AM, from November 2008 to September 2010.

For human landing collections (HLC), two persons at each site [one inside a house (indoors) (Figure 5) and one outside a house (outdoors) (Figure 6)] sat and exposed their legs as bait. The collection time for each hour was divided into 45 minutes for collection and l5 minutes resting for the 12 hour period.

For animal collections, one cow was kept under a large net that allowed mosquitoes to enter for 12 hour period (Figure 7). A collector captured mosquitoes inside the net for 15 minutes each hour. All collected mosquitoes were identified to by species. Adults caught hourly was held in separate cups, supplied with sustenance (5% sucrose solution) and transported to the insectary. Mosquito species were identified initially using morphological keys (Rattanarithikul *et al*., 2006). Climatic data (temperature and relative humidity), were measured every 1 hour from 06:00 PM to 06:00 AM using digital thermometer and hygrometer, whereas rainfall data were obtained from the Western Thai Meteorological Department, Mae Sot District, Tak Province. The year was divided into three seasons following the designation by the Thai Meteorological Department, which bases its records on rainfall and temperature values: hot season (mid February to mid May), rainy season (mid May to mid October), and dry-cool season (mid October to mid February).

Figure 5 Human Landing Indoor collections

Figure 6 Human Landing Outdoor collections

Figure 7 Cow Bait collections

1.3 Morphology and molecular identification of anophelines

All specimens of *Anopheles* mosquitoes were first identified into species by using the morphological traits following the conventional key of Rattanarithikul and Panthusiri, (1994). Female of *An. minimus* complex were morphologically identified, kept, transferred to laboratory, and placed into micro tubes before storing in refrigerator until use. Both of 2 species were presented character that could differentiate the two species. *An. minimus* may present a presector pale spot (PSP) on costal wing vein, whereas *An. harrisoni* may present both presector pale and humeral pale spots (HP) (Figure 1) (Sungvornyothin *et al*., 2006a). All of specimens of *An. minimus* complex were confirmed with specific molecular method (Garros *et al*., 2004a; Sungvornyothin *et al*., 2006b).

The Allele Specific Polymerase Chain Reaction (AS-PCR) procedure was used for molecular identification of *An. minimus* and *An. harrisoni***.** DNA extraction was done on individual adult mosquitoes, whole specimen or portions of mosquitoes, such as head, legs and thorax, according to Linton *et al*., (2001) and using the AS-PCR assay developed by Garros *et al*., (2004a). The PCR method confirmed the identification of *An. minimus* and *An. harrisoni* in Minimus Complex.

Genomic DNA was extracted from each adult mosquito followed by Linton et al. 2001, amplification by PCR and sequencing of the Internal Transcribed Spacer 2 (ITS2) region. The PCR mixture contained 17.75 µl ultrapure distilled water, 2.5 µl of 10X reaction buffer (Qiagen, Valencia, CA), 10 mM of each dNTP , 10µM of primer, 0.5 units of *Taq* DNA polymerase and 0.5 µl of DNA template. After an initial denaturation step at 94°C for two min, 40 amplification cycles were programmed as follows: 94°C for 30 sec, 54°C for 30 sec, 72°C for 40 sec, and a final extension step at 72°C for five min. One negative control was included per test run. Products were visualized by electrophoresis on a 2 % agarose gel. Primer names, sequences, and sizes of the PCR products are shown in Table 2.

Table 2 Primers designed for *Anopheles* species diagnostic assay with respective.

Species	Primer name	Sequence (5' to 3')	Size of the product (bp)	Tm (^{o}C)
Universal forward primer	ITS2	TGT GAA CTG CAG GAC ACA T		54.5
Anopheles pampanai	PAM	TGT ACA TCG GCC GGG GTA	90	56.0
Anopheles aconitus	ACO	ACA GCG TGT ACG TCC AGT	200	58.2
Anopheles harrisoni	MIC	GTT CAT TCA GCA ACA TCA GT	180	53.2
Anopheles varuna	VAR	TTG ACC ACT TTC GAC GCA	260	53.7
Anopheles minimus	MIA	CCC GTG CGA CTT GAC GA	310	57.6

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

In Garros *et al*., (2004a), the ITS2A is the universal primer that binds to the same position on the ITS DNA for 10 species in the Funestus Group (including African species *Anopheles leesoni, Anopheles funestus, Anopheles vaneedeni, Anopheles rivulorum and Anopheles parensis*), while the specific primers (PAM to MIA) bind at different locations on the ITS2 DNA of the corresponding species.

1.4 Data analysis

Seasonal abundance and biting activity of *An. minimus* was separated by the 3 season. Analysis of Variance (ANOVA) using SAS program package (SAS Release 6.01, SAS Institute, Cary, NC, USA) was used to compare variations in activity patterns and abundance by hour time of year and collections method even by collection were divided into 3 hour quarters (early evening, late evening, pre-dawn and dawn) for analysis. The level for statistical significance for all tests was set at 0.05 % (*P* value < 0.05).

2. Insecticide induced behaviors in two sibling species within the Minimus Complex, malaria vectors in Thailand.

2.1 Mosquito populations

Anopheles minimus (laboratory colony: AM-L). This colony was maintained in the laboratory more than 15 years. It was originally collected from animal quarters in Rong Klang District, Prae Province, northern Thailand in 1993 and subsequently maintained in the insectary at the Vector Borne Disease Bureau, Department of Disease Control (DDC), Ministry of Public Health, Nonthaburi, Thailand since 1995. The colony has been maintained in the insectary at Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand since 2001.

Anopheles minimus (field population: AM-F). *Anopheles minimus* was collected from the Ban Tum Sua (16^{o} 41 N 98^{o} 41 E), Mae Sot District, Tak Province,

western Thailand (Figure 4). This area is surrounded by fruit orchards on the east and by the intact native forest on the west. There is a 2 meter wide running fresh water stream bordered by a mix of native vegetations all along the margins.

Anopheles harrisoni (field population: AH-F). *Anopheles harrisoni* was captured from Ban Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14^{o} 17 N 99^{o} 11 E) (Figure 8). This area is located in a mountainous terrain completely surrounded by intact native forest. A 2 meter wide slow running stream with native vegetation along its margins is the potential habitat of *Anopheles harrisoni* (Sungvornyothrin *et al*., 2006b). Female mosquitoes were collected from cow baited traps during the evening hours (1800-0600 hr).

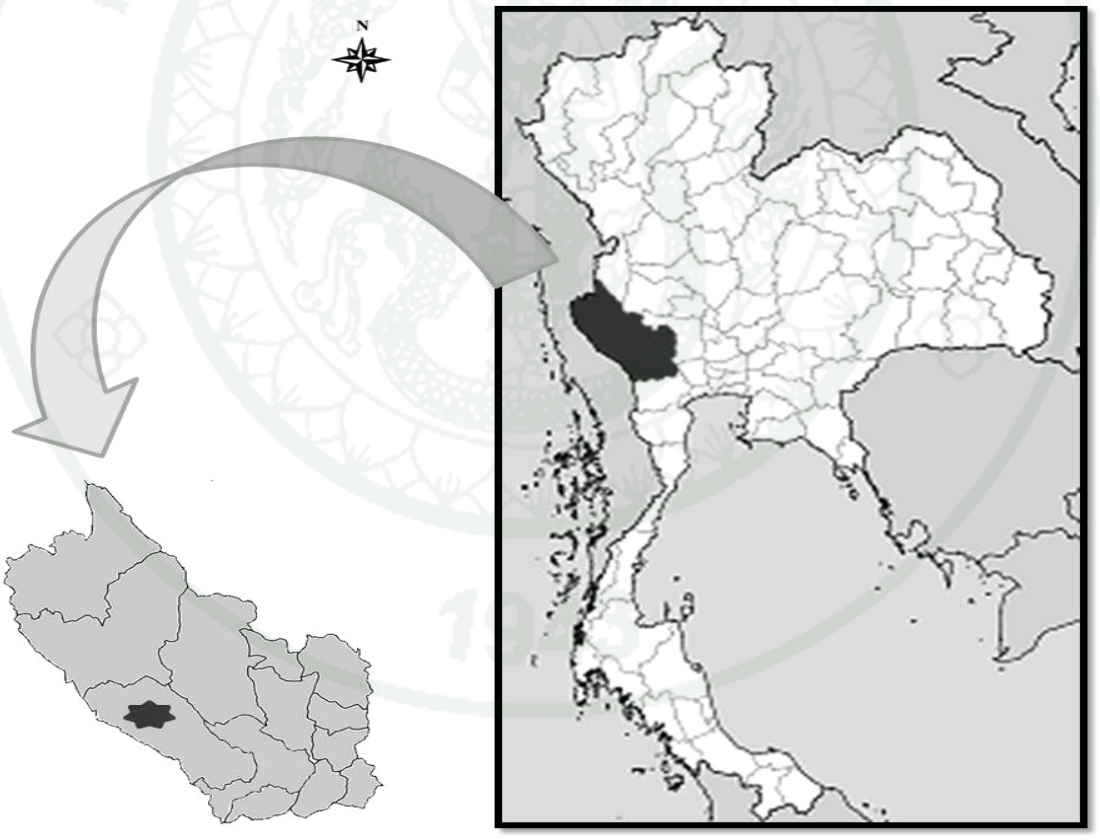


Figure 8 Collection site of Ban Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand

2.2 Morphological and molecular *Anopheles* species identification

Mosquitoes were initially identified using the morphological keys of Rattanarithikul *et al*., 2006. Specimens belonging to *An. minimus* were identified based on the humeral pale spot (HP) being absent on costal vein of both wings vein and *An. harrisoni* when the HP is present on at least on one costal vein. Subsequently, all specimens were individually processed for DNA extraction Collins *et al*., 1987. Molecular analysis was performed by the Allele-Specific assay as previously described (Garros *et al*., 2004b; Sungvornyothin *et al*., 2006a).

2.3 Insecticide impregnated papers

Filter papers, measuring 12 cm x 15 cm and 14.7 cm x 17.5 cm were prepared for WHO susceptibility tests and for excito-repellency assays, respectively. All test papers were impregnated with analytical grade bifenthrin at diagnostic doses of 0.03% using acetone as diluent for both susceptibility and behavioral tests systems (25 mg a.i./m^2, recommended field dose) (WHO, 2001). In addition, test paper were impregnated with DEET (*N*,*N*-diethyl-meta-toluamide) at 5% prepared with absolute ethanol in the same manner. All impregnated papers were prepared using diluents according to WHO protocol and treated at the rate of 2.0 ml of insecticide solution per 180 cm^2. Control papers were impregnated with diluents only. The papers were left to air dry 24 hr and all tested papers were prepared 48 hr before use.

2.4 Dose response data

Three populations of mosquito (*Anopheles minimus*; laboratory colony, *Anopheles minimus*; field population and *Anopheles harrisoni*; field population) were exposed with 0.069% of bifenthrin. For each test, 25 mosquitoes were used in each cylinder and five cylinders were used for each trials (two cylinders for control and three cylinders for treatment). Impregnated papers with only diluents were contained to control cylinders and impregnated papers with chemical were contained to treatment cylinders. Mosquitoes were introduced into holding tube for 1 hr and after

that all mosquitoes were then transferred to exposure tube for 1 hr then transferred back to holding tube again and a 10% sugar solution soak in cotton pad were provided. After 24 hr, all live and dead mosquitoes were recorded.

2.5 Behavioral tests

Experiments were designed to compare two wild-caught populations of *An. minimus* and *An. harrisoni* and a long-established laboratory colony of *An. minimus* in contact versus non contact exposures using a recommended field dose of bifenthrin. Identical test chambers (four per test trial) were used for all excito-repellency assays as previously described (Noosidum *et al*., 2008).
Only female specimen was used in excito-repellency tests. Comparisons were made with response to contact irritancy and noncontact repellency to bifenthrin and DEET. The test system was developed by Chareonviriyaphap *et al*., (2002). The stainless steel outer chamber device measures 34 cm x 32 cm x 32 cm is made up of several components numbered from 1 to 7; 1) a rear door cover, 2) an inner Plexiglas glass panel with a rubber latex-sealed door, 3) a Plexiglas holding frame, 4) a screened inner chamber, 5) an outer chamber, 6) a front door, and 7) an exit portal slot. (Figure 9) Mosquitoes were deprived of all sustenance for 24 hrs before the experiment. All assays were performed during daylight hours between 0900 and 1600 hrs with each test replicated four times. Tests were performed during an exposure period of 30 min with one-minute observation intervals to record escape of mosquitoes into exit container. After each test was completed, mortality and knockdown of all test specimens was recorded. Specimens that escaped and those remaining in expose chamber test from the treated and control chamber were held separately for 24-hour observation on mortality. Additional details on excito-repellency test system are available in previous work (Thanispong *et al*., 2009).

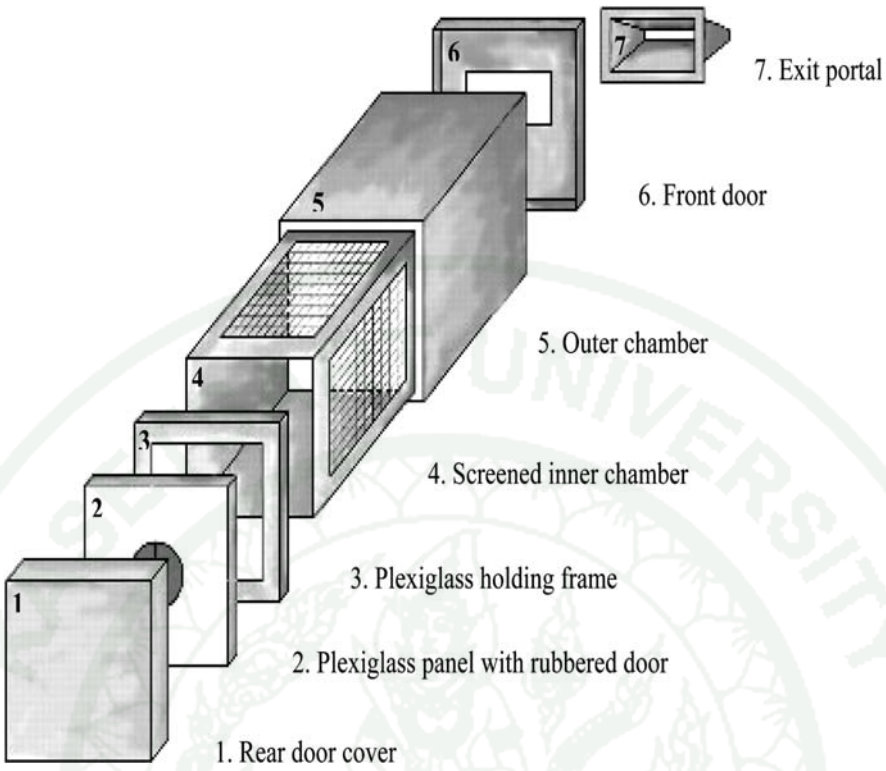


Figure 9 Excito-repellency test chamber used to observe insecticide behavioral responses

2.6 Data analysis

For susceptibility tests, the resistance status of adult mosquitoes were categorized based on Based on World Health Organization criteria (WHO, 1981)

98-100%	mortality indicates complete susceptibility
80-97%	mortality suggests the possibility of resistance ("Tolerance") that needs to be confirm and monitored by repeat testing
< 80 %	mortality strongly suggests resistance

For excito-repellency test.

Data a Kaplan-Meier survival analysis method was used to analyze the treated and patterns of escape in control assays based on 1-min intervals between populations and concentrations of bifenthrin and DEET (Kleinbaum, 1995). The time in minutes for 25% (ET25), 50% (ET50) and 75% (ET75) of test population to escape was estimated using the life table method. A log-rank method (Mantel and Haenzel, 1959) was used to compare patterns of escape behavior using SAS 6.10 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Results

Total of specimens, 360 mosquitoes of *An.minimus* from Tak Province and 206 mosquitoes of *An. harrisoni* from Kanchanaburi Province were identified using both morphological and molecular methods. All specimens from Tak Province were morphologically identified as *An. minimus,* and confirmed as *An. minimus* by molecular methods. Specimens from Kanchanaburi Province were determined as *An. harrisoni* by morphology and confirmed at the molecularly level.

Molecular methods identified the two sibling species of the Minimus Complex, *An. minimus* and *An. harrisoni*. The AS-PCR assay is used to distinguish *An. minimus* and *An. harrisoni*. Results are show lengths of amplified species-specific products as 310 bp as *An. minimus*, whereas lengths of amplified species-specific products were 180 bp for *An. harrisoni* in lanes 2-4 and first lane is shown negative control. The flagment sizes of the DNA ladder is indicated in basepairs (bp) on the lane 5 (Figures 18 and 19).

1. Seasonal abundance and biting activity of *Anopheles minimus* (Diptera: Culicidae) in western Thailand

The survey of *Anopheles minimus* in Tak Province, resulted in 3,859 *Anopheles minimus* being collected. The data regarding mosquitoes captured from November 2009 through September 2010 at Ban Tum Sua, Mae Sot District is presented in Table 4. A total of 876 (22.70% of all captures) *An. minimus* were collected from cow-bait collection

A total of 7,663 mosquitoes were collected during this study to included *An. minimus* complex, Dirus Complex, Maculatus Complex and other species. Number of mosquitoes in Minimus Complex were captured in very high (50.36% of total) follow

by Maculatus Complex (19.68% of total), Dirus Complex (0.33% of total) and 29.63% of total for other species (Table 3).

An. minimus was found to be significantly more abundant during the hot season to wet season, especially from March to May during both years. Human landing collections (HLC) produced more outdoor (46.28%) than indoor (31.02%) mosquitoes ($P < 0.0001$). Hourly feeding activity for *An. minimus* was observed for all collection methods (Table 4). A lower number of mosquitoes captured from cow-bait collections compared to indoor and outdoor HLC respectively. The activity peaks of indoor HLC occurred from 0100-0200 hr and 0300-0400 hr during year one, whereas only 1 peak (0100-0200) was observed in year two. Activity patterns are similar for both years (Figure 10). Peak of outdoor HLC occurred from 2100-2200 and 0100-0200 hr in year 1. In year 2, peak occurred from 2300-2400 and 0100-0200 hours (Figure 11). For cow-bait captures, peak activity occurred from 2300-2400 hours in year 1, 0100-0200 hours and 0300-0400 hours in year 2 (Figure 12). Total number of landing mosquitoes/h were analyzed by three-way ANOVA , comparing season (dry, hot ,wet), time period (early evening, late evening, predawn, dawn) and types of collection (indoor, outdoor, animal bait) (Table 5). There were significant differences in number of mosquitoes captured between first year and second year ($F = 18.99$, df = 1, $P < 0.0001$), between seasons ($F = 35.83$, df = 2, $P < 0.0001$), between time period ($F = 9.85$, df = 3, $P < 0.0001$) and between human and cow baited collections ($F = 14.49$, df = 2, $P < 0.0001$). Statistical analysis revealed a positive association between season and time intervals ($F = 2.99$, df = 6, $P = 0.0083$) and between season and collection methods ($F = 15.10$, df = 4, $P < 0.0001$). There were no apparent significant relationship between time period and collection methods ($F = 1.25$, df = 6, $P = 0.2835$) and between season and time period and collection methods ($F = 1.41$, df = 12, $P = 0.1629$).

The activity patterns by collection type of *An. minimus* are shown in Figures 4, 5 and 6. The total number of mosquitoes collected from bi monthly period. By month, the largest + number of mosquitoes captured occurred in May in second year (995 mosquitoes, 25.78% of total of year two). Temperature, relative humidity and rainfall

are shown in Table 4. *An. minimus* densities were associated with decrease in rainfall during the collection days but not correlative with temperature and relative humidity.

Table 3 Total numbers of *Anopheles* Complex captured from Ban Tum Sua, Mae Sot District.

Month	Species Complex			
	Minimus	Dirus	Maculatus	Others
FIRST YEAR				
November‘ 08	608	1	84	202
January‘ 09	190	-	54	58
March‘ 09	272	-	50	90
May‘ 09	366	4	81	205
July‘ 09	30	3	9	269
September‘ 09	6	8	9	123
SECOND YEAR				
November‘ 09	117	-	62	21
January‘ 10	258	1	38	82
March’ 10	429	-	23	20
May’ 10	995	-	992	66
July’ 10	303	-	84	781
September’ 10	285	8	22	336
Total	3859	25	1508	2271

Table 4 Total numbers of *Anopheles minimus* captured from Ban Tum Sua, Mae Sot District.

Month	*An. minimus*				T[1]		RH[2]		R[3]
	In	Out	Cow	Total	Indoor	Outdoor	Indoor	Outdoor	
FIRST YEAR									
November‘ 08	164	170	274	608	19.9	19.6	77.8	76.6	0
January‘ 09	58	46	86	190	21.55	21.6	77.4	71.55	0
March‘ 09	64	130	78	272	23.9	25.6	64.2	74.85	0
May‘ 09	103	181	82	366	28.85	26.6	76.45	66.35	4.0
July‘ 09	15	0	15	30	31.1	23.2	77.5	70.7	31.2
September‘ 09	3	1	2	6	24.25	24.5	79.9	79.95	10.4

Table 4 (Continued)

Month	*An. minimus*				T^1		RH^2		R^3
	In	Out	Cow	Total	Indoor	Outdoor	Indoor	Outdoor	
SECOND YEAR									
November‘ 09	63	32	22	117	18.7	17.9	90.15	91.85	0
January‘ 10	126	73	59	258	18.3	19	88.8	89.25	0
March’ 10	77	229	123	429	23.6	23.6	82.8	84.6	0
May’ 10	249	658	88	995	23.8	23.85	93.65	92	4.4
July’ 10	149	141	13	303	25.35	23.95	92.45	93.3	2.1
September’ 10	126	125	34	285	18.8	23.7	94.4	97.25	12.9
Total	1197	1786	876	3859					

[1] T : Temperature (^{o}C)

[2] RH : Humidity (%)

[3] R : Rainfall (mm)

Table 5 Three-way ANOVA of total number landing mosquitoes/h, seasons (dry, hot, and wet), collection methods (indoor and outdoor human bait, and cattle bait) and time intervals (early evening, late evening, predawn, and dawn) as discriminating factors.

Source	df	Sum of squares	Mean squares	F	Significant
Year[1]	1	3876.04167	3876.04167	18.99	<.0001
Season[2]	2	14627.62037	7313.81019	35.83	<.0001
Time period[3]	3	6030.12500	2010.04167	9.85	<.0001
Collection method[4]	2	5916.95370	2958.47685	14.49	<.0001
Season x Time period	6	3662.86111	610.47685	2.99	0.0083
Season x collection method	4	12326.99074	3081.74769	15.10	<.0001
Time period x collection method	6	1529.63889	254.93981	1.25	0.2835
Season x Time period x Collection method	12	3463.41667	288.61806	1.41	0.1629

[1] Year = Year 1 and 2

[2] Season = Dry, Hot, Wet

[3] Time period = Early evening, late evening, predawn, dawn

[4] Types of collection = Indoor, outdoor, cow

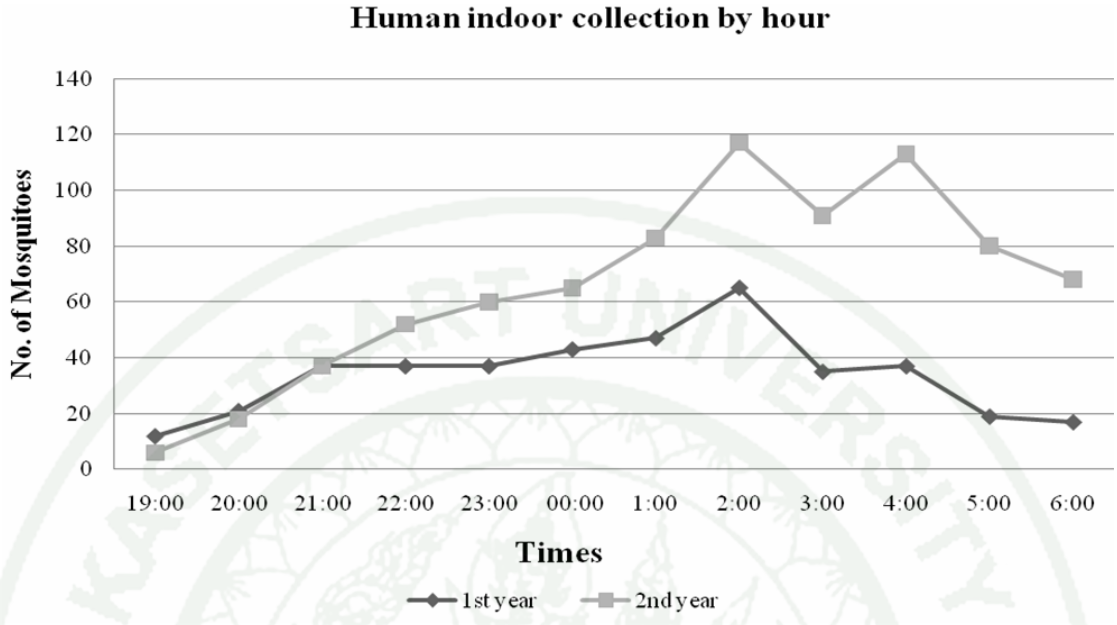


Figure 10 Temporal patterns of *An. minimus* blood feeding activity for indoor human landing collections.

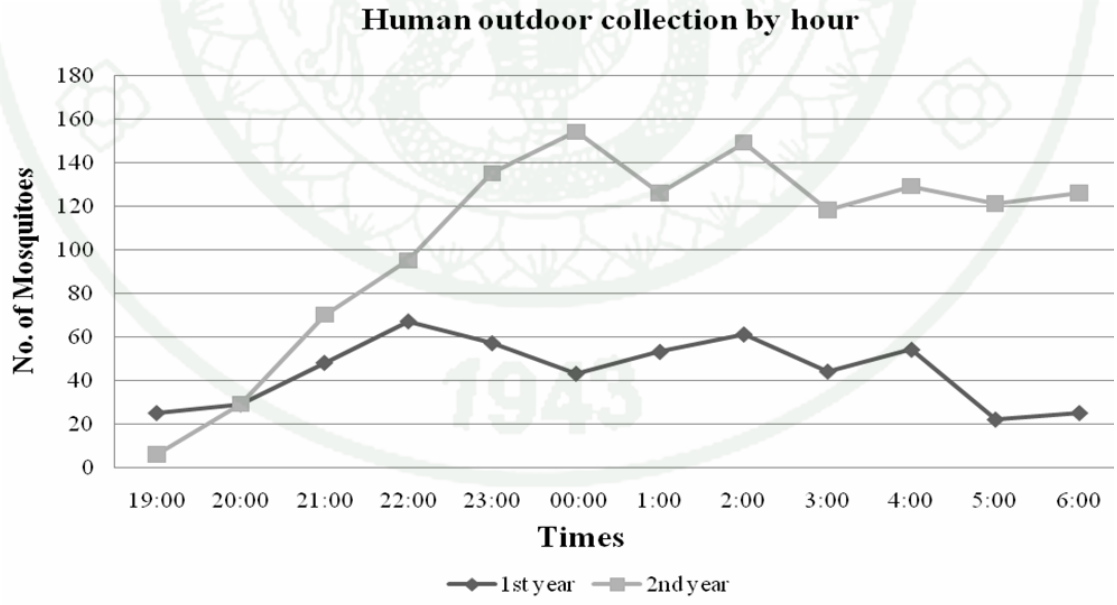


Figure 11 Temporal patterns of *An. minimus* blood feeding activity for outdoor human landing collections.

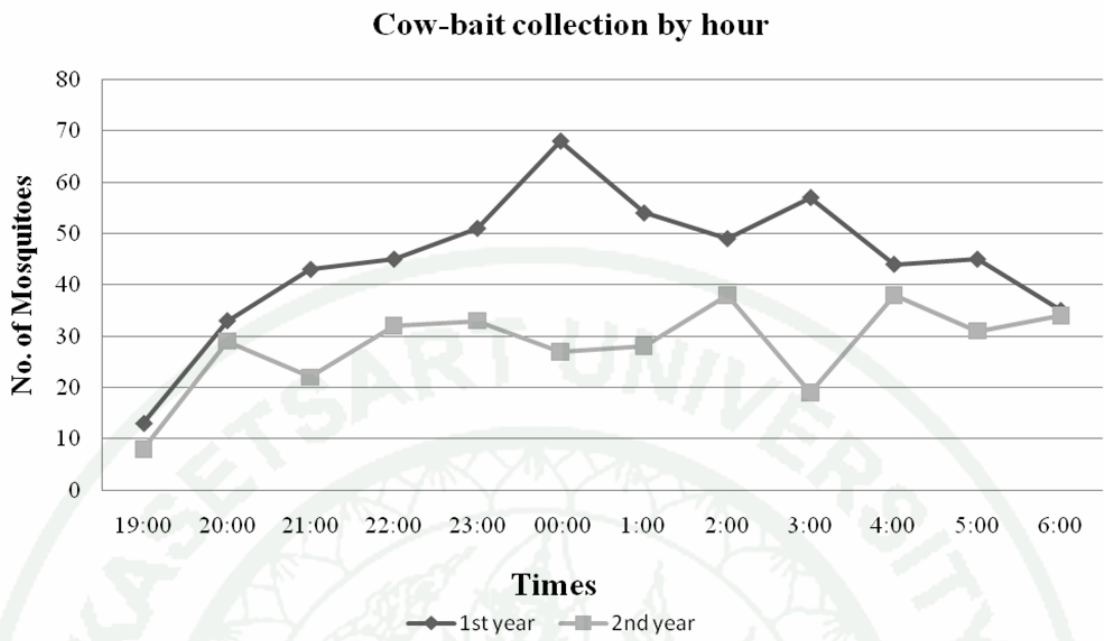


Figure 12 Temporal patterns of *An. minimus* blood feeding activity for cow-bait collections.

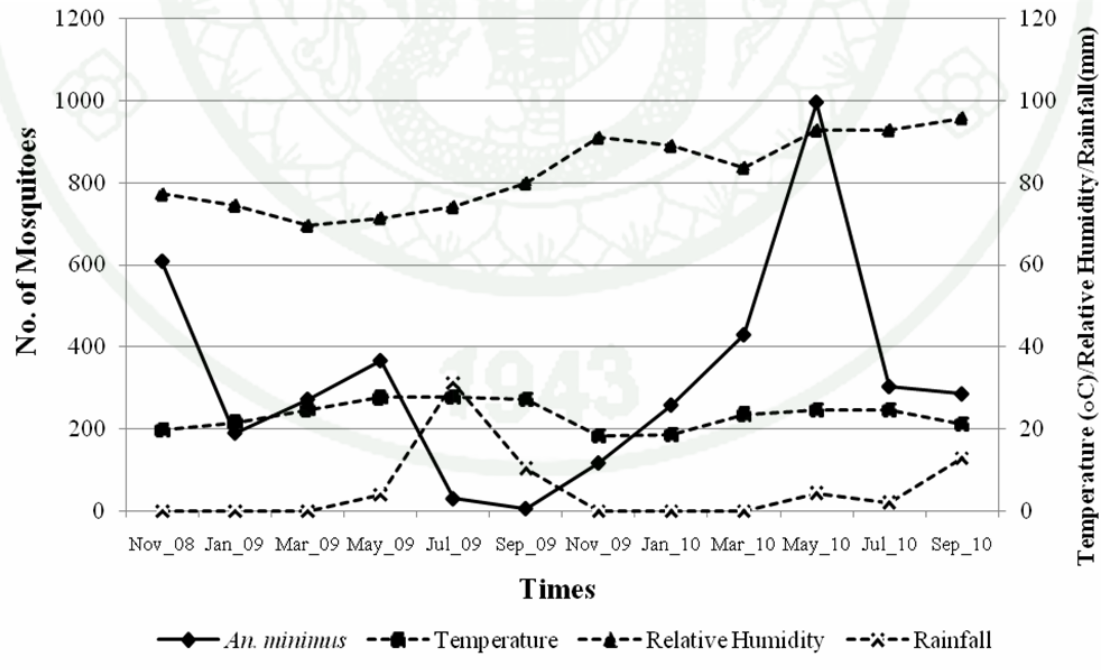


Figure 13 Monthly collection of *An. minimus* in relation to average ambient air temperature and relative humidity in Ban Tum Sua, Mae Sot District, Tak Province.

2. Behavioral responses of *An. minimus* and *An. harrisoni* (Diptera: Culicidae) to bifenthrin and DEET

2.1 Insecticide susceptibility assays

All three test populations were exposed to recommended (WHO, 2009) operational field dose of bifenthrin (0.069%) were found completely susceptible after 1 hr contact. DEET was not tested for contact toxicity without focus on only excitation and repellency. Mean percent escape and mortality to bifenthrin was separated by contact 'irritancy' (excitation) and noncontact repellency (Table 6).

2.2 Behavioral assays

Bifenthrin: Comparing the two field populations, the escape response in the bifenthrin contact trials was significantly stronger ($P = 0.0002$) in *An. harrisoni* (80% escape) within 30 min compared to *An. minimus* (40% escape). Greater escape response in the contact trials was also observed from the *An. minimus* laboratory colony (81.03%) (Figure 14). Percent lethality of escaped mosquitoes were low (0 - 4.5%), whereas those that remained in the treated chambers produced a higher range of mortality (1.7 - 83.3%). All control chambers produce low mortality (0 – 3.4%). Similarly, noncontact mortalities were low (0 – 7.7%) as were all mosquitoes in matched control chambers (0 – 5.3%). In noncontact trials, significantly stronger ($P < 0.0001$) escape response (repellency) was observed in the laboratory colony (66.7%) than either of the two field populations *An. minimus* (17.86%) and *An. harrisoni* (22.41%) (Figure 15).

DEET: The escape response (excitation) from contact trials was significantly ($P < 0.0001$) stronger in *An. minimus* (77.6%) than *An. harrisoni* (27.6%) (Figure 16). Percent escape and mortality of the three test populations exposed to 5% concentration DEET (182 mg/m^2 equivalent) responses in both contact and noncontact tests are summarized in Table 2. The *An. minimus* laboratory colony

showed the strongest 30-min escape response in contact trials (86.7%) compared to field populations. Overall, percent mortalities of escaped and nonescape mosquitoes from treated contact chambers were nil except for one test series involving *An. harrisoni* (6.25%), however none were significantly different from matched controls. In noncontact trials, *An. minimus* showed much stronger repellency (54.2 - 56.9%) compared to *An. harrisoni* (24.1%) (Figure 17). Post-exposure mortalities of escape and nonescape females in noncontact exposure and control chambers low (< 5%). For all three populations, no mortality and knockdown was observed from those mosquitoes that successfully escaped from the noncontact DEET-treated chambers.

Escape time: Table 8 summarizes the escape patterns by time and percent exiting chemically-treated chambers expressed in 1-min intervals for 25, 50, and 75% (ET_{25}, ET_{50}, and ET_{75}) of the test population to exit successfully test chambers. In contact trials, the time duration to escape was longer for the *An. minimus* laboratory colony with bifenthrin than DEET. For the *An. minimus* field population, the escape times with exposure with DEET were prolonged, whereas escape time estimates for *An. harrisoni* could not be calculated because of insufficient numbers of mosquitoes exiting during the 30-min test. Similarly, the ET_{50} and ET_{75} for *An. minimus* field population in the contact trial could not be estimated. For noncontact trials, DEET escape time estimates could only be generated for the *An. minimus* lab and field strains (ET_{25} and ET_{50} values only) and only for the laboratory colony when exposed to bifenthrin (Table 8). The remaining noncontact trials produce insufficient numbers of escaped mosquitoes.

Probability of escape: There was a marked escape response in contact trials using bifenthrin or DEET but variation between the 3 test populations. Non contact repellency was less dramatic but still significant compared to matched controls. Figures 9-12 show the proportions of mosquitoes remaining in the excito-repellency test chambers treated with bifenthrin after contact (Figure 14) and noncontact exposure (Figure 15), and DEET contact (Figure 16) and noncontact exposure (Figure 17). Proportions were used to construct the probabilities of escape from test chambers in the different assay designs. Stronger contact excitation was

seen with *An. minimus* (lab) against bifenthrin and DEET; whereas, *An. harrisoni* demonstrated strong responses to bifenthrin and *An. minimus* (field) against DEET. As with contact test findings, the lab colony showed stronger repellent reaction in escape with bifenthrin or DEET (Figures 15 and 17), while only *An. minimus* (field) presented a similar strong response when exposed to DEET (Figure 17). In all noncontact trials, there were significant differences in escape response compared with paired controls ($P < 0.05$).

Log-rank tests of significance are presented in Table 9 comparing each chemical and test format (contact and noncontact assay) within and between test populations. Within population comparisons, in all cases but one (DEET and *An. harrisoni*), there were significant differences between contact and noncontact escape responses. Multiple comparisons between the 3 populations, 2 chemicals and contact/noncontact test designs gave varying levels of significance. There was no difference in either excitation or repellency seen between colony and field strain of *An. minimus* exposed to DEET. All but two bifenthrin combinations showed differences in escape response between contact and noncontact tests. No significant difference was seen in contact escape between *An. minimus* (lab) and *An. harrisoni* or repellency response between the two field species and bifenthrin.

Table 6 Percentage escape and 24 hr mortality of *Anopheles minimus* and *Anopheles harrisoni* exposed to bifenthrin (25 mg a.i./m^2) in contact and noncontact trials.

Condition	Test Population	Bifenthrin (No. of tests) N=60	Number escaped (%)	% mortality	
				Escaped	Remain
Contact	AM-L[1]	Treatment	47 (81.03)	0	1.72
		Control	7 (11.67)	0	3.40
	AM-F[2]	Treatment	24 (40.00)	4.54	13.63
		Control	0 (0)	0	0
	AH-F[3]	Treatment	48 (80)	2.08	83.33
		Control	5 (8.62)	0	0
Noncontact	AM-L	Treatment	40(66.67)	3.33	0
		Control	9(15.79)	0	5.26
	AM-F	Treatment	10(17.86)	0	8.33
		Control	2(3.45)	0	0
	AH-F	Treatment	13(22.41)	7.69	13.33
		Control	5 (8.62)	0	4.08

[1] *Anopheles minimus* (laboratory) = AM-L

[2] *Anopheles minimus* (Tak) = AM-F

[3] *Anopheles harrisoni* (Kanchanaburi) = AH-F

Table 7 Percentage escape and mortality of *Anopheles minimus* and *Anopheles harrisoni* exposed to 5 percent DEET (182 mg/m^2) in contact and noncontact trials.

Condition	Test Population	DEET (No. of tests) N=60	Number escaped (%)	% mortality	
				Escaped	Remain
Contact	AM-L[1]	Treatment	52 (86.67)	0	0
		Control	5 (8.62)	0	0
	AM-F[2]	Treatment	45 (77.59)	0	3.45
		Control	5 (8.62)	5.17	5.08
	AH-F[3]	Treatment	16 (27.59)	6.25	2.38
		Control	1 (1.72)	0	0
Noncontact	AM-L	Treatment	32(54.24)	0	0
		Control	3(5.17)	0	3.45
	AM-F	Treatment	33 (56.90)	0	0
		Control	3 (5.17)	0	3.45
	AH-F	Treatment	14 (24.14)	0	4.45
		Control	1 (1.69)	0	0

[1] *Anopheles minimus* (laboratory) = AM-L

[2] *Anopheles minimus* (Tak) = AM-F

[3] *Anopheles harrisoni* (Kanchanaburi) = AH-F

Table 8 Mean escape time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of *Anopheles minimus* and *Anopheles harrisoni* to escape from excito-repellency chambers containing bifenthrin or DEET at 30 minutes of exposure.

Test condition	Bifenthrin			DEET		
Population	ET_{25} [1]	ET_{50} [2]	ET_{75} [3]	ET_{25}	ET_{50}	ET_{75}
Contact						
AM-L	8	14	25	2	7	16
AM-F	12	*	*	1	22	29
AH-F	6	12	26	*	*	*
Noncontact						
AM-L	13	21	*	9	22	*
AM-F	*	*	*	5	22	*
AH-F	*	*	*	*	*	*

* Insufficient number of mosquitoes escape from test chamber; ET: Escape Time: *Anopheles minimus* (laboratory): AM-L; *Anopheles minimus* (Tak): AM-F; *Anopheles harrisoni* (Kanchanaburi): AH-F

[1] ET_{25} = Escape time = Time in minutes for 25% of female mosquitoes to escape from excito-repellency test chambers

[2] ET_{50} = Escape time = Time in minutes for 50% of female mosquitoes to escape from excito-repellency test chambers

[3] ET_{75} = Escape time = Time in minutes for 75% of female mosquitoes to escape from excito-repellency test chambers

Table 9 Log-rank tests of significance comparing each chemical and test format (contact and noncontact test) within and between test populations.

Species	Bifenthrin		DEET	
	Contact	Noncontact	Contact	Noncontact
AM-L[1] vs. AM-F[2]	<0.0001	<0.0001	0.3273	0.7174
AM-L vs. AH-F[3]	0.4779	<0.0001	<0.0001	0.0004
AM-F vs. AH-F	0.0002	0.5969	<0.0001	0.0002

Chemical	AM-L	AM-F	AH-F
	Contact vs Noncontact	Contact vs Noncontact	Contact vs Noncontact
Bifenthrin	0.0184	0.0160	<0.0001
DEET	<0.0001	0.0118	0.6209

Significance set at $P < 0.05$

[1] *Anopheles minimus* (laboratory) = AM-L

[2] *Anopheles minimus* (Tak) = AM-F

[3] *Anopheles harrisoni* (Kanchanaburi) = AH-F

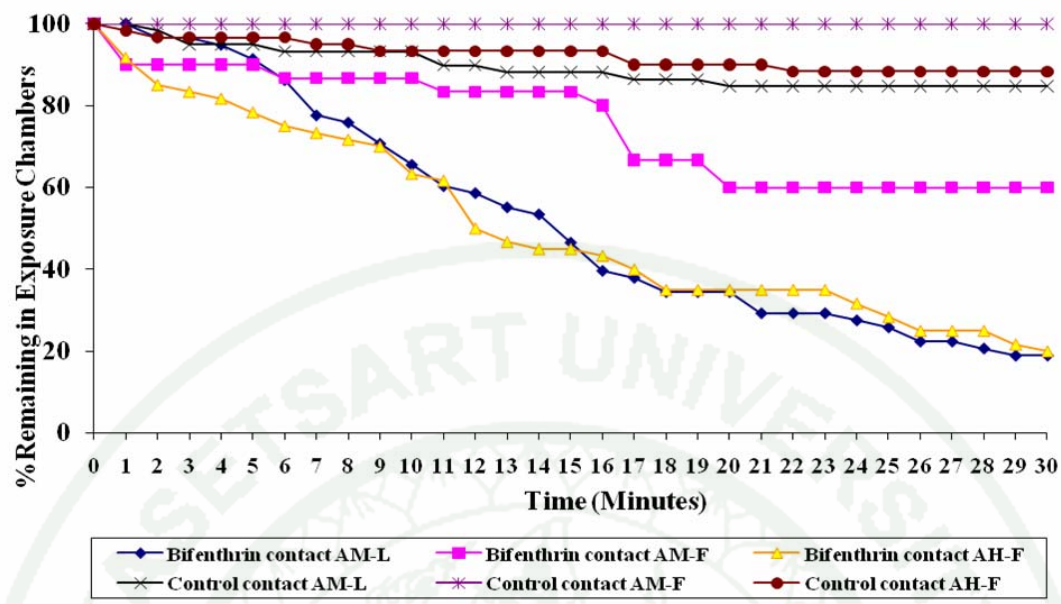


Figure 14 Escape probabilities of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to bifenthrin (25 mg/m^2) for treatment and control contact trials.

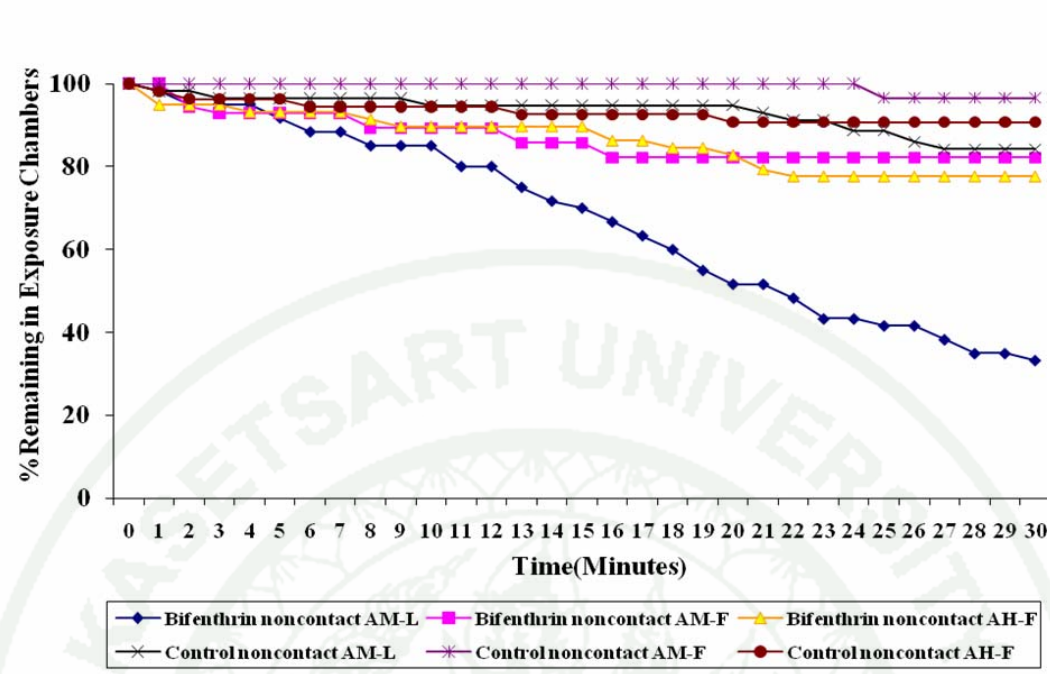


Figure 15 Escape probabilities of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to bifenthrin (25 mg/m^2) for treatment and control noncontact trials.

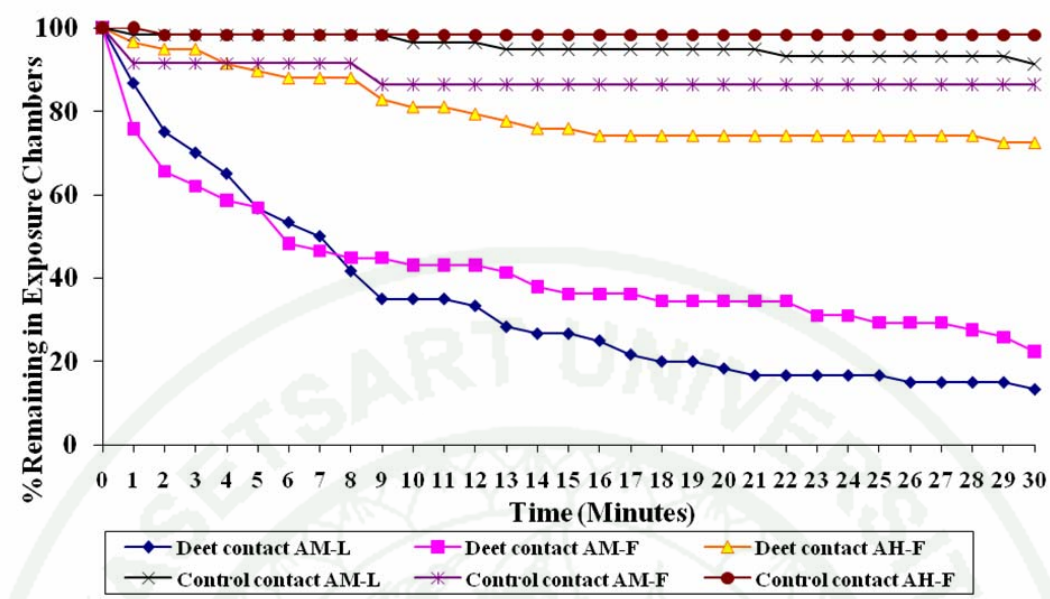


Figure 16 Escape probabilities of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to 5 percent DEET for treatment and control contact trials.

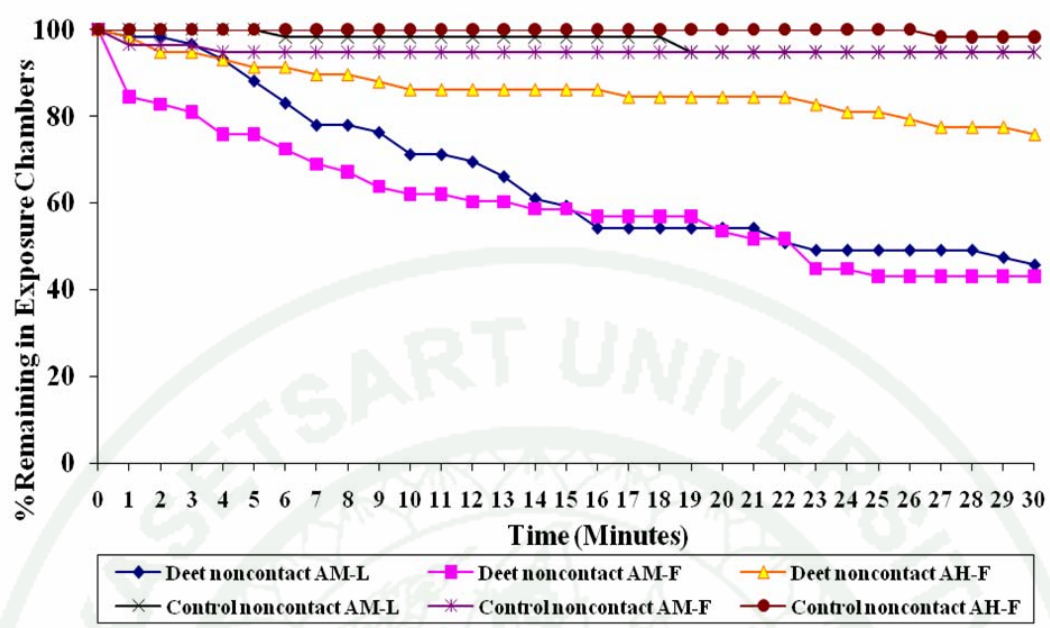


Figure 17 Escape probabilities of *Anopheles minimus* (Lab), *Anopheles minimus* (Tak) and *Anopheles harrisoni* (Kanchanaburi) exposed to 5 percent DEET for treatment and control noncontact trials.

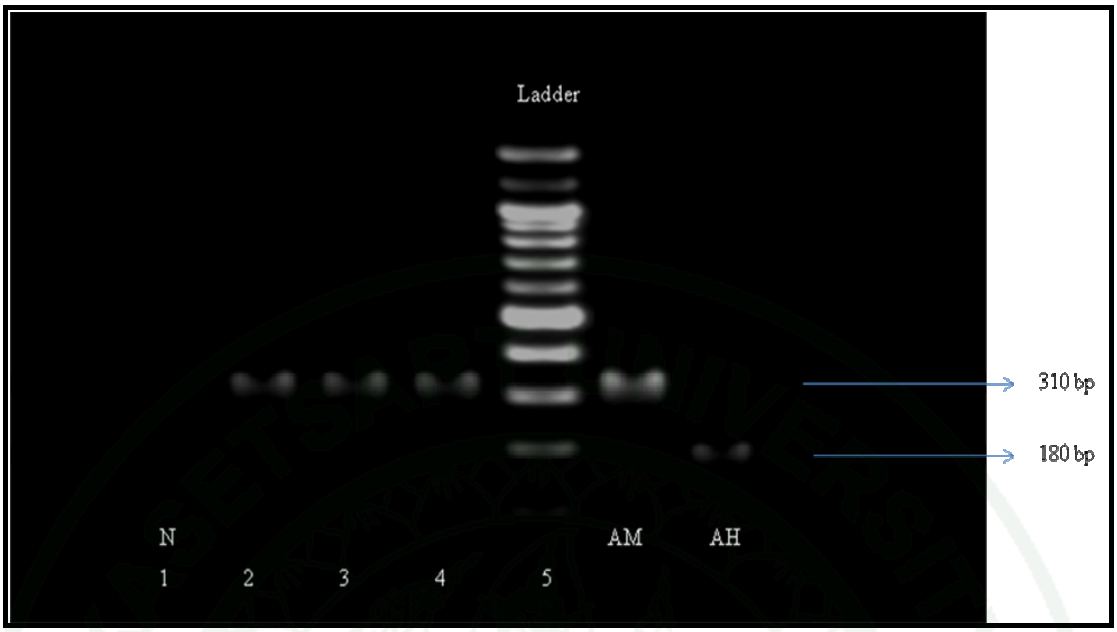


Figure 18 Multiplex Allele-Specific PCR assay. Lane 1: N = negative control, lanes 2-4: *An. minimus* from Tak Province, lane 5: 100 bp molecular ladder, AM: *An. minimus*, AH: *An. harrisoni*.

Figure 19 Multiplex Allele-Specific PCR assay. Lane 1: N = negative control, lanes 2-4: *An. harrisoni* from Kanchanaburi Province, lane 5: 100 bp molecular ladder, AM: *An. minimus,* AH: *An. harrisoni*.

Discussion

1. Seasonal abundance and biting activity of *Anopheles minimus* (Diptera: Culicidae) in western Thailand

An. minimus is one of the major vectors of malaria in hill-forest regions containing numerous small, cool freshwater streams. At Ban Tum Sua, we observed the biting activity and host preference of *An. minimus* females for 2 nights during the third week of every two months in the first year and during periods of full moon day in second year.

Ban Tum Sua, Mae Sot District, Tak Province is located in close with Thai-Myanmar border, have high risk area of malaria and have high malaria incidence rates. They have been found 31,658 cases of malaria in Tak Province from January 1999 to December 2004 (Sriwattanapongse *et al*., 2008). There are two main species of malaria in western of Thailand are *Plasmodium falciparum* and *P. vivax* (White *et al*., 1999).

In our study, *Anopheles minimus* was the most commonly captured mosquito from both human and animal- baited captured sampling methods in 2 years of collections. The biting activity and host preference of *An. minimus* were recorded. The total number is 3,859 of *An. minimus.* A larger number (77.3% of total) of *An. minimus* were captured from either human bait collections. *An. minimus* showed a considerably greater predilection for human than domestic animal hosts and slightly greater preference to biting on human outdoors (46.3%) than humans indoors (31.0%) and animal bait (22.7). The blood feeding activity of *An. minimus* s.l. (presumable *An. minimus*) has been reported in Thailand. In Mae Tha Waw Village, Tak Province, this species exhibited feeding activity throughout the night with peaks between 2100-2200 hr (Harbach *et al*., 1987). Ratanatham *et al*., (1988) reported two feeding peaks for *An. minimus* collected in Pakchong District, Nakhon Ratchasima Province; the first and largest peak occurring during early-evening (1900-2200 hr), and a second, weaker morning peak occurring near dawn (0500 hr). Rattanarithikul *et al*., (1996) also

reported two outdoor feeding periods for *An. minimus* from southern Thailand, one beginning from 1800 to 2300 hrs, and a second, more moderate, peak beginning at 0100 hours and declining slowly throughout the second half of the night. In our study, peaks of indoor collections occurred slightly later in the evening from 0100-0200 hr and 0300-0400 hrs and outdoor collections from 2100-2200 hr and 0100-0200 hr. For cow-bait captures, peaks occurred from around midnight (2300-2400 hr) in year 1 to 0100-0200 hr and 0300-0400 hr in year 2. Peak outdoor biting activity was observed earlier from 1800 to 2100 hr, with maximum activity at 1900-2000 hr at Ban Pu Teuy, Tri Yok District, Kanchanaburi Province (Chareonviriyaphap *et al*., 2003). There was only observation in biting activity one similar with our study from Mae Tha Waw Village, Tak Province showing a peak between 2100-2200 hr. *An. minimus*, peak feeding activity showed after 22.00 hr (Trung *et al.*, 2005).

An. minimus and *An. harrisoni* present a zoophilic behavior as they mostly feed on cattle (Sungvornyothin *et al*., 2006). *An. harrisoni* have been found higher exophagic and zoophilic behavior compared to *An. minimus* (Manguin *et al*., 2008) and *An. minimus* have been found more efficient malaria vector than *An. harrisoni* (Trung *et al*.,2004; Garros *et al*., 2005).

An. minimus and *An. harrisoni* have been found in sympatry and widespread in hilly forested region (Sungvornyothin *et al*., 2006b). In our study *An. minimus* densities was found positively associated with decreased rainfall in rainy season in year 1 whereas adult mosquitoes abundance of both species have been found increase during rainy season (Rattanarithikul *et al*., 1996; Chareonviriyaphap *et al*., 2003). The larvae mainly inhabit of *An. minimus* prefer edges of slow-running stream (Chareonviriyaphap *et al*., 2003; Sungvornyothin *et al*., 2006b) and are less dependent on rain fall

Bidlingmayer, 1967 has been shown a higher level of flight activity from suction traps at full moon that at new moon. In this study, Year 2, moon phase was recorded and considered in determining time of collections but not in year 1(third

week of month). Number of mosquito has been shown in year (2,387) higher than year 1 (1,472) due to the influence of moonlight and dark period collection.

Understanding the biting activity of *An. minimus* is important for proper application of control measures. Bionomics aspects defining the vector capacity and relative risk for disease transmission associated with a particular species can assist in prioritization and design of appropriate vector prevention and control strategies.

2. Behavioral responses of *An. minimus* and *An. harrisoni* (Diptera: Culicidae) to bifenthrin and DEET

In this study, two species within the Minimus Complex, representing three known test population species, were used to compare the behavioral responses to bifenthrin, a synthetic pyrethroid residual insecticide toxicant, and DEET, a common active ingredient used for mosquito bite protection by repellency. Previous studies have demonstrated strong refractory responses of *An. minimus* s.l. populations exposed to various insecticides in Thailand (Chareonviriyaphap *et al*., 2001, 2004; Pothikasikorn *et al*., 2005, 2007). However, one of the limitations from those earlier finding was a reliance on morphological characters for identification of *An. minimus* and *An. harrisoni* that likely resulted in a certain level of species misidentification (Sungvornyothin *et al*., 2006a, 2006b). This study overcame the limitations of morphology between these two species by reliably identifying populations based on DNA using a RFLP-PCR technique (Sawabe *et al*., 2003; Garros *et al*., 2004).

Significant behavioral responses were documented in both species using an excito-repellency test system; however, the degree of escape responses was different between the three test populations. In general, the long-standing *An. minimus* laboratory colony was the most responsive to contact excitation and noncontact repellency to both chemicals compared to the 2 field populations. Interestingly, significant differences in escape responses were observed between the two field populations, displaying more varied responses depending on the chemical and test format. *Anopheles minimus* was less responsive in both test designs contact and noncontact with bifenthrin compared to DEET, whereas *An. harrisoni* showed a much stronger contact excitation response with bifenthin and a much lower excitation and repellency response to DEET compared to *An. minimus* (Table 6 and 7). Repellency action (escape) with bifenthrin was comparatively weak compared to contact tests, for both field populations. With only one exception (*An. harrisoni* + DEET), significant differences in escape responses were seen between all paired contact and noncontact trials. All paired noncontact tests were significant different in escape response compared to pair controls showing repellency with occur with both compounds.

Contact excitation responses in this study were similar to those of previous reports in laboratory and field populations of *An. minimus* s.l. (Chareonviriyaphap *et al*., 2001, 2004; Sungvornyothin *et al*., 2001; Pothikasikorn *et al*., 2005, 2007). The repellency response to bifenthrin was pronounced in *An. minimus* (lab) but not the field populations, whereas both lab and field populations had very similar escape patterns to DEET. Similarly, weak repellency of *An. minimus* s.l. (approx. 95% morphologically identified as *An. harrisoni*) to pyrethroids has been reported (Chareonviriyaphap *et al*., 2001; Pothikasikorn *et al*., 2005). The comparatively weak repellency response in *An. harrisoni* may be associated with the evolutionary or innate processes in detecting chemical signals different from those in *An. minimus* (Pothikasikorn *et al*., 2005). Pu Teuy Village (predominately *An. harrisoni*) is considered a low risk area for malaria transmission in which indoor residual spray (IRS) with deltamethrin is rarely applied compared to more malaria prone areas (Mae Sot District) where *An. minimus* is abundant and a primary vector (MOPH 2008). This varying amount of previous exposure to IRS between the two field-collected populations used in this study may be partly responsible for the different avoidance behavior seen between these genetically-related species. Conversely, differences in behavior may reflectionate differences not associated with previous chemical exposure.

Within the Minimus Complex, the sibling species *An. minimus* and *An. harrisoni* have been identified in Thailand as competent vectors of malaria (Green *et al*., 1990; Sungvornyothin *et al*., 2006a; Manguin *et al*., 2010). Following the introduction of DDT in the late 1940s to control the vectors of malaria in Thailand (Chareonviriyaphap *et al*., 2000), selection pressure was reported to have modified some populations *An. minimus* s.l. to preferentially feed outdoors in proportionally greater numbers (Nutsathapana *et al*., 1986). As a consequence, insecticides may have a limited impact on populations that have the ability to alternate between indoor and outdoor feeding behaviors (Pothikasikorn *et al*., 2005, 2007). Although both species are primarily exophagic throughout their geographic range, a few populations of the Minimus Complex remain markedly predominately endophagic and display strong anthropophilic feeding preferences (Sungvornyothin *et al*., 2001, 2006b).

Behavioral diversity and innate heterogeneity in responses to insecticidal application within the complex may have had a profound influence on the ability of chemicals to interrupt malaria transmission. Therefore, careful observation of insecticide behavioral responses by geographical populations of species can assist the selection of appropriate vector prevention and control strategies to suit the locality.

In insecticide comparison tests, bifenthrin has shown relatively low irritant (excitant) and knockdown properties compared with permethrin and deltamethin (WHO 2001). These same studies concluded that an excito-repellency effect was present but bifenthrin still provided a consistent high kill by allowing mosquitoes to rest on treated surfaces for longer periods than deltamethrin as comparison. Bifenthrin also demonstrated similar airborne knockdown effects with other pyrethroids. DEET is the most widely used active ingredient in commercial topical insect repellents, showing broad effectiveness against many insect species, including mosquitoes (Rutledge *et al*., 1983), yet its mechanism of action and molecular target(s) remain unknown, or at best, unclear (Ditzen *et al*., 2008; Pickett *et al*., 2008; Syed and Leal, 2008). Although DEET vapor has long been regarded a repellent (i.e., a substance that causes orientation away from a source), more recent studies have concluded it acts as an inhibitor vice a true repellent (Dogan *et al*., 1999; Dogan and Rossignol, 1999). Based on electrophysiological responses, DEET has been shown to inhibit odor-evoked currents mediated by the insect odorant receptor complex (Ditzen *et al*., 2008), effectively inhibiting perception of host odors and chemo-attractant cues. Use of DEET in treated containers as a deterrent to induce anti-oviposition behavior on *Aedes albopictus* mosquitoes (Xue *et al*., 2001) may also be operating as an inhibitor of larval habitat chemical cues (attractants) rather than functioning as a true repellent.

Apart from insecticides used in public health control programs, other compounds, especially DEET are well known to exhibit profound behavioral responses (Surgeoner, 1995; Cox, 2005). Whether DEET performs as a true repellent or inhibitor is a question of mechanism and was not addressed in this study; however, the outcome as a deterrent (escape) was measured. There has been little published

information describing the two behavioral actions, contact excitation and spatial repellency, of DEET on anopheline mosquitoes. Assuming both contact excitation and spatial repellency are involved in the escape response seen in the contact chamber design, it is not therefore possible to clearly differentiate the specific actions of excitation and repellency. The differences in escape response between the two test designs (contact and noncontact) would presumably help separate and quantify the significance of either excitation or repellency actions alone. The same would apply in factoring out escape in paired controls with contact and noncontact tests to arrive at an adjusted percent excitation and repellency, respectively. Nevertheless, neither test design used a known attractant (e.g., host cue) inside the DEET-treated chamber, a requirement to determine if inhibition was a mode of action. Those mosquitoes that escape in the noncontact test designs appear to have been the result of repellency alone (minus a low percentage random act of escape not associated with DEET). Based on our study findings using the excito-repellency test system, DEET appears to act as contact stimulant (excitant) and a moderate spatial repellent.

The excito-repellency test system remains a useful tool for understanding how chemicals perform at sub-lethal concentrations as contact stimulants and repellents on mosquitoes (Roberts et al 1997). Together with a probability model for analyses and interpretation of data and the recent development of a high throughput screening system (Roberts *et al*., 2000; Grieco *et al*., 2007), there still remains much work ahead to describe response outcomes and mechanisms against a large array of different chemical compounds and other vector species. Experimental hut studies in field settings that include accurate sampling of house-entering vector populations are crucial for a meaningful assessment of spatial repellents as possible vector control strategies. Our findings demonstrate there are differences in behavior exposure to bifenthrin and DEET between the two species of the Minimus Complex in Thailand. Moreover, it illustrates caution in attempting to extrapolate results from colonized (selected) populations to more heterogeneous populations of the same species (or closely related) present in the field. These behavioral differences may be innately species-dependent or influenced by ecological and geographical variations between the test populations or related to exogenous factors including the relative exposure to

residual insecticides between the two collection sites, Pu Teuy and Mae Sot. Despite the many unanswered questions, we believe that our results can help optimize the use of currently available public health tools and spur the development of new ones.

CONCLUSION

1. Seasonal abundance and biting activity of *Anopheles minimus* (Diptera: Culicidae) in western Thailand

A total of 3,859 *Anopheles minimus* were collected from November 2009 through September 2010 at Ban Tum Sua, Mae Sot District. *An. minimus* was found to be significantly more abundant during the hot and wet seasons, especially from May to July periods. Peak of HLC outdoor collections occurred during the middle of the evening (2100-2200 and 0100-0200 hrs in Year 1) and shifted slighting in Year 2, peaking at 2300-2400 and 0100-0200 hrs. For cow-bait captured, peak activity occurred from 2300-2400 hours in Year 1 and shifted to later evening hours 0100-0200 and 0300-0400 hrs in Year 2. In general *An. minimus* were more abundant during the wet season compared to dry season and conditions. Findings were discussed relative to the importance of this vector for malaria transmission in the Ban Tum Sua.

2. Behavioral responses of *An. minimus* and *An. harrisoni* (Diptera: Culicidae) to bifenthrin and DEET

Behavioral responses of two species in the Minimus Complex exposed to an operational field dose of bifenthrin or DEET was described using an excito-repellency test system. Differences in response were seen between populations and species. For bifenthrin, the escape response in contact trials was significantly stronger (P = 0.0002) in *An. harrisoni* (80% escape in 30 min) than *An. minimus* (40%). Greater escape response with bifenthrin contact trials was also observed from the *An. minimus* laboratory colony (81.03%), very similar to *An. harrisoni*. For DEET, escape response in contact trial was significantly ($P < 0.0001$) stronger in *An. minimus* (77.6%) than *An. harrisoni* (27.6%) and the *An. minimus* laboratory colony showed the strongest escape response overall (86.7%) compared to both field populations. All test populations rapidly escaped after direct contact with surfaces treated with either

bifenthrin or DEET contact compared to match-paired untreated controls. Field collected *An. minimus* demonstrated a more rapid escape response to DEET than to bifenthrin, whereas *An. harrisoni* showed a converse response.

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