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TITLE: The Bionomics of *Anopheles* Species in Relation to Malaria Transmission Dynamics in Chang Island, Trat Province, Thailand, with Susceptibility and Behavioral Responses to Pyrethroids

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THESIS

THE BIONOMICS OF *ANOPHELES* SPECIES IN RELATION TO MALARIA TRANSMISSION DYNAMICS IN CHANG ISLAND, TRAT PROVINCE, THAILAND, WITH SUSCEPTIBILITY AND BEHAVIORAL RESPONSES TO PYRETHROIDS

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Observations on species diversity of *Anopheles* mosquitoes, biting patterns and seasonal abundance of potential malaria vectors were conducted in two villages on Chang Island, Trat Province, in eastern Thailand, one located near the coast and the other in the low hills of the central interior of the island. From 5,399 female anophelines, 70.25% were in the subgenus *Cellia* with remaining species in the subgenus *Anopheles*. Five important putative malaria vectors were molecularly identified, including *Anopheles epiroticus, An. dirus, An. sawadwongporni*, *An. maculatus,* and *An. minimus.* From both locations, a greater number of anophelines were collected during the dry season compared to the wet. *Anopheles epiroticus* found only along the coast showed greater exophagic and zoophilic tendencies with peak blood feeding occurring between 18:00 and 19:00. In contrast, *An. dirus* in the interior location demonstrated an activity peak between midnight and 1:00 h.

The insecticide susceptibility and behavioral responses of four wild-caught populations of female *An. epiroticus* to synthetic pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin) were assessed. Test populations were collected from different localities along the southern Thai coast, in Trat (TR), Songkhla (SK), and Surat Thani (ST) Phang Nga (PN) Provinces. All four populations were found completely susceptible to the synthetic pyrethroids. Behavioral responses using an excito-repellency test system found TR had the strongest contact irritancy escape response, followed by PN. Moderate noncontact repellency responses to all three compounds were observed in the TR population but comparatively weaker than paired contact tests. Few mosquitoes from the SK and ST populations escaped from test chambers, regardless of insecticide tested or type of trial.

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Student's signature Thesis Advisor's signature

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Wanapa Ritthison

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LIST OF ABBREVIATIONS

An.	=	Anopheles
AS-PCR	=	Allele-specific PCR
BBC	=	Buffalo-bait collections buffalo-bait collections
BVBD	=	Bureau of Vector Borne Diseases
ER	=	Excito-repellency
ºC	=	Degree(S) Celsius
cm	=	Centimeter
COI	=	Cytochrome oxidase one
Cyt-b	=	Cytochrome-*b*
dNTP	=	deoxynucleotide-5'-triphosphate
h	=	Hour
HLC	=	Human-landing collections
HLO	=	Human- landing outdoor
HLI	=	Human-landing indoor
IRS	=	Indoor residual spray
ITN	=	Insecticide treated nets
ITS2	=	Transcribed spacer region II
LLIN	=	Long-lasting insecticide treated netting
ml	=	Millilitre
mM	=	Millimolar
min	=	Minute
$MgCl_2$	=	Magnesium chloride
MOPH	=	Ministry of Public Health
P	=	Probability Value
PCR	=	Polymerase chain reaction
RH	=	Relative Humidity
rDNA	=	Ribosomal DNA
TAE	=	Tris-acetate-EDTA electrophoresis buffer solution

LIST OF ABBREVIATIONS (Continued)

VBDC	=	Vector Borne Diseases Control Center
WHO	=	World Health Organization
χ^2	=	Chi-Square
μl	=	Microlitre
μM	=	Micromolar

THE BIONOMICS OF *ANOPHELES* SPECIES IN RELATION TO MALARIA TRANSMISSION DYNAMICS IN CHANG ISLAND, TRAT PROVINCE, THAILAND, WITH SUSCEPTIBILITY AND BEHAVIORAL RESPONSES TO PYRETHROIDS

INTRODUCTION

Although malaria mortality and morbidity have been significantly reduced throughout most of the Kingdom of Thailand, the number of malaria cases remains unacceptably high in some areas, especially in the more underdeveloped and developing areas along and near the international borders with eastern Myanmar, western Cambodia and northern Malaysia. The combination of recurring parasite introduction by malaria-infected migrants and the presence of efficient malaria mosquito vectors continue to pose a major risk for these localities (Ministry of Public Health [MOPH], 2009). Based on routine surveillance activities in Thailand, recorded malaria cases peaked in 1988 with 349,291 cases countrywide and declined thereafter to 85,625 cases by 1995. Subsequently, detected malaria cases have been significantly reduced, declining from 81,692 in 2000 to only 26,150 cases in 2008 (WHO South-East Asia Regional Office, 2010). In 2013, there were 20,298 confirmed malaria cases and 47 malaria deaths (Bureau of Vector Borne Diseases [BVBD], 2013). This reduction in malaria cases has been partly the result of an effective, well-organized vector control program, especially wide coverage using indoor residual spray (IRS) and a greater distribution and availability of long-lasting insecticide treated nets (ITNs).

Effective control of malaria is not possible without a better understanding of the local vector identification, biology, ecology and behavior in relation to transmission dynamics and relative risk to the human population. A more comprehensive understanding of the epidemiology of disease transmission can lead to more efficient, targeted and site-specific vector control strategies. Detailed information on feeding and host-seeking behavior helps to define a particular species

capacity to acquire and transmit malaria, its contribution to the risk for disease transmission in the human population, and further assists in the design and implementation of appropriate vector prevention and control strategies. Studies on malaria vectors in Thailand, and elsewhere in Southeast Asia, have allowed recognition of numerous *Anopheles* 'cryptic' species and species complexes (Baimai *et al.*, 1989; Rattanarithikul *et al.*, 2006; Manguin *et al*., 2008b). Of the approximately 74 *Anopheles* species recognized in Thailand (Somboon and Rattanarithikul, 2014) only a small number are considered primary or secondary vectors of malaria; specifically, 7 species have been incriminated as major malaria vectors in Thailand: *Anopheles baimaii* (Green *et al*., 1991), *Anopheles dirus* (Rosenberg *et al*., 1990; Green *et al*., 1991), *Anopheles minimus* (Rattanarithikul *et al*., 1996), *An. maculatus* (Green *et al*., 1991; Rattanarithikul *et al*., 2006), *Anopheles pseudowillmori* (Green *et al*., 1991) *Anopheles aconitus* (Gould *et al*., 1967; Green *et al*., 1991; Maheswary *et al*., 1992) and *An. sawadwongporni* (Coleman *et al*., 2002; Somboon *et al.*, 1998). While most recent work has described various biological aspects of vectors and malaria epidemiology near the international borders with Thailand, comparatively few investigations have been performed on the status of malaria and related entomological aspects elsewhere in the country, particularly near the sea coasts. As example, Chang Island (*Ko Chang*) is one of the malaria endemic areas in eastern Thailand that has been relatively neglected regard detailed investigations. In addition, the location of Chang Island near the Cambodian border makes it susceptible for more malaria cases. Therefore, malaria transmission on this island has to be monitored on a regular basis and this work is the first baseline data for future investigations.

Chang Island is the second largest island of Thailand with 217 km^2 and located in Trat Province, approximate 350 km southeast from Bangkok and 120 km from Cambodian border.One portion of the island is a major tourist destination with nearly 251,000 visitors registered in 2009 (Department of National Park, Wildlife and Plant Conservation, 2009). In both tourist and the remainder of the island there are approximately 7,400 (Department of Provincial Administration, 2010) permanent residents over a total area covering approximately 217 km^2. Most of the island consists of either remote hill forests or coastal zones in which malaria remains a

significant health risk. The current malaria risk has been reduced over the years with only 5 malaria cases having been reported from the island in 2013 (BVBD, 2013). *Anopheles dirus* s.l., *An. minimus* s.l., *An. maculatus* s.l. and *An. sundaicus* s.l. have been reported from Chang Island based on morphological identification. However, there is little bionomic or ecological information about these mosquito vectors on the island. This study was designed to determine the species diversity, trophic behavioral patterns, (biting pattern and host feeding preferences) and behavioral responses to insecticide of potential mosquito vectors in relation to malaria transmission. Additionally, captured mosquitoes were assayed for possible infectivity for malaria parasites to both incriminate potential vector species and better define risk of infection in the human population.

OBJECTIVES

1. To determine adult mosquito feeding behavior and host preferences, biting pattern and seasonal changes in relative abundance of each putative *Anopheles* malaria vector.

2. To compare and contrast coastal and inland (hill-forest) vector ecologies and behavior in regards to malaria epidemiology and transmission risk.

3. To examine adult *Anopheles* females captured in human baited collections for *Plasmodium falciparum* and *Plasmodium vivax* parasites by PCR-based techniques.

4. To observe the behavioral responses of *An. epiroticus* to three synthetic pyrethroids (permethrin, deltamethrin and alpha-cypermethrin)

LITERATURE REVIEW

1. Malaria

Malaria is a vector-borne disease caused by *Plasmodium* protozoan parasites and transmitted by female *Anopheles* mosquitoes. Presently, there are five *Plasmodium* species that can commonly cause human malaria, namely *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Putaporntip *et al*., 2009). The majority of severe malaria cases and deaths are associated with *P. falciparum* infection, while *P. vivax* infection can also cause substantial severe infections and relapse multiple times and many months after the initial infection, but results in fatal outcomes far less frequently than *P. falciparum*. *Plasmodium ovale, P. malariae* and *P. knowlesi* are far less common than *P. falciparum* and *P. vivax* infections throughout the known range of these parasite species (Mendis *et al*., 2001).

Malaria remains a major global public health problem with more than a hundred countries (103) still reporting appreciable risk. The World Health Organization (WHO, 2013c) estimated that approximately 3.4 billion people worldwide live in malaria risk areas. There were an estimated 207 million cases and 627,000 deaths in 2012. Most cases (80%) and deaths (90%) continue to occur in the African region, followed by the South-East Asia region (13% cases and 7% deaths), the Eastern Mediterranean Region (6% cases and 3% deaths) and less still in the Western Pacific Region (0.5% cases and 0.6% deaths) and Region of the Americas (0.5% cases and 0.1% deaths), respectively (WHO, 2013c).

Thailand is one of several countries in South-East Asia region, where malaria remains endemic. Because of the efforts of the Thai National Malaria Control Programme, the numbers of malaria cases have progressively decreased due to organized mosquito control methods from 349,291 in 1988 to 85,625 cases in 1995. In 2013, there were 20,298 confirmed malaria cases and 47 malaria deaths attributed to the disease, with a species prevalence of approximately 45.7% *Plasmodium vivax,*

39.6% *P. falciparum* and 0.34% *P. malariae* (BVBD, 2013). Although malaria has dramatically declined in many areas of the country, some still remain prone to periodic malaria outbreaks that occur in high risk areas, especially along the international borders with Myanmar and Cambodia, areas typically associated with forest-fringe foothills and where the presence of efficient malaria vectors exist. The populations at risk along the borders represent both Thai (e.g. farmers, rubber and forestry workers, tourists, border patrol polices, soldiers, miners and hunters etc.) and non-Thai groups (primarily refugees, migrant laborers, and treatment-seeking groups along the border).

2. Malaria in Chang Island

Chang Island, the second largest island of Thailand, is located in along the east coast of the Gulf of Thailand. Most of the island consists of both hill forests and coastal zones were malaria remains a significant health threat. This island is malaria endemic, ranging from periodic (stable) transmission (designated 'A2' zone) to high risk (B1 zone) outside the main tourist area, depending upon location and season. Nine villages are designated as A2 and another 9 villages as B1 areas (BVBD, 2010). From 2002 to 2013, a total of 414 malaria cases, were indigenous case were recorded from the island (Table 1). During this period, the number of infections peaked in 2002 with 108 cases including 20 cases of *P. falciparum* and 87 cases of *P. vivax* infections (Vector Borne Diseases Control Center [VBDC] 3.4 Trat, 2013). Consequently, malaria situation has been reduced significantly. Only two malaria species have been reported on the island, *P. falciparum* and *P. vivax* commonly occur on the island. Malaria vector species, *Anopheles dirus* s.l., *An. minimus* s.l., *An. maculatus* s.l. and *An. sundaicus* s.l. have long been known to occur in Chang Island, but based on morphological identification alone (VBDC 3.4 Trat, 2010). However, there is little bionomic or epidemiological information about these mosquito vectors on the island. One of the primary objectives of this study was to determine the species diversity and trophic behavioral patterns (biting cycle and feeding preferences) of potential mosquito vectors in relation to malaria transmission.

Table 1 Number of malaria cases, malaria-attributable deaths and *Plasmodium* species in Chang Island from 2002-2013.

Year	Cases	Deaths	*P. falciparum*	*P. vivax*
2002	107	0	20	87
2003	48	0	25	23
2004	38	0	20	18
2005	20	0	8	12
2006	61	0	9	52
2007	35	0	11	24
2008	9	0	1	8
2009	24	0	4	20
2010	11	0	0	11
2011	29	0	1	28
2012	27	0	3	24
2013	5	0	0	5

Source: VBDC 3.4 Trat (2013)

3. Malaria vectors in Thailand

The efficiency of *Anopheles* mosquitoes for transmitting malaria parasites varies between different species and geographic locations. Throughout the world, there are 537 known species of *Anopheles* mosquitoes (Harbach, 2013) and approximately 41 of them play an important role as vectors of malaria (Sinka, 2013). Of these, about 35 species are within a species complex, which comprises collectively of around 145 sibling species members (Harbach, 2013).

Of the approximately 74 *Anopheles* species recognized in Thailand, consisting of 73 formally named species and a closely related species of *Anopheles gigas* s.l. Giles (Somboon and Rattanarithikul, 2014; Somboon *et al.*, 2011). Species include members in the Leucosphyrus Group (Neomyzomyia), Maculatus Group (Neocellia

series), Funestus Group (Neocellia series), Barborostris Group (Myzorhynchus Series), and Ludlowae Group (Pyretophorus Series), which include 9 species of mosquitoes that have been incriminated as important malaria vectors in Thailand (Green *et al.*, 1991; Rattanarithikul *et al*., 2006; Suwonkerd *et al.*, 2013), including *An. dirus* (Baimai *et al*., 1988; Rosenburg *et al.*, 1990), *An. baimaii*, (Baimai *et al*., 1988; Green *et al*., 1991) *An. minimus* (Ratanatham *et al.*, 1988; Rattanarithikul *et al*., 1996), *An. pseudowillmori*, *An. maculatus* (Cheong *et al*., 1968) and *An. aconitus* (Maheswary *et al*., 1992), *An. sawadwongporni* and *An. campestris* (Somboon *et al*.,1998; Coleman *et al*., 2002) which are all closely associated with hill forest and forest-fringe areas. Malaria also occurs along the coastal areas and islands where the brackish water breeding mosquito, *An. sundaicus* s.l. occurs (Sumruayphol *et al*., 2010). Those species within species complexes in Thailand and neighboring countries cluding Myanmar, Vietnam, Laos, Cambodia, Malaysia and Indonesia are shown in Table 2.

Table 2 Known and potential malaria vector species in Thailand.

Species complexes	Vector of human malaria (*P. vivax, P. falciparum, P. malariae and P. ovale*)		Vector of macaque malaria (*P. knowlesi*)	
	Thailand	Neighboring countries	Thailand	Neighboring countries
An. dirus complex				
An. dirus	+	+	-	-
An. baimaii	+	-	-	-
An. cracens	-	-	+	+
An. minimus complex & related species				
An. minimus	+	+	-	-
An. aconitus	+	+	-	-
An. maculatus group				
An. maculatus	+	+	-	-
An. pseudowillmori	+	-	-	-
An. sawadwongporni	+	-	-	-
An. sundaicus complex				
An. epiroticus	+	+	-	-
An. barbirostris complex				
An. campestris	+	-	-	-
An. leucosphyrus complex				
An. latens	-	-	+	+

+ malaria vector ; - not recorded as a malaria vector

Source: Saeung (2012)

3.1 *Anopheles* (*Cellia*) *dirus* Peyton & Harrison species complex

The Dirus Complex belongs to the subgenus *Cellia*, Leucosphyrus Group in the Neomyzomyia Series (Harbach, 2004). Eight species have been recognized within the Dirus Complex (Baimai *et al.*, 1988; Sallum *et al*., 2005b; Takano *et al*., 2010). In Thailand, five species have been recognized in the complex: *An. dirus* (formerly species A), *An. cracens* (formerly species B), *An. scanloni* (formerly species C), *An. baimaii* (formerly species D) and *An. nemophilous* (formerly species E) (Baimai *et al*., 1988). Within the Dirus Complex, *An. dirus* and *An. baimaii* have been incriminated as primary vectors of malaria in Thailand (Xu *et al*., 1998). Members of this complex are found in forested foothills, deep forests, and cultivated forests preferring shaded habitats. *Anopheles dirus* occurs throughout Thailand, while *An. baimaii* is more common in the west of country (Sallum *et al*., 2005a). *Anopheles cracens* is only known from southern (peninsular) Thailand. *Anopheles scanloni* occurs in western and southern Thailand and appears to be intimately linked to limestone environments, primarily restricted to 'islands'of limestone karst habitats (O'Loughlin *et al*., 2008). *Anopheles nemophilous* has an apparent patchy distribution along the Thai–Malay Peninsula and Thai border areas with Myanmar and Cambodia (Manguin *et al*., 2008a). Larvae of this species complex typically inhabit small, usually temporary, mostly shaded bodies of fresh, stagnant water, including pools, puddles, animal footprints, margins of small streams, and sometimes domestic wells. These species are typically found in hilly or mountainous regions with primary or secondary evergreen and deciduous forests, bamboo forests, and fruit and rubber plantations (Baimai *et al*., 1988; Prakash *et al*., 2001). Sungvornyothin *et al.* (2009) found *An dirus* s.l. was more abundant during the wet season compared with the dry and hot seasons in Sai Yok District, Kanchanaburi Province, Thailand, while adult densities have been positively associated with increased rainfall (July to August) (Tananchai *et al*., 2012a). *Anopheles dirus* has shown relatively stronger zoophilic tendencies i.e., captured higher numbers on cattle (63.2%) compared to 36.8% from indoor and outdoor landing collections (Tananchai *et al.*, 2012b), a similar host pattern observed by Sungvornyothin *et al.* (2009). *An. dirus* at Sai Yok District showed a prominent indoor biting peak between 19:00 – 20:00 and a later outdoor

peak of 23:00 to 24:00 h, while cattle-baited collections showed one clear peak in the early evening (19;00 – 20;00 h) followed by a steady decline throughout the rest of the night (Tananchai *et al*., 2012b).

3.2 *Anopheles* (*Cellia*) *minimus* Theobald species complex

The Minimus Complex belongs to the subgenus *Cellia*, the Minimus Subgroup, the Funestus Group in the Myzomyia Series (Harbach, 2004), of which some members represent major malaria vectors throughout mainland Southeast Asia. Three sibling species members are within this complex, i.e., *An. minimus* (formerly species A), *An. harrisoni* (formerly species C) and *An. yaeyamaensis* (formerly species E), 2 of which (*An. minimus* and *An. harrisoni*) are present in Thailand. Only *An. minimus* has been definitively incriminated as a primary vector of malaria (Green *et al*., 1991) with sporozoite infection rates between 0.3-1% (Gingrich *et al*., 1990; Manguin *et al.*, 2008a). *Anopheles minimus* and *An. harrisoni* have been found sympatric in western Thailand (Garros *et al.*, 2006). *Anopheles minimus* is widespread throughout the country while *An. harrisoni* is confined in the western and northern subregions, including Tak and Chiang Mai Provinces (Rattanarithikul *et al*., 2006). Both species are typically found in forested hilly areas and principally used stream pools and still margins as larval habitats. Larvae of this species complex occur in cool unpolluted water with partial shade and grassy margins. Larvae are also found in ponds, lakes, palm swamps, seepage pools and springs, rock pools, small ditches, bogs and marshes, ground pools, and rice fields (including fallow fields and pools in dry fields) (Rattanarithikul *et al*., 2006). Seasonal *Anopheles minimus* and *An. harrisoni* have been found more prevalent during the hot and wet periods in Pu Teuy Village, Sai Yok District, Kanchanaburi Province (Sungvornyothin *et al.*, 2006). Both species demonstrate stronger exophagic and zoophilic tendencies. A more anthropophilic feeding behavior and preference to blood feed outdoors (approx. 60%) by *An. minimus* was observed in Mae Sot District, western Thailand (Tisgratog *et al*., 2012). For *An. harrisoni,* outdoor biting activity occurred throughout the night with one distinct biting peak immediately after sunset (18:00 h), whereas indoor biting showed two small peaks at 20:00 and 24:00 h. (Sungvornyothin *et al.*, 2006).

Tisgratog *et al.* (2012) observed indoor biting activity of *An. minimus* was pronounced in the early morning hours between 01:00 and 04:00 h with a peak at 02:00 h, whereas an outdoor biting surge began around 22:00 h, with a peak near midnight.

3.3 *Anopheles* (*Cellia*) *maculatus* Theobald species group

The Maculatus Group belongs in the Neocellia Series (Harbach, 2004). Nine species have been formally recognized within the Maculatus Group, of which seven species are found in Thailand, i.e., *An. sawadwongporni* (species A), *An. maculatus* (species B, plus Form E), *An. dravidicus* (species C), *An. notanandai* (species G), *An. willmori* (species H), *An. pseudowillmori* (species I) and *An. rampae* (*An. maculatus* metaphase karyotype Form K) (Rattanarithikul and Green, 1986; Green *et al*., 1992; Saeung, 2012; Suwonkerd *et al*., 2013; Harbach, 2013). *Anopheles pseudowillmori*, *An. sawadwongporni* and *An. maculatus* have been incriminated as either important or secondary malaria vectors in Thailand (Green *et al.*, 1991, 1992; Rattanarithikul *et al*., 1996; Saeung, 2012). Both *An. maculatus* and *An. sawadwongporni* are widely distributed throughout the country except in the far southern part of the country for *An. sawadwongporni*, whereas *An. maculatus* is found quite commonly throughout the peninsular region (Baimai *et al*., 1993; Rattanarithikul *et al*., 1996). *Anopheles pseudowillmori* is found predominately along the Thai-Myanmar border and far northern areas of the country (Green *et al*., 1992; Rattanarithikul *et al.*, 1995). Members of this species group are found in or near hilly and mountainous areas. Larvae typically occupy fresh water ponds, lakes, swamps, ditches, pits wells, pools (grassy, sand, ground, flood, stream), stream margins, seepage springs, rice fields, animal foot prints, wheel tracks, artificial containers, tree holes and bamboo stumps (Rattanarithikul *et al.*, 2006). Muenworn *et al.* (2009) found that *An. maculatus* and *An. sawadwongporni* were more abundant during the wet season at Pu Teuy (Kanchanaburi Province). Peak biting activities of both species occurred between 20:00-23:00 h and a smaller peak between 01:00-03:00 h with a greater tendency to feed on cattle than humans.

3.4 *Anopheles* (*Cellia*) *sundaicus* Rodenwaldt species complex

The Sundaicus Complex belongs to the subgenus *Cellia* and the Ludlowae Group in the Pyretophorus Series (Harbach, 2004). This complex is regarded as the principal vector of malaria along many coastal areas in Southeast Asia (Adak *et al*., 2005; Alam *et al*., 2006; Dusfour *et al*., 2007a). The species complex is widely distributed from northeastern India, eastwards to southern Vietnam (south of the 11th parallel) and southwards to the Andaman and Nicobar Islands (India), Malaysia (peninsular and northern Borneo), and Indonesia (Java, Sumatra, Sulawesi, and Lesser Sunda Island group) (Linton *et al*., 2001; Dusfour *et al*., 2004a). At least four sibling species are recognized in the complex, *An. epiroticus* (formerly *An. sundaicus* species A), *An. sundaicus* s.s., *An. sundaicus* species E and *An. sundaicus* species D (Dusfour *et al*., 2007b; Alam *et al*., 2006). In Thailand, only *An. epiroticus* is now regarded as present and is found along the coastal regions of the Indian Ocean and Gulf of Thailand and scattered islands in eastern and southern regions (Scanlon *et al*., 1968; Sukowati *et al*., 1996, 1999; Linton *et al*., 2005; Rattanarithikul *et al*., 2006) and has been incriminated as a secondary malaria vector (Gould *et al*., 1966; Harinasuta *et al*., 1974; Chowanadisai *et al*., 1989). The larvae of the Sundaicus Complex generally require sunlit habitats of pooled stagnant water with green algae and non-invasive, floating vegetation. *Anopheles epiroticus* larvae are typically associated with brackish water habitats (Linton *et al*., 2001; Dusfour *et al*., 2007b). Rao (1984) reports that the most suitable breeding places contain brackish water. The major breeding places include coastal shrimp/fish ponds and inland sea-water canals, but immature stages also inhabit ponds, swamps, blocked lagoons, open mangrove, and rock pools (Dusfour *et al*., 2004a; Harinasuta *et al*., 1974). Larvae of *An. epiroticus* have been found in cement tanks with a pH range of 8.2-8.7 in Rayong Province. The biting pattern of *An. epiroticus* was shown to increase steadily between 18:00-24:00 h with a peak of biting activity around midnight (Sumruayphol *et al*., 2010). The behavioral patterns of the *An. sundaicus* complex vary by geographical location (Dusfour *et al*., 2004a). In Thailand, Gould *et al*., (1966) observed that *An. sundaicus* s.l. (*An. epiroticus*) had a greater outdoor biting frequency and feeding preference on cows, indicating a more pronounced exophagy and zoophily.

4. Molecular identification of species complexes

The majority of the malaria vectors in Thailand belong within species complexes or a closely-related group wherein species are morphologically very similar that are impossible to distinguish (Harbach, 2004; Manguin *et al*., 2008a). Accurate identification is essential to estimate species composition at each area and to determine which species are responsible for malaria transmission. In general, individual species cannot be reliably separated by morphological criteria alone and require alternative methods for identification. Molecular-based methods are widely used for species identification especially the utilization of the polymerase chain reaction (PCR) method, which is reasonably rapid, reliable, and extremely sensitive. The major advantage of PCR is that it requires only miniscule amounts of DNA for amplification (detection) analysis. The allele-specific PCR (AS-PCR) assay is a variation of the polymerase chain reaction which is used to identify or utilize single base differences in DNA which requires the sequence of the target DNA sequence, including differences between the alleles. AS-PCR has been used to identify members of *Anopheles* complexes in Southeast Asia using ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) (Manguin *et al*., 2002; Walton *et al*., 1999; 2007; Garros *et al*., 2004; Dusfour *et al*., 2004b; 2007a; Linton *et al*., 2001, 2005; Takano *et al*., 2010). The ribosomal DNA internal transcribed spacer region II (ITS2) and mitochondrial cytochrome oxidase one (COI) loci are quickly evolving gene regions that are commonly used to discriminate between anopheline species at the molecular level (Beebe and Saul, 1995). AS-PCR is more frequently used to differentiate members within *Anopheles* species complexes in Thailand. This assay was developed to distinguish and unambiguously identify members of the Dirus Complex using ITS2 sequences (Walton *et al.*, 1999). Tananchai *et al.* (2012a) subsequently used this protocol to identify *An. dirus* and *An. baimaii* in Kanchanaburi Province. For the Minimus Complex, AS-PCR is frequently used to distinguish *An. minimus* and *An. harrisoni*, and ITS2 for closely related species such as *An. aconitus, An. pampanai*, and *An. varuna* n (Garros *et al*., 2004; Manguin *et al.*, 2008b). Populations of *An. minimus* from Tak and Kanchanaburi Provinces have been examined by the same method (Sungvornyothrin *et al.*, 2006; Tisgratog *et al*., 2012). ITS2 (rDNA) was used

to distinguish five species in the *An. maculatus* group in northwestern Thailand (Walton *et al*., 2007). Additionally, AS-PCR using cytochrome oxidase I (COI) and cytochrome-*b* (Cyt-b) of mitochondrial DNA can distinguish between *An. epiroticus*, *An. sundaicus* B and C, and *An. sundaicus* E belong to Sundaicus Complex (Dusfour *et al.,* 2007a).

5. Malaria vector control and insecticide resistance

Malaria vector control in Thailand has relied heavily on two methods based on insecticides comprised of indoor residual spraying (IRS) and long-lasting insecticide treated netting (LLIN) to reduce vector survival and biting densities, thus suppressing human vector contact (Chareonviriyaphap *et al*., 2013). These methods can be effective for 3-6 months (IRS, depending on insecticide used or for up to 4-5 years in the cases of LLINs and material durability). Beginning in the 1950s, DDT was the chemical of choice and was used extensively in malaria endemic areas. DDT use was gradually phased out in Thailand between 1995 and 2000 and replaced by two pyrethroids, deltamethrin and permethrin (Chareonviriyaphap *et al*., 1999, 2000). Consequently, synthetic pyrethroids have gained general acceptance for use in both IRS and LLIN (Chareonviriyaphap *et al*., 2001) due to their relatively low mammalian toxicity but high invertebrate potency at low concentrations resulting in rapid immobilization (knockdown) and killing (toxicity) action. Deltamethrin has been used primarily IRS of house indoor surfaces and permethrin for LLINs and window and door curtains (Chareonviriyaphap *et al*., 2004). Additionally, alpha-cypermethrin has gradually become more commonly used for protection against indoor and outdoor biting mosquitoes, including *Anopheles* (Grieco *et al.*, 2007; Mongkalangoon *et al*., 2009). As pyrethroids have begun to lose effectiveness globally against mosquitoes that have developed significant resistance against them in many areas (Corbel and N'Guessan, 2013), it is crucial to continue monitoring how mosquitoes respond to synthetic pyrethroids and other compounds (old, new and novel), including behavioral responses to sub-lethal concentrations. In Thailand, the resistance of *Anopheles* to synthetic pyrethroids has not yet been reported. However,

resistance to DDT has been documented in two species, *An. annularis* s.l. and *An. minimus* s.l. in the northern of Thailand (Prapanthadara *et al*., 2000).

6. Behavioral response to insecticides

Behavioral responses by mosquitoes to insecticides have been recognized for many decades (Kennedy, 1947; Davidson, 1953). In the past, chemicals that produced avoidance responses in mosquitoes were often regarded as inferior attributes when selecting compounds for vector control programs. Virtually all work focused on the direct insecticidal (toxic) action on insect populations as the primary, if not only, means to control vectors and transmission. Until recently, relatively few investigations have concentrated on behavioral responses, specifically avoidance or deterrence of mosquitoes exposed to chemicals. In addition to toxicity, at least two different forms of behavioral responses, broadly defined as 'excito-repellency' include contact excitation (irritancy) and noncontact spatial repellency (Roberts *et al*., 2000). Irritant escape responses follow direct physical contact with an active ingredient (e.g., a chemically-treated surface); whereas repellency results when an insect spatially detects and avoids a space containing an active ingredient without making physical contact (Roberts and Andre 1994; Chareonviriyaphap *et al*., 1997). Both types of behavioral responses can be experimentally differentiated by using an excito-repellency (ER) test system (Chareonviriyaphap *et al*., 1997; Tanasinchayakul *et al*., 2006). The system for describing and quantifying the excitatory effects of insecticides on mosquitoes was first developed in the early 1960's and has been modified over subsequent decades (Rachou *et al*., 1963; Evans, 1993; Chareonviriyaphap *et al*., 1997; Roberts *et al*., 2000). An improved ER box system developed by Tanasinchayakul *et al*.(2006) for testing both contact irritancy and noncontact repellency has been modified further into a collapsible chamber designed for greater ease of use and set-up in the field and laboratory (Kongmee *et al.*, 2012b). Numerous studies have shown clear behavioral avoidance in various *Anopheles* species to synthetic pyrethroids (Sungvornyothrin *et al*., 2001; Chareonviriyaphap *et al*., 2002, 2006; Pothikasikorn *et al*., 2005; Muenworn *et al*., 2006). In general, behavioral responses of *Anopheles* to pyrethroids have consistently resulted in

significantly stronger contact irritant responses when compared to spatial repellency activity. Additionally, a modular, high-throughput laboratory-based assay system for screening of irritancy, repellency and toxicity has been developed (Grieco *et al*., 2007). Since the development of these two independent test systems and a quantified mathematical framework for analysis and interpretation, published accounts on mosquito behavioral responses to public health insecticides and potential topical repellent compounds have progressively increased (Sungvornyothin *et al*., 2001; Kongmee *et al*., 2004; Chareonviriyaphap *et al*., 2001, 2004; Grieco *et al*., 2005, 2007; Pothikasikorn *et al*., 2005, 2007; Muenvorn *et al*., 2006; Polsomboon *et al*., 2008; Monkalangoon *et al*., 2009; Thanispong *et al*., 2010; Tisgratog *et al*., 2011; Tananchai *et al*., 2012b).

7. Detection of *Plasmodium* species in *Anopheles* mosquitoes

Human malaria is caused by five *Plasmodium* parasites: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. *Plasmodium falciparum* and *P. vivax* cause the majority of malaria infections in humans worldwide, wherein *Plasmodium falciparum* is responsible for the vast majority of the severe infections and malaria-associated mortality (Sachs and Malaney, 2002). The sporozoite rate (*Plasmodium* infections in the salivary glands of *Anopheles*) has long been used to evaluate the status of malaria vectors and transmission intensity in r malaria studies and control programs. Traditionally, sporozoites in *Anopheles* have been detected by microscopic dissection, a laborious, pain-staking process with a high degree of error. However, this technique requires skilled personnel to perform accurately several methods have been available for the detection of malaria parasites in vectors, most notably use the circumsporozoite enzyme-linked immunosorbent assay (Wirtz *et al*., 1985) The detection of *Plasmodium* in the mosquito can also use PCR, DNA-based methods which are more sensitive and specific than all other methods (Snounou *et al*., 1993; Mahapatra *et al*., 2006; Bass *et al*., 2008). PCR amplification of mitochondrial DNA can detect *P. falciparum* and *P. vivax* in *Anopheles* and was used to test specimens collected in Chang Island (Cunha *et al*., 2009).

MATERIALS AND METHODS

1. Host feeding activity and seasonal abundance of *Anopheles* mosquitoes in Chang Island, Trat Province, eastern Thailand

1.1 Study site

Chang Island is located approximately 350 km from Bangkok in Trat Province, eastern Thailand near the border of Cambodia. Chang Island is the 2nd largest island in the Gulf of Thailand with an area of approximately 217 km^2 and a population of 7,646 inhabitants. Most of the island consists of both forested foothills and coastal zones bordered by mostly by native rainforest or commercial tree plantations. *Anopheles* mosquitoes were collected from two different locations on the island, a coastal site at Khlong Yuan Village (12° 02'N, 102° 23'E) with 47 inhabitants and an inland forest site near Khlong Jao Lueam waterfall (12° 06'N, 102° 18'E) with 30 inhabitants (Figure 1). Khlong Yuan Village is close to the sea (<500 m), approximately 39 m above sea level and surrounded by fruit orchards and rubber plantations along with native mangrove, whereas Khlong Jao Lueam is near a waterfall and surrounded by deep forests and steep hills. Khlong Jao Lueam site is approximately 2.5 km distance from the sea and 71 m above sea level.

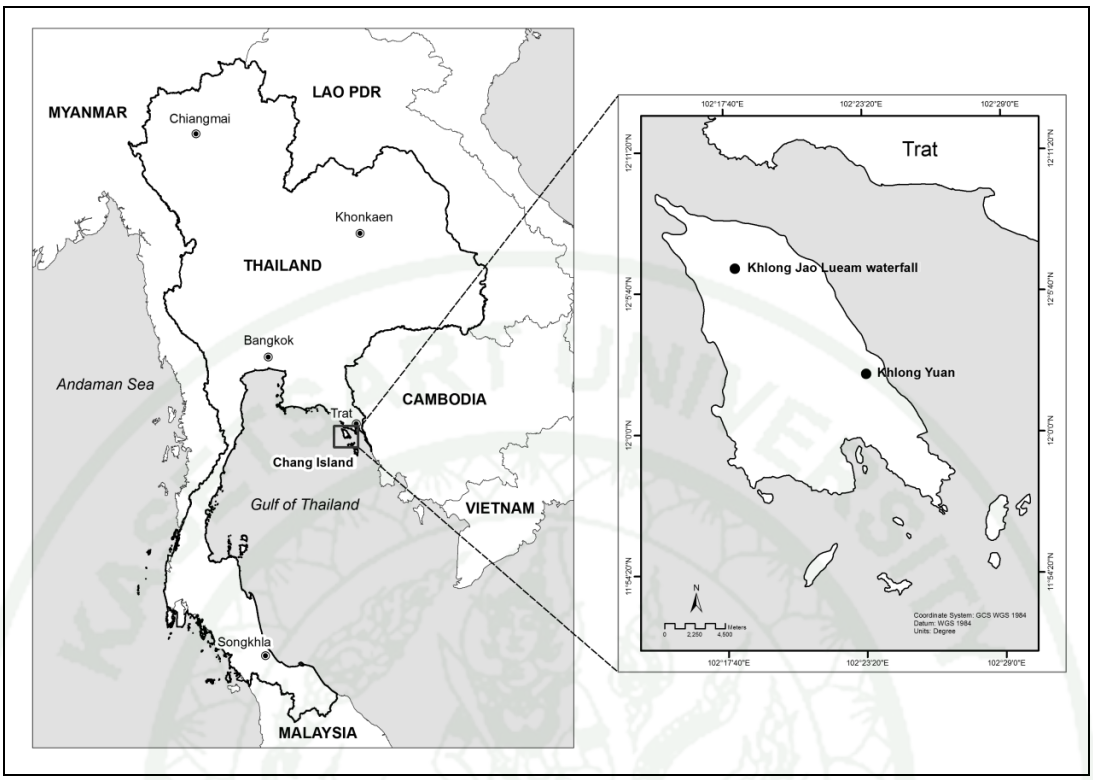

Figure 1 Location of Chang Island, Trat Province, Thailand, and study locations.

1.2 Collection methods

Adult mosquitoes were collected once every two months during three consecutive nights for a period of two years from 2011 to 2012. Mosquito collections were carried out based on three methods, human-landing indoor (HLI), human-landing outdoor (HLO) and buffalo-bait collections (BBC) as applicable (Figures 2 and 3). Human-landing collections (HLC) were performed in a local house for each site. Each study site had two mosquito collection teams and each team was divided into two groups of two collectors each collecting for 6 hours (entomology technicians were from the local malaria control sector), positioned either indoor or outdoor from sentinel house. The teams collected mosquitoes from 18:00 to 06:00 h with a first team involved from 18:00 to 24:00 h, followed by a second team beginning at midnight to 06:00 h. Every hour the team members rotated between indoor and outdoor collection sites to avoid potential collector bias. HLC were done for 45 min each hour (e.g., 18:00-18:45 h) with a 15-min rest period between hours. Each

collector captured landing mosquitoes using a flashlight and a mouth aspirator. Protocol review and approval for HLC activities were provided from the Ethics Review Committee for Research Involving Human Subjects, Health Science Group of Faculties, Colleges and Institutes, Chulalongkorn University, Thailand (Approval COA No. 167/2013). The BBC method involved using one tethered animal to attract host-seeking mosquitoes allowing them to blood feed and subsequently rest on the inside surface of the netting set up from ground level to 0.3 m height and placed around the periphery of the bait animal holding area. BBC was carried at Khlong Yuan Village only due to the presence of only one buffalo on the entire island BBC was performed 15 min each hour from 18:00-06:00 h by the collector who collected in outdoor. All *Anopheles* females were held in a plastic cup, labeled by hour and site of collection and covered with netting and a cotton pad saturated with 10% sugar solution before returning mosquitoes to a central location for morphologically sorting the specimens to species or species complex. Ambient air temperature and relative humidity were recorded from indoor and outdoor locations at time of collections. At the BBC site, during each hour of the collection, a manual thermo-hygrometer (BARICO GmbH, Villingen-Schwenningen, Germany) was used to collect the ambient air temperature and relative humidity. Rainfall data was obtained from the Trat meteorological station located on the nearby mainland.

Figure 2 Human-landing collections.

Figure 3 Buffalo-bait collections.

1.3 Morphological identification

Anopheles females were sorted out in the field using the morphological identification key for Thai anophelines (Rattanarithikul *et al*., 2006). Afterward, all specimens were preserved in liquid nitrogen and returned to the laboratory at Kasetsart University for molecular identification, *Plasmodium* detection, and further analysis.

1.4 Molecular identification

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

1.4.1 DNA extraction

Female *Anopheles* mosquitoes were extracted genomic DNA by adjusting procedures of Linton *et al.* (2001) and Manguin *et al.* (2002). Individual mosquito was homogenized in 50 μl of extraction buffer (0.2 M sucrose, 0.1M Tris-HCl at pH 8.0, 50 mM EDTA and 0.5% SDS). The tube was incubated at 65 °C for 30 min, add 11 μl 5 mM potassium acetate (pH 9.0) and the tube placed on ice for 30 min. Centrifuge at 12,000 rpm for 20 min and remove supernatant to a clean tube and add 100 μl of 100% ethanol then place into the 4^{o}C refrigerator for 10 min. Centrifuge at 12,000 rpm for 20 min at 4^{o}C to pellet the DNA. Wash the pellet with 150 μl of 70% ethanol and centrifuge at 12,000 rpm for 5 min at 4^{o}C. Wash again with 100% ethanol and centrifuge at 12,000 rpm for 5 min at 4^{o}C and then the pellet dry at room temperature. Put 100 μl of TE buffer and store at -20 °C.

1.4.2 Amplification by PCR

Minimus Complex: The ITS2 region was used to amplify the genomic DNA by the AS-PCR assay following the protocol of Garros *et al*. (2004). In a final volume of 25 µl, PCR amplification conditions were as follows: 1x reaction buffer (Invitrogen), 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.2 µM of each primer, 2.5 units of *Taq* DNA polymerase (Invitrogen) and 1 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for two min, follow by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 sec, and extension at 72°C for 40 sec, and a final extension at 72°C for 10 min (Table 3).

Table 3 Primers and fragment size of PCR product for identification within the Minimus Complex.

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

Source: Garros *et al*. (2004)

Dirus Complex: The rDNA ITS2 was used to amplify the genomic DNA of members of the Dirus Complex by the AS-PCR assay following the protocol of Walton *et al*. (1999). In a final volume of 25 µl, PCR amplification conditions are as follows: 1x reaction buffer (Invitrogen), 0.2 mM of dNTPs, 2 mM of $MgCl_2$, 1 µM of each primer, 2 units of *Taq* DNA polymerase (Invitrogen), 10% dimethylsulphoxide

(DMSO) and 3 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for 5 min, follow by 32 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 10 min (Table 4).

Table 4 Primers and fragment size of PCR product for identification Dirus Complex.

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

Source: Walton *et al*. (1999)

Maculatus Group: The ITS2 of rDNA primers were use to amplify the genomic DNA of Maculatus Group by the AS-PCR assay following the protocol of Walton *et al*. (2007). In a final volume of 25 µl, PCR amplification conditions are as follows: 1x reaction buffer (Invitrogen), 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, 0.2 mM of primers 5.8F, MAC, DRAV, K and 0.1 mM of primers SAW and PSEU, 2 units of *Taq* DNA polymerase (Invitrogen) and 2 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 10 min (Table 5).

Table 5 Primers and fragment size of PCR product for identification of Maculatus Group.

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

Source: Walton *et al*. (2007)

Sundaicus Complex: The mtDNA was used to amplify the genomic DNA of members of the Sundaicus Complex by the AS-PCR assay following the protocol of Dusfour *et al*. (2007a). In a final volume of 20 μL, PCR amplification conditions are as follows: 1X reaction buffer, 0.2 mM dNTPs, 1.5 mM of $MgCl_2$, 0.2 μM each primer, 2.5 U of *Taq* polymerase and 2 μl of DNA template diluted 1/10. The PCR cycles are as follows: one cycle at 94°C for 5 min, follow by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 10 min (Table 6).

Table 6 Primers and fragment size of PCR product for identification within the Sundaicus Complex.

Species	Primer	Sequence (5´ to 3´)	Size of PCR product (bp)
Universal primer	CBsunA	AAT GTT ACA AGA ATT CA	
Universal primer	CBsunB	TTA GCT ATA CAT TAT GC	575 bp
An. sundaicus s.s.	SunSS	TAT CAT TCT GAG GAG CC	313 bp
An. sundaicus E	SunE	ATG ATT TTT ACG AAT TTG C	498 bp
Universal primer	SpCO	GAA CGG TTT ATC CTC CT	
An. epiroticus	Epi	TAT TCG ATC TAA AGT AAT C	167 bp

Source: Dusfour *et al*. (2007a)

1.4.3 PCR product analysis

PCR product was detected by electrophoresis of 10 µl from the reaction mix 1 µl loading dye 10X on 2% agarose gels. The gels were made from 1 g of SeaKem® LE agarose (USA) in 50 ml of 1 X TAE buffer. 5 µl of GelStar® Nucleic Acid Gel Stain put into the melted agarose with approximately 55 °C. The electrophoresis was run on 100 Volt for 25 min. The DNA was visualized and photographed on Gel Documentation the sizes of the PCR products are as follows:

-Dirus Complex

An. dirus	562 bp
An. scanloni	349 bp
An. baimaii	306 bp

-Minimus Complex

An. minimus	310 bp
An. harrisoni	180 bp
An. aconitus	200 bp
An. pampanai	90 bp
An. varuna	260 bp

-Maculatus Group

An. maculatus	180 bp
An. sawadwongporni	242 bp

-Sundaicus Complex

An. sundaicus species E	498 bp
An. sundaicus s.s.	313 bp
An. epiroticus	167 bp

1.5 Data analysis

Data collected based on time of year, collection hours, locations and methods used were analyzed. Seasonal periods were separated to include 'wet' (May to October) and 'dry' (November to April) seasons, collection time periods were classified as early evening (18:00–21:00 h), late night (21:00–24:00 h), pre-dawn (24:00–03:00 h) and dawn (03:00–06:00 h), and collection types were listed as HLI, HLO and BBC. The evening biting behavior of *An. epiroticus* was tabulated by averaging the number of *Anopheles* landing per hour per human at indoor and outdoor locations and by averaging the number of mosquitoes captured per buffalo per hour (15-min collection each hour). Comparisons of landing data were analyzed by non-parametric Kruskal-Wallis tests, Wilcoxon and Mann-Whitney. The accepted level of significance was set at 0.05% (Pvalve < 0.05), followed by correlation coefficient (r) analysis taking into account the correlation between specimen captured and environmental variables. All data were analyzed using the SPSS statistical package (version 17.0, SPSS, Chicago, IL).

2. Detection of *Plasmodium* parasites in *Anopheles* mosquitoes

Molecularly identified *An. epiroticus* and *An. dirus* were tested for *Plasmodium* infection using the PCR assay method of Cunha *et al.* (2009) for *P. falciparum* and *P. vivax* (Table 7). No attempts were made to detect *P. malariae* and *P. ovale* as these parasites had not been reported on Chang Island and even if present would have been very rare. A total volume of 20 µl was used for the reaction mixture including 2 µl of DNA, 1.8mM $MgCl_2$, 250 µM of each dNTPs, 250 pmole of each primer, 2 µl of PCR buffer (10mM Tris–HCl, pH8.3, 50mM KCl), 1.0 unit of Taq DNA polymerase. The amplification conditions are: one initial denaturation cycle at 96 °C for 10 min; 30 cycles of denaturation at 95 °C for 1 min and annealing at 60 °C for 5 min; and a final extension at 60 °C for 1 h. PCR product was run on 2% agarose gel. The expected sizes of species amplifications were 273 bp for *P. falciparum* and 290 bp for *P. vivax.*

Table 7 Primers and expected fragment size for *Plasmodium falciparum* and *Plasmodium vivax.*

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

Source: Cunha *et al.* (2009)

3. Insecticide susceptibility and behavioral avoidance in *Anopheles epiroticus*, malaria vector in Thailand

Anopheles epiroticus was evaluated for susceptibility and behavioral responses to pyrethroid insecticides using the standard World Health Organization susceptibility test and an excito-repellency test system to compare four wild-caught populations of *An. epiroticus* in Thailand

3.1 *Anopheles epiroticus* test populations

Four wild populations of *An. epiroticus* were collected for testing

1) The Trat population (TR) was obtained using a live buffalo as bait and a mouth aspirator to collect resting mosquitoes off nearby netting in Klong Yuan Village, Ko Chang District, Trat Province, southeastern Thailand (12 ^{o} 02’ N, 102 ^{o} 23’ E).

2) The Songkhla population (SK) was obtained from a cow-bait collection method using mouth aspirators to capture mostly blooded resting mosquitoes in Bang Not Nai Village, Hat Yai District, Songkhla Province, southern Thailand (7 ^{o} 46’ N, 100 ^{o} 28’ E)

3) The Surat Thani population (ST) was obtained using a cow-bait method and mouth aspirator in Laem Sui Village, Chaiya District, Surat Thani Province, southern Thailand (9^{o} 24$^{'}$N, 99^{o} 18$^{'}$E).

4) The Phang Nga population (NG) was collected using cattle as bait and mouth aspirator in Laem Pakarang Village, Takua Pa District, Phang Nga Province, southern Thailand (8^{o} 43$^{'}$N, 98^{o} 14$^{'}$E).

The buffalo/cow-baited capture method involved using one or more animals to attract host-seeking mosquitoes allowing them to first blood feed and subsequently rest on netting set up from ground level to a 0.3 m height and placed around the periphery of the animal holding areas. Collections were conducted 15 min each hour from 18:00-06:00. All female mosquitoes were held in a plastic cup covered with

netting and provided a cotton pad saturated with 10% sugar solution while awaiting species identification and processing for testing.

3.2 Morphological and molecular anopheline species identification

Female mosquitoes were identified to either species or species group using the morphological keys of Rattanarithikul *et al*. (2006) before testing and subsequently, all test specimens initially identified as *Anopheles sundaicus* complex and used in the experiments were individually identified using DNA extraction procedures of Linton *et al*. (2001). Molecular analysis for sibling species identification within the *An. sundaicus* complex was performed by AS-PCR described by Dusfour *et al*. (2007a). Following each susceptibility bioassay, mosquitoes were frozen using liquid nitrogen and stored individually in cryo-tubes to prevent cross-contamination until molecular identification.

3.3 Insecticides

The three pyrethroid insecticides selected for susceptibility assays and excito-repellency tests were:

1. Deltamethrin [(*S*)-alphacyano-3-phenoxybenzyl (1*R*, 3R)-3-(2,2-dibromovinyl)-2,2 dimethylcyclopropanecarboxylate] provided by BASF, Aktiengescllshaft Bangkok, Thailand in August, 2008.

2. Permethrin [(3-phenoxybenzyl (1 *RS*, 3 *RS*, 1*RS*, 3 *SR*)-3 - (2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate)] provided by Ladda Company, Bangkok, Thailand in October 2010.

3. Alpha-cypermethrin ((*R*)-cyano (3-phenoxyphenyl) methyl (1*S*, 3S)-*rel*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) received from BASF Aktiengescllshaft,, Thailand in August 2010.

3.4 Insecticide treated papers

Insecticide treated papers were produced at the Department of Entomology, Kasetsart University based on WHO procedures and specifications (WHO 1998, 2013a). For susceptibility tests, test papers of each active ingredient were prepared using Whatman® No. 1 paper at 12 x 15 cm size using the standard operational diagnostic doses of 0.05% deltamethrin and 0.75% permethrin (WHO, 2013a). Alpha-cypermethrin was tested at a concentration of 0.082%. For excito-repellency tests, all test papers (15x 17.5 cm size) were individually and uniformly treated with standard field doses of 0.02 g/m^2 of deltamethrin, 0.3 g/m^2 of permethrin, and 0.03 g/m^2 of alpha-cypermethrin (WHO, 2009). Control papers were impregnated with solvent only (silicone oil with acetone).

3.5 Insecticide susceptibility tests

The World Health Organization commercial test kits were used in this study (WHO, 1981, 1998). Twenty-five wild-caught, predominantly blooded female mosquitoes were introduced into each respective holding tube configuration containing either an insecticide-treated paper and non-treated holding paper or a control with a non-insecticide paper treated with diluents carrier only and a non-treated holding paper. Following a brief holding period of approximately 5 min all mosquitoes were exposed for 60 min to either treated or control paper surfaces. Immediately after exposure, the number of knockdown mosquitoes in each test was recorded and all specimens transferred into corresponding clean holding tubes and provided with 10% sucrose-soaked cotton pads. Mortality was recorded at 24 h post-exposure and expressed as a direct percentage or 'corrected' percent mortality using Abbott's formula (Abbott, 1925) depending on the background mortality (between 5 and 20%) seen in the matched control test. Four replicates of paired controls and treatments for each insecticide were performed. The susceptibility of each test population to each insecticide was evaluated on the following WHO revised criteria (WHO, 2013a) (Figure 4).

$$\text{Abbott's formula} = \frac{\text{\% Test mortality - \% Control mortality}}{\text{100 - \% Control mortality}} \times 100$$

Figure 4 Insecticide susceptibility test set-up.

3.6 Excito-repellency tests

An excito-repellency test system was used to evaluate the behavioral responses of *An. epiroticus* to a discriminating concentration of each pyrethroid insecticide. The system of excito-repellency test consisted of two treatment chambers containing insecticide-treated papers and two control chambers containing only oil-based carrier treated papers described in Chareonviriyaphap *et al*. (2002) (Figures 5 and 6). All tests were performed between 08:00 and 16:30 h under natural field conditions. For each test, 15 female mosquitoes were released into each of four chambers. Mosquitoes were allowed to acclimate to the inside of the chamber for approximately three minutes before the exit portal was opened. At the beginning of the test, the number of mosquitoes that escaped from the respective chambers into the attached receiving cage was recorded at one-min intervals for a period of 30 min. All mosquitoes escaping during each one-minute interval were transferred to individual clean cups. At the end of 30 min exposure, the number of dead or knockdown mosquitoes was recorded separately from inside each exposure chamber and external

holding cage for both treatment and controls. All live mosquitoes from each chamber were held separately and provided with 10% sugar solution as nutrition. After 24 hr, mortality was recorded and corrected as above using Abbott's formula, if applicable.

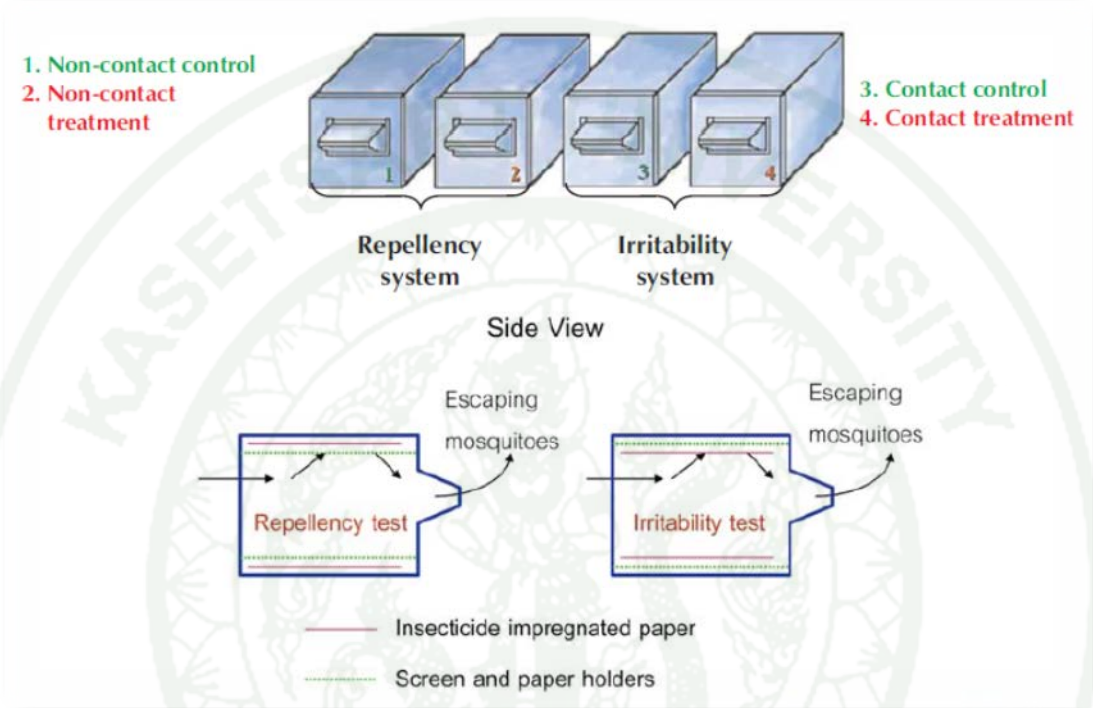

Figure 5 Excito-repellency test chamber used to study insecticide behavioral responses.

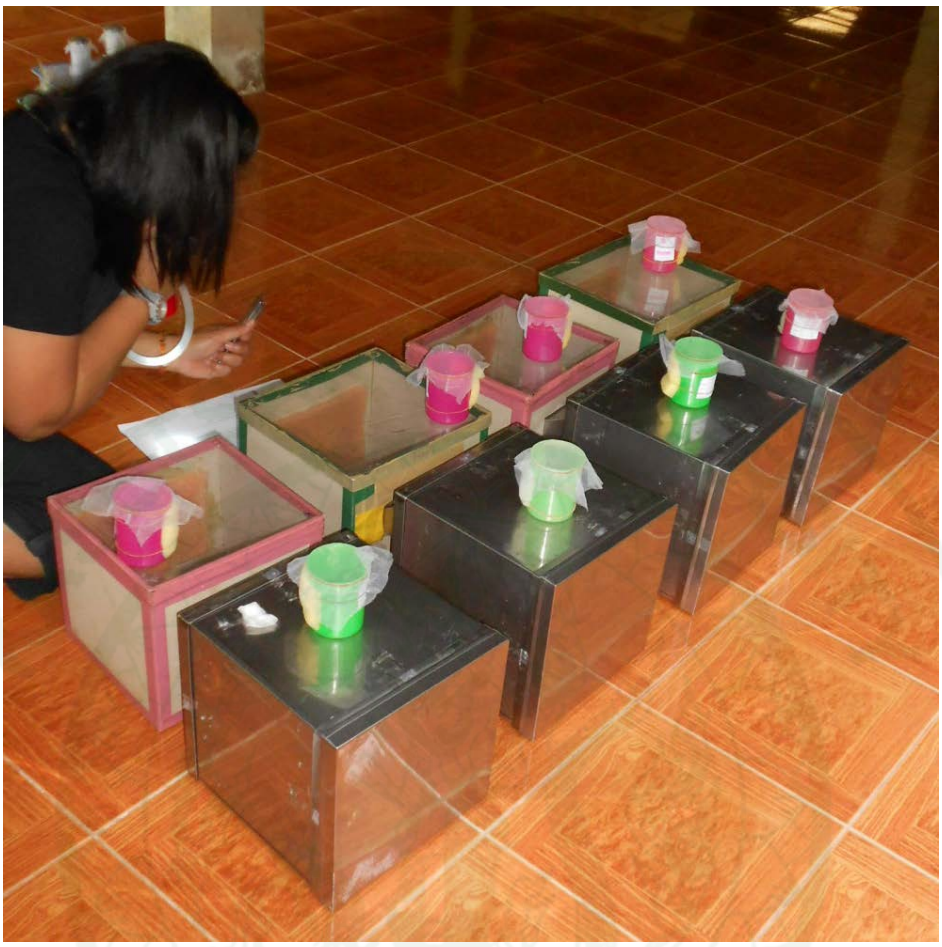

Figure 6 Excito-repellency test set-up

3.7 Data analysis

The susceptibility to each insecticide was evaluated on the following WHO revised criteria (WHO, 2013a) as follows: final mortality between 98 and 100%, a test population is considered "susceptible"; mortality between 90 and 97% is either treated as "suggestive of the existence of resistance" and requiring further investigation, i.e., additional bioassays or determination of mechanism(s); and mortality below 90% is an indication of outright "resistance" in the population tested. If the observed mortality (corrected if necessary) is between 90% and 97%, the presence of resistant genes in the vector population should be confirmed (not conducted in this study). If at least two additional tests consistently show mortality below 98%, then resistance is confirmed. Lastly, if mortality is less than 90%,

confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary provided a minimum of 100 mosquitoes of each species (population) is tested.

Percentage of escape obtained from the treated exposure chambers was adjusted based on matched-paired control escape responses using Abbott's formula. Likewise, when control mortality was between 5 and 20%, the final mortality percent in insecticide exposed samples was also adjusted accordingly. Kaplan-Meier survival (life table) analysis was used to estimate mosquito escape rates over time for each test configuration (i.e., by chemical, contact and noncontact configurations, respectively). Survival analysis was used to analyze the rates of escape by combining data from each trial chamber configuration (Kleinbaum 1995, Roberts *et al*., 1997). Survival analysis was also used to estimate the escape time (ET) at which 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of the test populations escaped from the chamber. Patterns of escape behavior were evaluated within the test cohorts and between difference treatment groups using a log-rank method (Mantel and Haenzel, 1959). Statistical significance for all tests was set at $P < 0.05$.

RESULTS AND DISCUSSION

Results

1. Host feeding activity and seasonal abundance of *Anopheles* mosquitoes in Chang Island, Trat Province, eastern Thailand

In the two-year period (2011- 2012), combined study sites collected a total of 5,399 anophelines , comprising 14 *Anopheles* taxa separated within two subgenera, *Cellia* and *Anopheles* representing 9 (n=3,793, 70.25%) and 5 (n= 1,606) species, respectively. From all collections, 97.5% (5,264) were captured from the coastal site of Klong Yuan and only 2.5% (135) were collected from the inland forested location (Table 8). However, buffalo-baited trapping only occurred in Khlong Yuan and represented 83.2% (4,494) of all anophelines collected. Excluding the BBC data, when comparing only HLC data, Khlong Yuan still produced the majority (85.1%) of captured anophelines compared inland Khlong Jao Lueam. Within the subgenus *Cellia,* 3,444 specimens (90.8%) belonged to the Sundaicus Complex, while 140 (3.7%) where in the Dirus Complex, 43 (1.13%) in the Maculatus Group, and 9 (0.24%) in the Minimus Complex. Many members within these 4 taxonomic assemblages are regarded as potential malaria vectors in Thailand (Saeung, 2012). Additionally, typical non-malaria vectors within the *Cellia* subgenus included *Anopheles jamesii***,** *An. kochi***,** *An. karwari***,** *An. vagus* and *An. philippinensis* (Table 8). Five taxa within the subgenus *Anopheles* were identified, including *An. umbrosus*, *An. barbirostris* group, *An. aitkenii* group, *An. hyrcanus* group and *An. peditaeniatus* (Table 8). Only members in the Barbirostris and Hyrcanus Groups have been found naturally infected with either *P. falciparum* and/or *P. vivax* parasites (Rattanarithikul *et al*., 1996).

Only specimens from the four putative malaria vector species complexes or group in the subgenus *Cellia* were subjected to further and definitive species identification using the appropriate multiplex AS-PCR assay (see Materials &

Methods). Five important species were identified, including *An. dirus* (former *An. dirus* species A) (3.85% of samples assayed), *An. minimus* (former *An. minimus* species A) (0.25%), *An. sawadwongporni* (0.77%), *An. maculatus* (0.41%) and *An. epiroticus* (former *An. sundaicus* species A) (94.72%) (Table 9 and Figures 9-12). The initial morphological identification showed 14 species or assemblages, whereas PCR allowed the identification of *An. sawadwongporni* (Maculatus Group) resulting in a total of 15 species collected on Chang Island during this study period.

In Klong Yuan, *An. epiroticus* was the most abundant (65.4%) of the 15 *Anopheles* species and when compared with the other four key potential malaria vectors present on the island, it contributed to 98.4% of the total collection (Tables 8 and 9). By contrast, Khlong Jao Lueam did not have evidence of *An. epiroticus,* whereas *An. dirus* was the predominate malaria vector species (94.1%), followed by *An. minimus* (5.9%), the only 2 anophelines captured in the village (Table 8).

In this study, *An. epiroticus* was the most abundant species in Chang Island nearer the coastline and therefore was further investigated regard adult biting activity, host preference and density. The majority of *An. epiroticus* from Klong Yuan were captured in the buffalo trap (78.4%) compared to Human Landing Collection (HLC). The distribution of this species in the HLC found 65.5% outdoors compared to time-matched indoor collections (Tables 9 and 12). With 21.6% of *An. epiroticus* collected on humans either indoors or outdoors, this species demonstrated some degree of anthropophily but appears attracted to both humans and buffalo as blood sources. The *An. epiroticus* feeding patterns by hour and collection method are shown in Figure 7. The indoor and outdoor human biting activity presented a fairly even distribution over the entire evening period. Because the number of mosquitoes captured per person hours was very small (generally less than 2 per person/hr) any perceived rise in activity are considered insignificant and it appears this species is active throughout the evening, both indoors and out. In contrast, the mosquito activity patterns associated with the buffalo bait showed clearly the largest peak in the beginning of the evening from 18:00 to 19:00 h and declining progressively thereafter throughout the night.

A greater number of *An. epiroticus* were collected during the dry season (November to April) with a notable peak in the early dry season from November to January (Table 12 and Figure 8). In contrast, adult *An. epiroticus* was found in relatively low densities during the wet months (May to October). In the inland forest site, both *An. dirus* and *An. minimus* were collected in greatest number during the dry season (peak January-March); however, the total collection numbers (n=135) make it difficult to draw any definitive conclusions regarding seasonality and species abundance (Tables 10 and 11).

Comparisons of HLC data were analyzed using non-parametric Kruskal-Wallis, Wilcoxon and Mann-Whitney tests. A strong significant difference in the number of *An. epiroticus* was found between seasons (Z= -4.696, $P < 0.05$) (Table 13), and between indoor, outdoor HLC and BBC methods (F = 5.319, df=2, $P < 0.05$). The Wilcoxon pairwise comparison between indoor *versus* outdoor collections (Z = -2.803, $P = 0.005$), between indoor *versus* buffalo (Z = -2.936, $P = 0.003$), and between outdoor *versus* buffalo (Z = -2.994, $P = 0.003$) were statistically different from one another (Table 14). There was no significant difference in the number of *An. epiroticus* collected between the four quarterly evening time intervals as analyzed in this study ($\chi^2 = 0.04$, $df = 3$, $P = 0.998$ indoor; $\chi^2 = 0.91$, $df = 3$, $P = 0.823$ outdoor and $\chi^2 = 0.579$, $df = 3$, $P = 0.903$ buffalo) (Table 15). Data from all collection methods were pooled to determine the correlation between mosquito abundance and measured environmental variables (Table 12 and Figure 8). Results indicated that *An. epiroticus* densities were strongly correlated with rainfall patterns ($r = -0.667$; $P = 0.009$) and relative humidity ($r = -0.640$; $P = 0.012$), but were not associated with relative minimum or maximum ambient air temperatures ($P > 0.05$) (Table 16).

Larval surveys were carried out by dipping method in and around Khlong Yuan and Khlong Jao Lueam from January 2011 to September 2012. A total of 43 anopheline larvae were collected from the two villages. Four species, *An. hyrcanus* group, *An. barbirostris* group, *An. jamesii* and *An. umbrosus* were found in swamp at Khlong Yuan, while only one species, *An. minimus* was collected in stream margins at

Khlong Jao Lueam. So, in this study, immature stages of malaria vector species were not found in Khlong Yuan.

Table 8 Total *Anopheles* mosquitoes collected based on morphological analysis at Chang Island, Trat Province, from January 2011 to Novemeber 2012.

Anopheles	Khlong Yuan		Khlong Jao Lueam	Total
	Human bait	Buffalo bait	Human bait	
Subgenus *Cellia*				
An. dirus s.l.	5	8	127	140
An. minimus s.l.	1	0	8	9
An. maculatus s.l.	0	43	-	43
An. sundaicus s.l.	743	2,701	-	3,444
An. jamesii	-	25	-	25
An. kochi	6	90	-	96
An. karwari	1	32	-	33
An. vagus	-	1	-	1
An. philippinensis	-	2	-	2
Subgenus Anopheles				
An. umbrosus	14	382	-	396
An. barbirostris group	-	12	-	12
An. aitkenii group	-	175	-	175
An. hyrcanus group	-	997	-	997
An. peditaeniatus	-	26	-	26
Total	770	4,494	135	5,399

Table 9 Numbers of putative malaria vector species based on molecular analysis collected at Chang Island, Trat Province, between January 2011 and November 2012.

Anopheles	Khlong Yuan			Khlong Jao Lueam		Total
	Buffalo	In	Out	In	Out	
An. dirus	8	0	5	58	69	140
An. minimus	0	0	1	8	0	9
An. sawadwongporni	28	0	0	0	0	28
An. maculatus	15	0	0	0	0	15
An. epiroticus	2,701	256	487	0	0	3,444
Total	2,752	256	493	66	69	3,636

In = Indoor collection

Out = Outdoor collection

Buffalo = Buffalo bait collection

Table 10 Monthly numbers of *Anopheles* mosquitoes collected at Khlong Yuan,Chang Island, Trat Province,January 2011-November 2012.

Month	*Anopheles* species															Total
	An. dirus			*An. minimus*			*An. maculatus*			*An. sawadwongporni*			*An. epiroticus*			
	In	Oot	Buf	In	Oot	Buf	In	Out	Buf	In	Out	Buf	In	Out	Buf	
Year 1																
Jan 11	0	0	0	0	0	0	0	0	4	0	0	10	40	52	310	416
Mar 11	0	0	0	0	0	0	0	0	0	0	0	0	29	33	395	457
May 11	0	0	2	0	0	0	0	0	9	0	0	11	18	27	45	112
Jul 11	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	3
Sep 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3
Nov 11	0	1	0	0	0	0	0	0	1	0	0	0	77	179	583	841
Year 2																
Jan 12	0	0	0	0	0	0	0	0	0	0	0	0	76	119	716	911
Mar 12	0	4	5	0	0	0	0	0	0	0	0	0	8	28	32	77
May 12	0	0	0	0	0	0	0	0	0	0	0	7	1	3	10	21
Jul 12	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Sep 12	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	5
Nov 12	0	0	1	0	1	0	0	0	1	0	0	0	6	43	602	654
Total	0	5	8	0	1	0	0	0	15	0	0	28	256	487	2,701	3,501

In = Indoor collection, Out = Outdoor collection, Buffalo = Buffalo bait collection

Table 11 Monthly numbers of *Anopheles* mosquitoes collected at Khlong Jao Lueam, Trat Province, January 2011-November 2012.

Month	*Anopheles* species				Total
	An. dirus		*An. minimus*		
	Indoor	Outdoor	Indoor	Outdoor	
Year 1					
Jan 11	3	25	0	0	28
Mar 11	5	4	8	0	17
May 11	2	3	0	0	5
Jul 11	0	1	0	0	1
Sep 11	0	0	0	0	0
Nov 11	2	1	0	0	3
Year 2					
Jan 12	2	2	0	1	5
Mar 12	42	33	0	1	76
May 12	2	0	0	0	2
Jul 12	0	0	0	0	0
Sep 12	0	0	0	0	0
Nov 12	0	1	0	0	1
Total	58	70	8	2	138

Table 12 Total monthly captures of *Anopheles epiroticus* from three collection methods in Chang Island, Trat Province.

Month	In	Out	Buffalo	Total	T	H	R
Year 1							
Jan 11	40	52	310	402	26.8	65	0
Mar 11	29	33	395	457	27	78	235.6
May 11	18	27	45	90	28.4	83	353.5
Jul 11	1	0	2	3	27.1	86	895.8
Sep 11	0	0	3	3	26.6	88	1446.7
Nov 11	77	179	583	839	28.2	73	9.5
Year 2							
Jan 12	76	119	716	911	27.3	76	141.1
Mar 12	8	28	32	68	28.1	80	136.5
May 12	1	3	10	14	27.6	86	622.9
Jul 12	0	1	0	1	27	87	857.4
Sep 12	0	2	3	5	26.3	89	1311
Nov 12	6	43	602	651	27.7	84	392.8
Total	256	487	2,701	3,444			

In = Indoor collection
Out = Outdoor collection
Buffalo = Buffalo bait collection
T = Temperature (^{o}C)
H = Humidity (%)
R = Rainfall (mm)

Table 13 Mann-Whitney test of seasons (wet and dry as discriminating factors of *Anopheles epiroticus*.

Season	Mann-Whitney
wet	Z= -4.696,P<0.05
dry	(P=.000)

Table 14 Wilcoxon tests of collection methods (indoor and outdoor human bait, and buffalo bait) as discriminating factors of *Anopheles epiroticus*.

Collection method	Wilcoxon
Indoor vs. Outdoor vs. Buffalo	F = 5.319, df=2, P < 0.05 P=0.010
Indoor vs. Outdoor	Z = -2.803, P = 0.005
Indoor vs. Buffalo	Z = -2.936, P = 0.003
Outdoor vs. Buffalo	Z = -2.994, P =0.003

Table 15 Kruskal-Wallis tests of time collections (early evening, late evening, predawn, and dawn) within each collection methods (indoor and outdoor human bait, and buffalo bait) as discriminating factors of *Anopheles epiroticus*.

Collection methods	Time	Mean ± Std. Error	Kruskal-Wallis Test
Indoor	Early eve	5.50 ± 2.641	χ^2=0.04, *df*=3, *P*=0.998
	Late eve	3.92 ± 1.401	
	Pre-dawn	6.17 ± 3.005	
	Dawn	5.75 ± 3.102	
Outdoor	Early eve	8.83 ± 3.481	χ^2=0.91, *df*=3, *P*=0.823
	Late eve	12.25 ± 5.610	
	Pre-dawn	10.42 ± 3.767	
	Dawn	9.25 ± 4.418	
Buffalo	Early eve	94.00 ± 34.435	χ^2=0.579, *df*=3, *P*=0.903
	Late eve	53.08± 21.924	
	Pre-dawn	44.25 ± 20.657	
	Dawn	33.75 ± 11.527	

Table 16 Correlation between total numbers of *Anopheles epiroticus* and rainfall, ambient temperatures and relative humidity in Chang Island, Trat Province.

Variables	r	r^2	Mean	Sig.(P)
Humidity	−0.640	0.41	81.25	0.012
Temperature	0.267	0.071	27.34	0.201
Rain fall	−0.667	0.445	533.5667	0.009

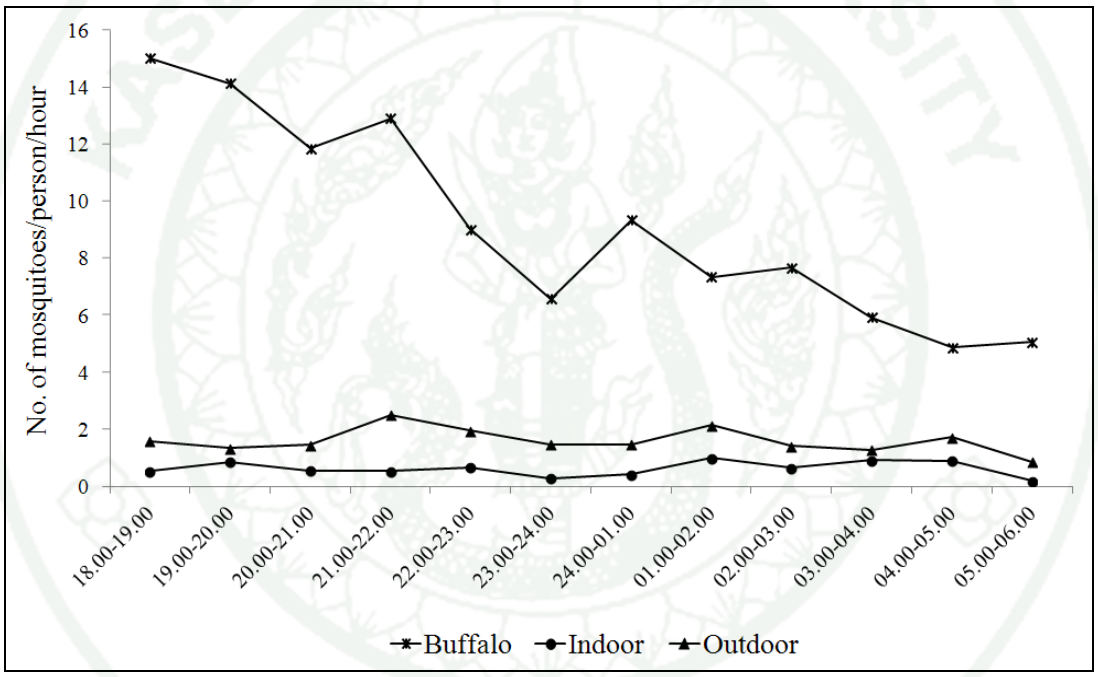

Figure 7 Hourly densities of *Anopheles epiroticus* by collection method as human-baited indoor and outdoor collections and buffalo-baited trap in Khlong Yuan.

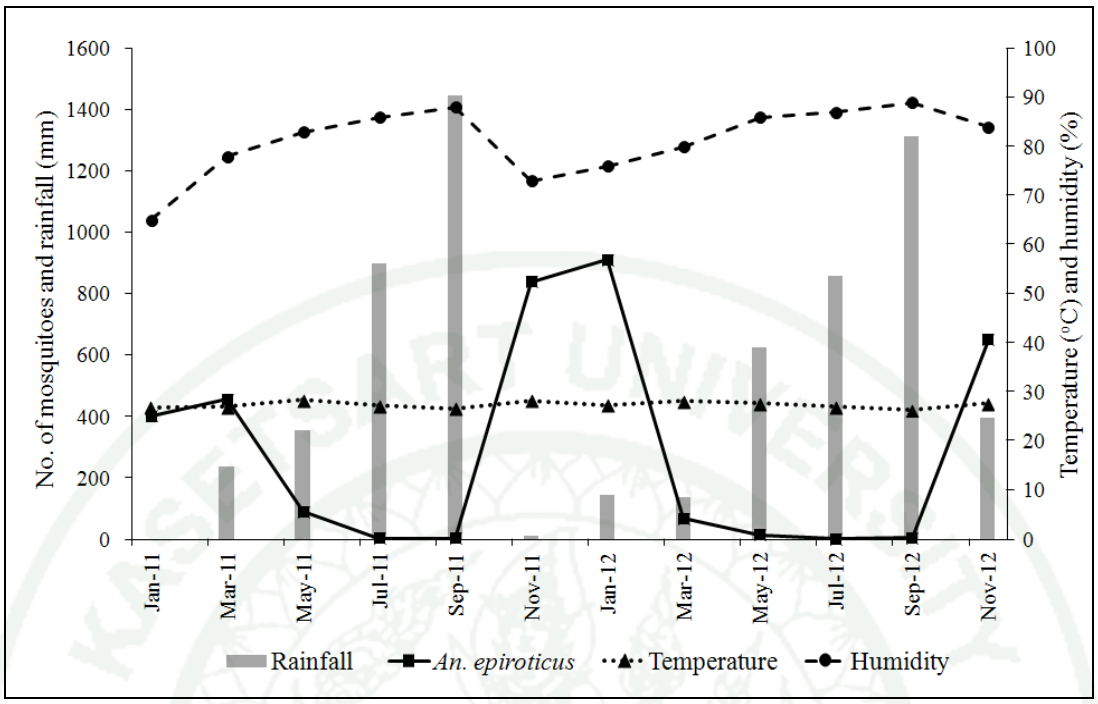

Figure 8 Seasonal abundance of *Anopheles epiroticus* in relation to precipitation and percent relative humidity on Chang Island, Trat Province during collection period.

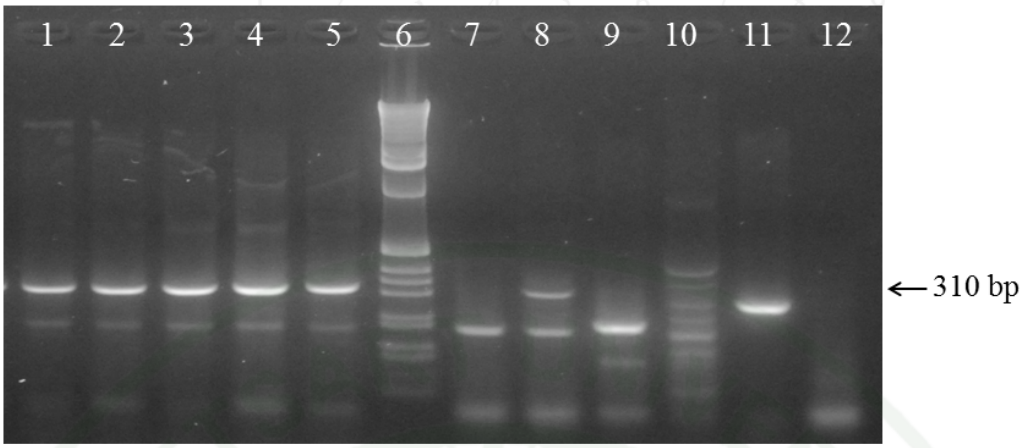

Figure 9 Amplified fragments using Allele-Specific PCR assay for identifying members of the Minimus Complex:
lane 1-5 = *An. minimus* samples
lane 6 = 100 bp DNA ladder
lane 7= *An. harrisoni* positive control
lane 8= *An. minimus* positives control
lane 9= *An. aconitus* positives control
lane10= *An. pampanai* positives control
lane11= *An. varuna* positives control
lane12 = negative control

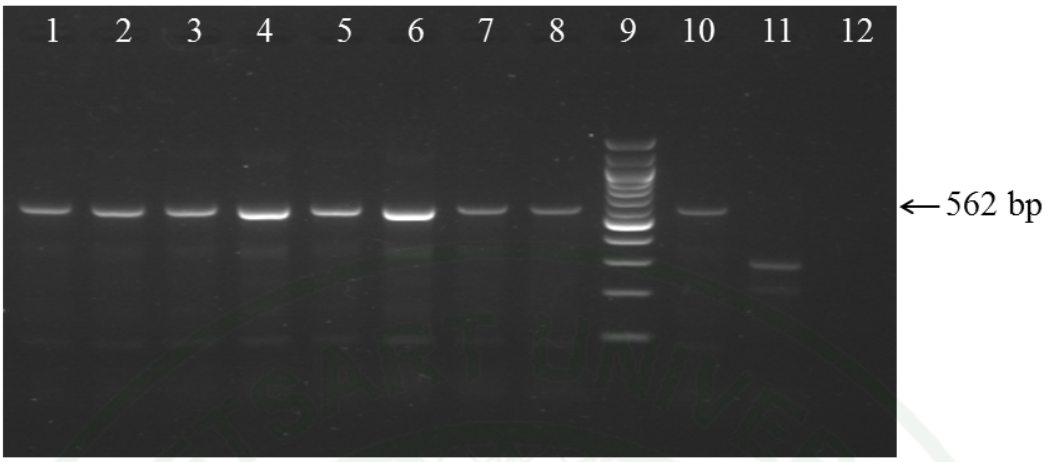

Figure 10 Amplified fragments using Allele-Specific PCR assay for identifying members of the Dirus Complex:
lane 1-8 = *An. dirus* samples
lane 9 = 100 bp DNA ladder
lane 10 = *An. dirus* positives control
lane 11 = *An. baimaii* positives control
lane 12= negative control

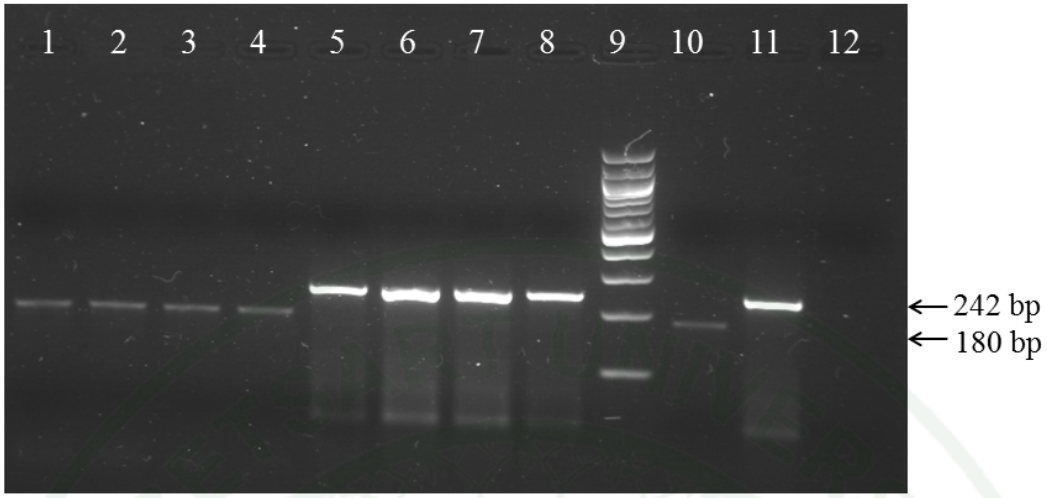

Figure 11 Amplified fragments using Allele-Specific PCR assay for identifying members of the Maculaus Group:

lane 1-4 = *An. maculatus* samples

lane 5-8 = *An. sawadwongporni* samples

lane 9 = 100 bp DNA ladder

lane 10= *An. maculatus* positives control

lane 11= *An. sawadwongporni* positives control

lane 12 = negative control

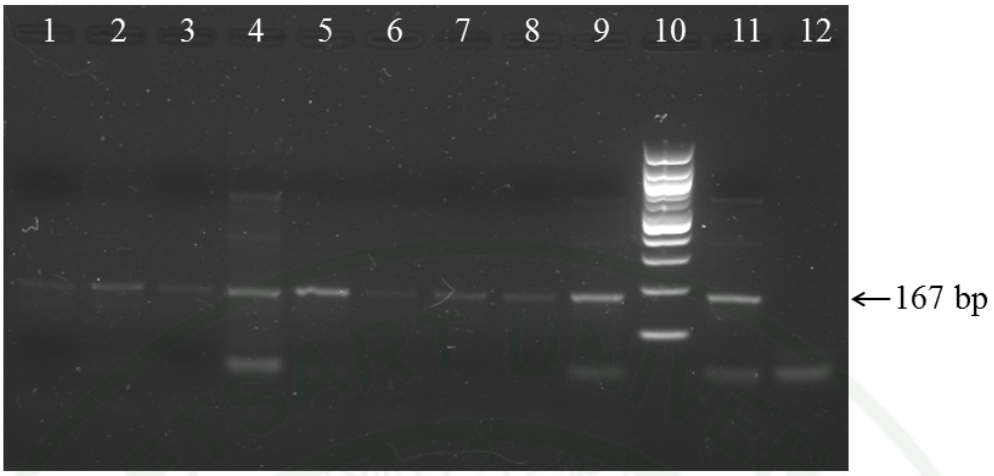

Figure 12 Amplified fragments using Allele-Specific PCR assay for identifying members of the Sundaicus Complex:
lane 1-9 = *An. epiroticus* samples
lane 10 = 100 bp DNA ladder
lane 11 = *An. epiroticus* positives control
lane 12 = negative control

2. Detection of *Plasmodium* parasites in *Anopheles* mosquitoes

Anopheles epiroticus and *An. dirus* are the putative dominant vectors present in Chang Island. Out of a sample of 743 and 132 specimens of each species respectively, collected from human-baited collections (HLC), a total of 730 individuals of both species consisting in 640 *An. epiroticus* and 90 *An. dirus* were subsequently processed for the detection of *P. falciparum* and *P. vivax* infection using PCR method by Cunha *et al*. (2009). Initially, the results showed all *An. epiroticus* failed to amplify any *Plasmodiu*m product, whereas amplification products were obtained from 36 specimens of *An. dirus* (Table 17 and Figure 13). The bands of the PCR products obtained from *An. dirus*, all specific for *P. vivax* were sequenced to confirm the amplification specificity for *P. vivax*. Unfortunately, the vast majority of PCR products failed to provide confirmatory sequences due to the low concentration of DNA available for analysis and that additional DNA of specimens were not available for repeat testing. Only two *P. vivax* positive specimens were detected upon

retesting and subsequently tested again by using a nested PCR developed by Singh *et al.* (1999) with the kind assistance of the Department of Protozoology, Mahidol University, Bangkok, Thailand. The nested PCR assay consisted of two rounds of amplification wherein the first PCR amplification, *Plasmodium*-specific primers were used. The PCR product of the first round served as DNA template for four separate second round PCR amplifications with primers specific for each of the 4 human malaria parasites of interest (*P. falciparum, P. vivax, P. ovale* and *P. malariae*). This method produced no amplification in the two suspect samples (Figure 14).

Table 17 PCR assay detection of *Plasmodium* in *Anopheles epiroticus* and *Anopheles dirus*.

Species	No. of positive/ No. of tested	
	PCR (Cunha *et al*., 2009)	Nested PCR (Singh *et al*., 1999)
An. epiroticus	0/640	-
An. dirus	36/90	0/2

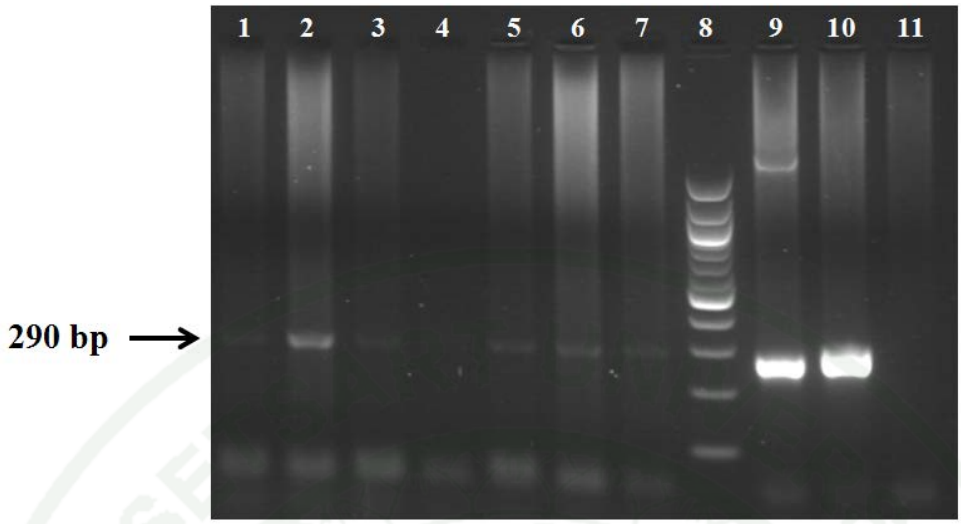

Figure 13 Amplified fragments using PCR assay for detection of *Plasmodium* parasites in *An. dirus*.:
lane 1-3, 5-7 = suspected like *P. vivax* samples
lane 8 = 100 bp DNA ladder
lane 9 = *P. falciparum* positive control
lane 10 = *P. vivax* positive control
lane 11 = negative control

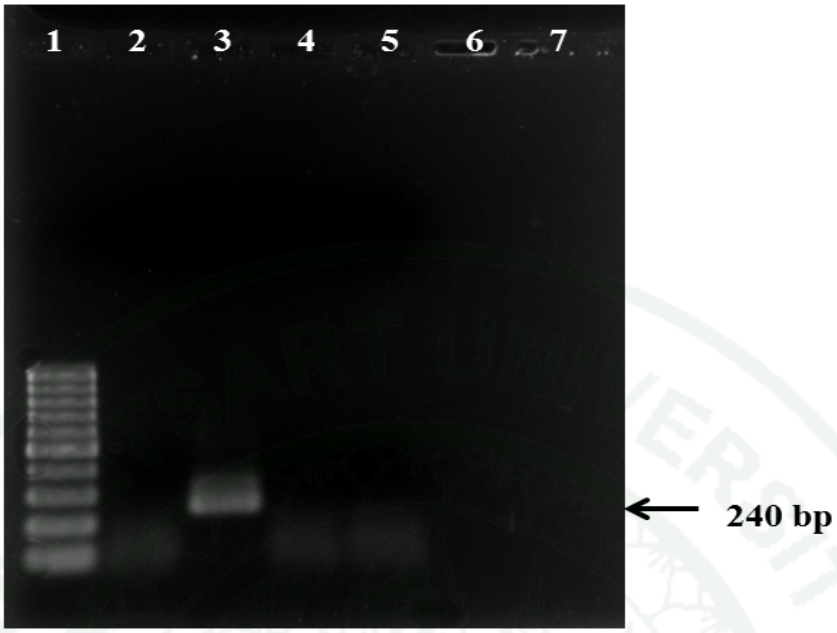

Figure 14 Amplified fragments using nested PCR assay for detection of *Plasmodium* parasites of *An. dirus*:
lane 1= 100 bp DNA ladder
lane 2 = negative control
lane 3 = *P. vivax* positive control
lane 4-5 = *An. dirus* samples

3. Insecticide susceptibility and behavioral avoidance in *Anopheles epiroticus*

The insecticide susceptibility of field populations of *An. epiroticus* (100 females each) were exposed to the standard diagnostic doses of deltamethrin (0.05%), permethrin (0.75%) and alpha-cypermethrin (0.082%) treated papers to access adult female susceptibility to each compound. All four populations demonstrated complete (100%) susceptibility to all three compounds and matched control mortalities were negligible (0-4%).

The percentage of *An. epiroticus* that escaped within a 30-min exposure period to deltamethrin, permethrin or alpha-cypermethrin in contact and noncontact trials are presented in Tables18 and 19. Among the four populations, TR demonstrated the strongest escape responses in both contact (78.4% alpha-cypermethrin, 76.8%

deltamethrin and 74.1% permethrin) and noncontact trials (46.3% for alpha-cypermethrin,17.5% deltamethrin and 37.6% permethrin), followed by the PN population in contact trials (contact: 34.4% alpha-cypermethrin, 24.4% deltamethrin, and 35.0% permethrin). Conversely, the ST displayed no or low escapes responses to all three compounds in both contact (0.1% alpha-cypermethrin, 3.4% deltamethrin and 0% permethrin) and noncontact trials (1.7% alpha-cypermethrin, 0% deltamethrin and permethrin) trials. SK showed no escape behavioral responses to deltamethrin in contact trials and to permethrin in noncontact trials. Overall, there was relatively low number of mosquitoes that escaped from the control chambers in both the contact (0-16.1%) and noncontact configurations (0-17.0%).

Mortality of mosquitoes after a 24-hr holding period from treated and untreated chambers compared with matched controls are provided in Tables 18 and 19. Figures for treatment escape and mortality percentages have been adjusted using Abbott's correction formula based on respective paired control responses. In general, for both TR and PN populations, higher mortalities were observed in contact *versus* noncontact trials as well as higher in noncontact tests compared to matched controls (control data not shown). Higher mortalities were typically observed in contact tests among nonescaped mosquitoes compared to those that escaped; the lone exception was the PN population exposed to alpha-cypermethrin. Among nonescaped mosquitoes in contact trials, the SK population had the highest mortalities (84% alpha-cypermethrin, 61% deltamethrin and 65% permethrin). Lower percent mortalities were observed from escaped mosquitoes in all populations except the SK population in which 61% succumbed to alpha-cypermethrin and 67% to permethrin (albeit total escape numbers were low that influenced final percent kill). In noncontact trials, mortalities of escaped and nonescaped mosquitoes were generally low, ranging between 0-40% for those that successfully escaped and between 0-37% for nonescaped specimens. The control percent mortality in contact trials was generally low but appears inflated in a few instances because of low numbers escaping the chambers. With the exception of a few trials involving SK and PN populations, control mortality in escaped and nonescaped mosquitoes in noncontact trials was generally much lower that paired treatment tests. Notable exceptions included SK

control mortality with alpha-cypermethrin (17 and 65%) and permethrin (40 and 33.3%), and PN in escaped and nonescaped tests with deltamethrin (5.1 and 30.5%), respectively. Times in minutes for mosquitoes to escape from treated chambers are given in Table 20. Escaped mosquitoes that departed from insecticide-treated chambers within 30 min are grouped as 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of total test sample leaving the chambers. In general, ET_{25}, ET_{50} and ET_{75} values for the TR population in contact trials were comparatively low for all 3 insecticides (1, 5, and 17 min for alpha-cypermethrin, 1, 2 and 16 min for deltamethrin and 1, 4, and 16 min for permethrin, respectively). For noncontact trials ET_{25} values for TR population were 10, 29, and 19 min for alpha-cypermethrin, deltamethrin, and permethrin, respectively. The PN population showed ET_{25} on contact trials only of 18, 6 and 10 min for alpha-cypermethrin, deltamethrin and permethrin, respectively. Escape times could not be determined for SK and ST populations due to insufficient numbers of mosquitoes escaping during the test period (Table 20).

Log-rank comparisons between the three insecticides and test conditions (contact and noncontact trials) are presented in Tables 21 and 22. Significant differences in escape responses were observed when controls are compared to contact and contact compared to noncontact trials in the TR and PN populations ($P < 0.05$). A statistical difference was observed between paired control and noncontact tests in the TR population for alpha-cypermethrin. No significant differences in escape responses were seen between paired controls and contact and noncontact trials, and similarly when contact was compared with noncontact trials with the ST population ($P < 0.05$). No significant differences were seen between paired controls and noncontact tests in SK and PN populations, with the single exception for SK population tested against deltamethrin (Table 21). There was no significant difference in escape responses in contact trials when test compounds were compared within populations. Likewise, no significant difference in escape responses were observed when test compounds were compared in noncontact trials, except deltamethrin compared to alpha-cypermethrin and permethrin in the TR population, and deltamethrin and permethrin in the PN population ($P < 0.05$) (Table 22).

The comparisons of escape probabilities based on survival analysis showing proportions of mosquitoes remaining in treatment and control chambers at 1 min intervals over a 30 min period in contact and noncontact trials are presented in Figures 15 – 20. The observed patterns are indicative of escape probabilities between contact and noncontact activity for deltamethrin (Figures 15 and 16), permethrin (Figures 17 and 18) and alpha-cypermethrin (Figures 19 and 20) of the 4 populations of *An. epiroticus*. There were significant differences in excitation seen in all contact and paired controls for TR and PN exposed to deltamethrin (TR $P < 0.0001$ TR, PN $P = 0.0008$), permethrin (TR $P < 0.0001$, PN $P < 0.0001$) and alpha-cypermethrin (TR $P < 0.0001$, PN $P < 0.0001$). Patterns of escape were not significantly different between contact and controls for SK and ST populations for deltamethrin ($P = 0.7687$SK, $P = 0.3014$ ST) and alpha-cypermethrin ($P = 0.7902$ SK, $P = 0.9691$ ST).

Table 18 Percentage escape and 24-h mortality of *Anopheles epiroticus* exposed to alpha-cypermethrin (0.03 g/m^2), deltamethrin (0.02 g/m^2) and permethrin (0.3 g/m^2) in contact trials.

Chemicals	Populations	No. Mosquitoes	% Escaped*	% Mortality*	
				Escaped	Remain
alpha-cypermethrin	TR	Treatment (59)	78.4	22.9	36.4
		Control (58)	13.8	0	0
	SK	Treatment (59)	1.8	61.4	84
		Control (58)	16.1	22.2	46.8
	PN	Treatment (59)	34.4	25.4	17.8
		Control (57)	7.0	0	8.8
	ST	Treatment (58)	0.1	0	62.1
		Control (60)	3.3	1.7	0
deltamethrin	TR	Treatment (60)	76.8	10.6	60.8
		Control (59)	6.8	0	1.8
	SK	Treatment (60)	0	4.0	60.9
		Control (59)	10.0	16.7	14.8
	PN	Treatment (60)	24.4	22.3	49.4
		Control (58)	5.2	3.4	27.6
	ST	Treatment (60)	3.4	5.0	53.5
		Control (62)	1.6	0	3.2
permethrin	TR	Treatment (59)	74.1	0	13.7
		Control (60)	15.0	11.1	2.0
	SK	Treatment (59)	14.3	66.7	64.5
		Control (60)	1.7	0	41.4
	PN	Treatment (60)	35.0	15.0	28.1
		Control (60)	0	0	5.0
	ST	Treatment (60)	0	0	37.3
		Control (60)	1.7	0	1.7

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani

*Treatment percent escape and mortality adjusted based on control responses.

Table 19 Percentage escape and 24-h mortality of *Anopheles epiroticus* exposed to alpha-cypermethrin (0.03 g/m^2), deltamethrin (0.02 g/m^2) and permethrin (0.3 g/m^2) in combined noncontact trials.

Chemicals	Populations	No. Mosquitoes	% Escaped*	% Mortality*	
				Escaped	Remain
alpha-cypermethrin	TR	Treatment (56)	46.3	0	0
		Control (59)	10.2	0	1.9
	SK	Treatment (56)	3.2	40.0	36.6
		Control (59)	10.9	16.7	65.3
	PN	Treatment (60)	1.7	0	15.5
		Control (62)	1.6	0	11.3
	ST	Treatment (58)	1.7	0	0
		Control (60)	0	0	0
deltamethrin	TR	Treatment (60)	17.5	0	0
		Control (56)	9.1	0	2.0
	SK	Treatment (60)	11.7	28.5	11.8
		Control (56)	0	0	3.7
	PN	Treatment (58)	2.2	0	7.5
		Control (59)	5.1	5.1	30.5
	ST	Treatment (60)	0	0	8.7
		Control (59)	1.7	0	5.1
permethrin	TR	Treatment (59)	37.6	0	0
		Control (53)	17.0	0	0
	SK	Treatment (59)	0	0	0
		Control (53)	8.5	40.0	33.3
	PN	Treatment (60)	0	0	22.2
		Control (60)	5.0	0	10.0
	ST	Treatment (60)	0	0	0
		Control (60)	1.7	0	3.3

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani

* Treatment percent escape and mortality adjusted based on control responses.

Table 20 Escape time in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of *Anopheles epiroticus* to escape from chambers treated with insecticides during 30 min of exposure.

Populations	Test condition	Alpha-cypermethrin			Deltamethrin			Permethrin		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
TR	Contact	<1	5	17	<1	2	16	1	4	16
	Noncontact	10	29	-	29	-	-	19	-	-
SK	Contact	-	-	-	-	-	-	-	-	-
	Noncontact	-	-	-	-	-	-	-	-	-
PN	Contact	18	-	-	6	-	-	10	-	-
	Noncontact	-	-	-	-	-	-	-	-	-
ST	Contact	-	-	-	-	-	-	-	-	-
	Noncontact	-	-	-	-	-	-	-	-	-

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani

(-) Insufficient number of mosquitoes escaped from test chamber

Table 21 Comparison of escape patterns of *Anopheles epiroticus* between testconditions (paired contact, noncontact and control trials).

Populations	Insecticides	Treatment pairs		
		Control vs. Contact	Control vs. Noncontact	Contact vs. Noncontact
TR	alpha-cypermethrin	< 0.0001	< 0.0001	< 0.0001
	deltamethrin	< 0.0001	0.0282	< 0.0001
	permethrin	< 0.0001	0.0012	< 0.0001
SK	alpha-cypermethrin	0.7902	0.6467	0.5505
	deltamethrin	0.7687	0.0066	0.5561
	permethrin	0.0070	0.4732	0.0617
PN	alpha-cypermethrin	< 0.0001	0.5434	< 0.0001
	deltamethrin	0.0008	0.9301	0.0029
	permethrin	< 0.0001	0.1521	< 0.0001
ST	alpha-cypermethrin	0.9691	0.3091	0.5626
	deltamethrin	0.3014	0.3132	0.0807
	permethrin	1.0000	1.0000	1.0000

TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani

Table 22 Comparison of escape responses of *Anopheles epiroticus* between insecticides in paired contact and noncontact trials.

Chemicals	Contact				Noncontact			
	TR	SK	PN	ST	TR	SK	PN	ST
alpha-cypermethrin vs. deltamethrin	0.9112	0.1461	0.2132	0.6941	0.0034	0.7604	0.3566	0.3091
alpha-cypermethrin vs. permethrin	0.8160	0.7333	0.5955	0.5427	0.4959	0.1077	0.1556	0.9808
deltamethrin vs. permethrin	0.7040	0.2397	0.4611	0.3173	0.0209	0.1908	0.0358	0.3173

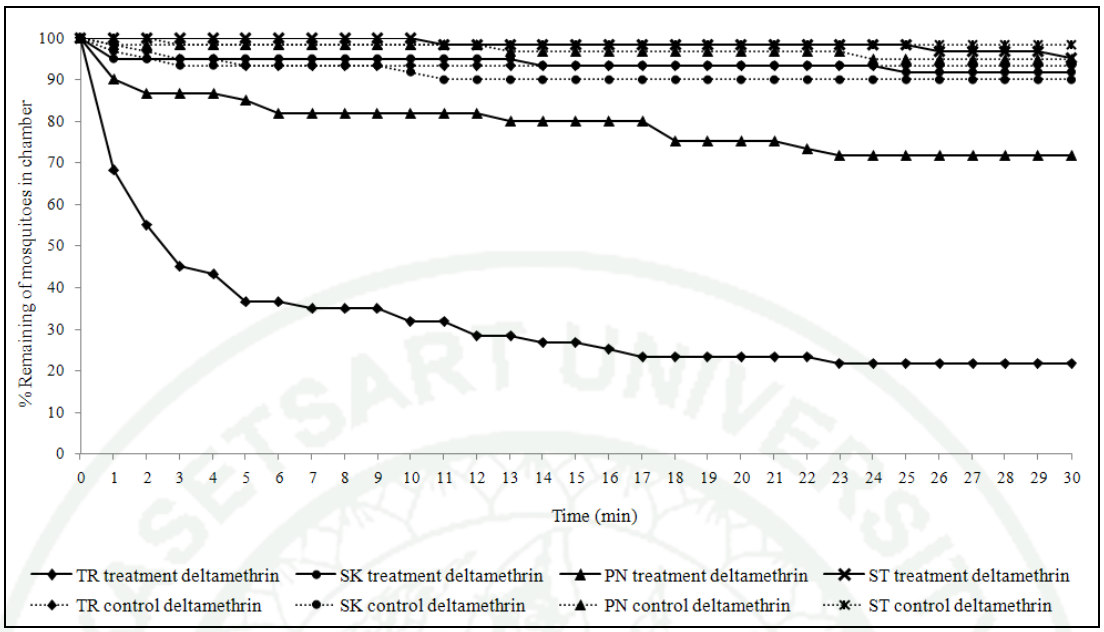

Figure 15 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.02 g/m^2 of deltamethrin in treatment and control contact trials.

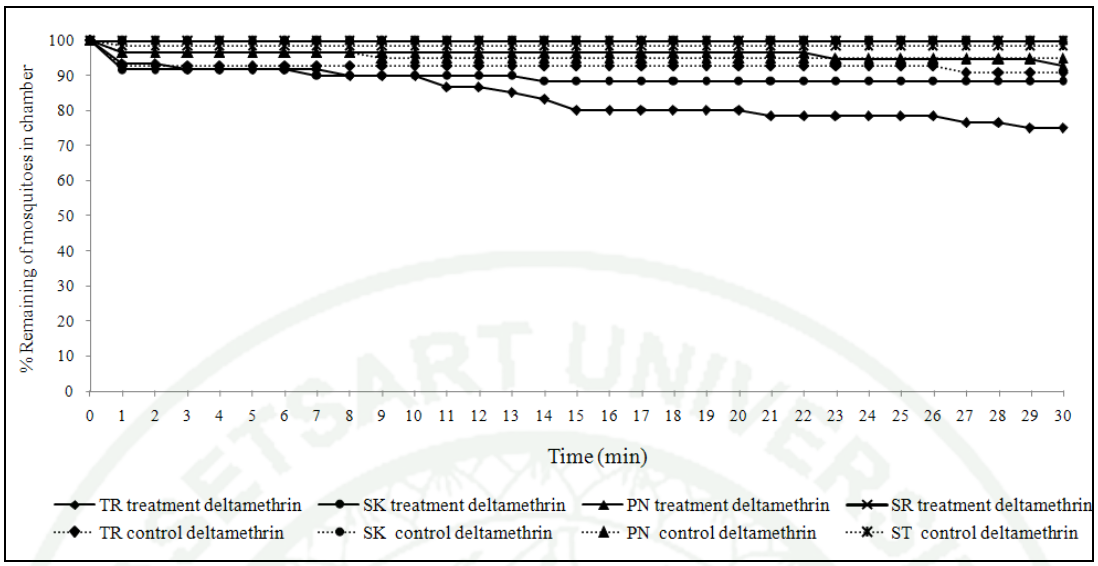

Figure 16 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.02 g/m^2 of deltamethrin in treatment and control noncontact trials.

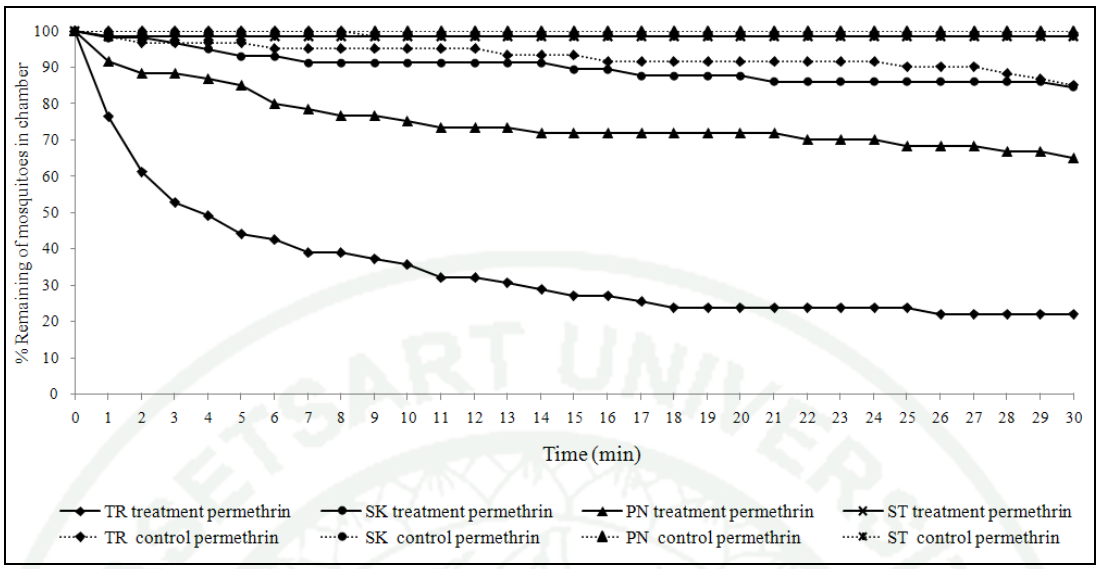

Figure 17 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.3 g/m^2 of permethrin in treatment and control contact trials**.**

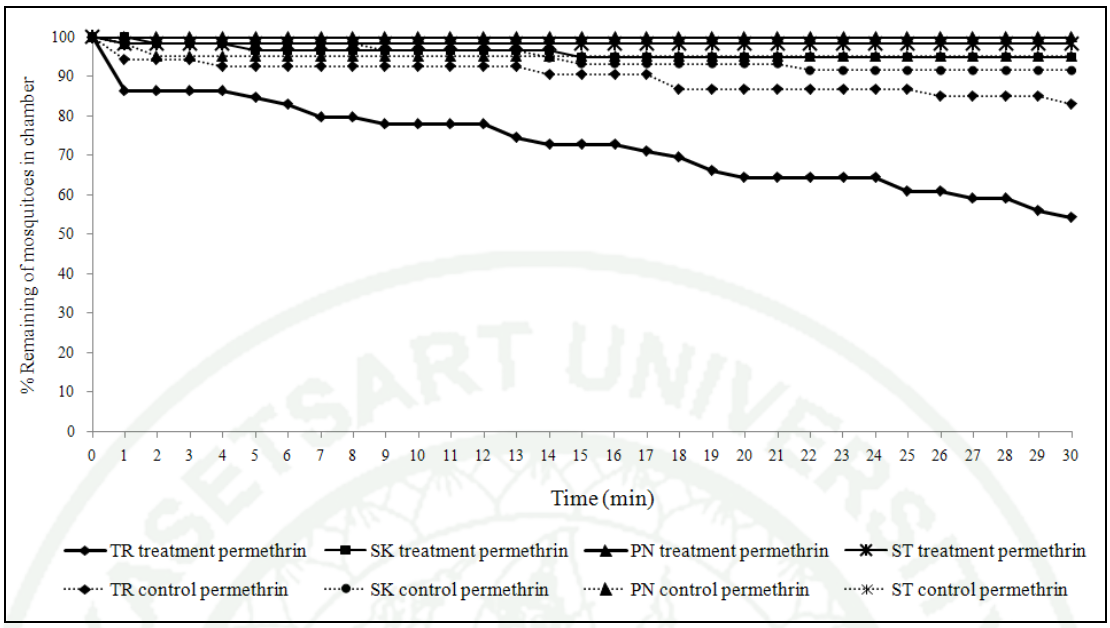

Figure 18 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.3 g/m^2 of permethrin in treatment and control noncontact trials.

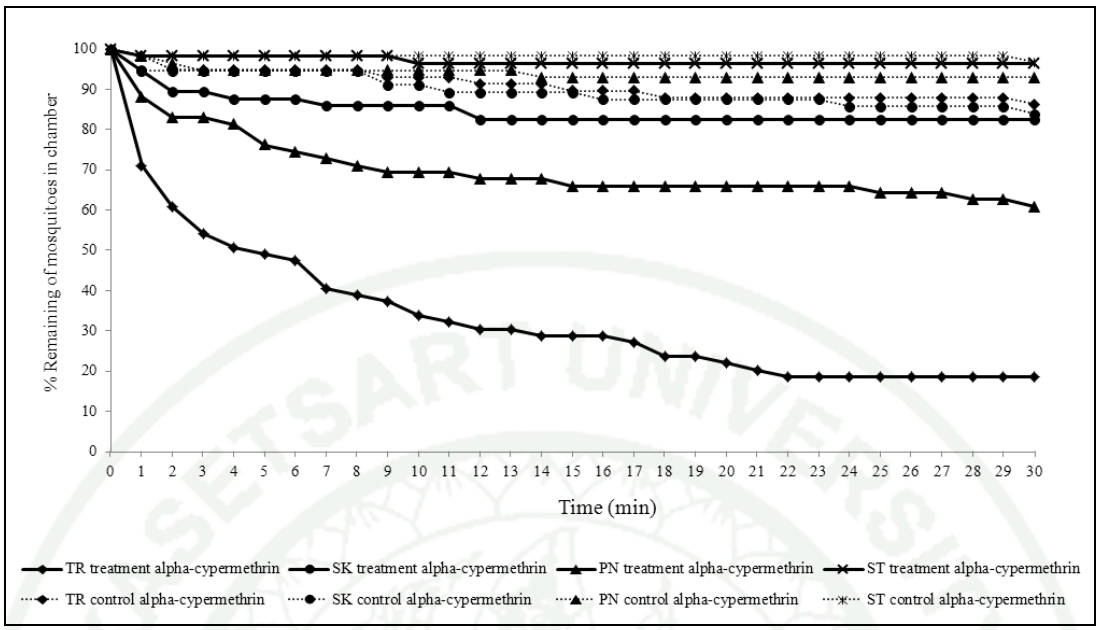

Figure 19 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.03 g/m^2 of alpha-cypermethrin in treatment and control contact trials.

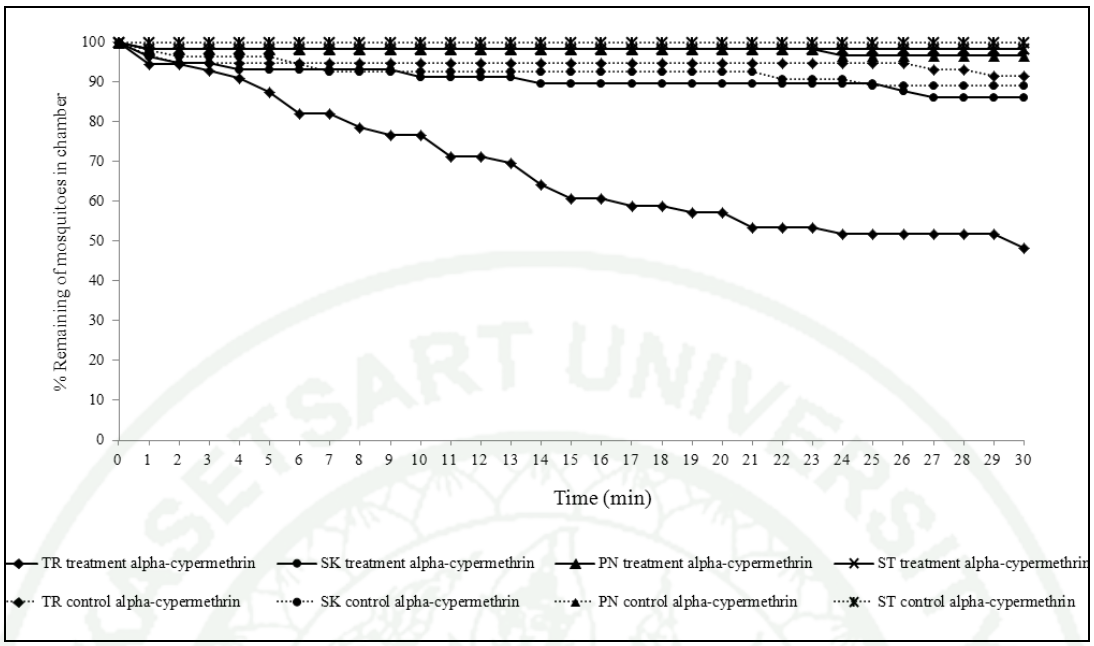

Figure 20 Escape probability of *Anopheles epiroticus* populations (TR = Trat; SK = Songkhla; PN = Phang Nga; ST = Surat Thani) exposed to 0.03 g/m^2 of alpha-cypermethrin in treatment and control noncontact trials.

Discussion

1. Host feeding activity and seasonal abundance of *Anopheles* mosquitoes in Chang Island

In this study, five important malaria vectors were molecularly identified as occurring on the island, including *An. dirus*, *An. minimus*, *An. maculatus*, *An. sawadwongporni* and *An. epiroticus*. *Anopheles epiroticus* was only found at Khlong Yuan, a site near the coast. In contrast, *An. dirus*, arguably the most efficient malaria vector in Thailand, was identified from Khlong Jao Lueam, an inland site in the forested lowland hills. Khlong Yuan contained five important malaria vector species in Thailand and showed much higher anopheline species diversity (15 species in all) compared to only two species captured in Khlong Jao Lueam of which *An. dirus* represented 94% of the catch. Khlong Yuan had several potential breeding habitats for *An. epiroticus*, a species that typically inhabits sunlit, mostly brackish water habitats containing floating algae (Sinka *et al*., 2011). Favorable habitats are abandoned or poorly maintained shrimp/fish ponds or inland seawater canals, but immature stages will also inhabit coastal ponds, swamps, mangrove, and rock pools (Manguin *et al*., 2008b). Despite the presence of potential larval habitats, sampling failed to identify the larval sites of *An. epiroticus* in and around Khlong Yuan. The inability to detect immature stages of this species is puzzling and something that was entirely unexpected given its relatively high adult abundance at the study site. A careful, systematic search for all possible larval habitats to include greater geographical coverage along the coastline of Chang Island is required.

The behavior of *An. sundaicus* sibling species can differ depending on the locality (Dusfour *et al*., 2004a; Linton *et al*., 2005). The biting activity of *An. sundaicus* complex typically occurs between 20:00 to 03:00 h (Sinka *et al.*, 2011). In Thailand, Gould *et al*. (1966) observed *An. sundaicus* s.l. (= *An. epiroticus*) greater outdoor biting frequency and feeding preference on cows, indicating more pronounced exophagy and zoophily. In contrast, the trophic behavior of *An. sundaicus* s.l. in Cambodia varied from exophagy to endophagy (Webster, 2000; Dusfour *et al*.,

2004a). In Rayong Province, Thailand, Sumruayphol *et a*l. (2010) observed *An. epiroticus* blood feeding predominantly between 18:00-24:00 h with a peak biting activity around midnight with a maximum of 6.6 bites/person/hour. In Chang Island, buffalo was found a strong attractant for *An. epiroticus* relative to human, indicating stronger zoophilic behavior. Buffalo-baited collections showed a peak activity during the early evening between 18:00 and19:00 h. The biting pattern of *An. epiroticus* in indoor and outdoor human landing collections were very similar, and activity densities stayed fairly uniform throughout the evening with only small increases of activity observed between hours of collection . The limited number of specimens of *An. dirus* (140), *An. minimus* (9), *An. maculatus* (15) and *An. sawadwongporni* (28), collected in this study did not allow for accurate interpretation of the host-seeking activity patterns in these malaria vector species.

The seasonal abundance of *An. epiroticus* in this study appeared to be influenced by several factors, most notably precipitation patterns. The population densities of *An. epiroticus* showed the greatest abundance during the dry season (November to April) for both the first and second years of observations. This species was found active at every collection period throughout the two years with the highest densities seen in November of both years and January 2012. In Indonesia, high densities of *An. sundaicus* s.l. were also associated with the dry season as lagoons and brackish water impoundments became more suitable habitats as water flow was impeded from entering the sea, creating large stagnant bodies of water with abundant floating algal mats (Sundararaman *et al*., 1957). However, in Rayong Province, *An. epiroticus* was found active throughout the year with the highest densities in the rainy season. During observations on Chang Island, a significant negative association with adult densities and higher mean ambient relative humidity was also noted.

Thailand faces recurring threats of emerging and re-emerging arthropod-borne diseases, especially malaria (Chareonviriyaphap *et al*., 2013). Malaria remains most prevalent along the less developed international borders of eastern Myanmar, northern Malaysia and western Cambodia, as well as coastal zones where *An. epiroticus* occurs. Chang Island is one of the most attractive and popular tourist sites in the

country. However, this island is still a malaria endemic area with an average of between 50-100 cases annually since the beginning of the 1990's. However, in 2013, only five malaria cases (all *P. vivax*) were detected, presumably transmitted on the island. The malaria risk areas are generally located in two relatively undeveloped (non-tourist) areas on the island, including a forest fringe in the interior low hills and coastal areas where *An. dirus* and *An. epiroticus* are present, respectively. The Chang National Park, on the eastern side of the island is primarily an inland forest with creeks, rivers, and waterfalls which provide many potential habitats for various malaria vectors.

Although malaria vectors have been identified on Chang Island (VBDC 3.4 Trat, unpublished data), no information about their biology and behavior (e.g., population dynamics, biting and host preference, and seasonal abundance) have been described from the island. Moreover, up until this study, there had been no attempt to identify the species present based on molecular methods. A critical component to understanding the local epidemiology is a precise identification of the vector species in various locales. The vectorial capacity of different sibling species can often vary in behavior, resulting in different capacities to transmit malaria. Such information is important to help identify the respective roles of each vector species in disease transmission and implementing the appropriate prevention and control strategies against specific targets.

Anopheles epiroticus belongs to the Sundaicus Complex in the Pyretophorus Series, a grouping of very important malaria vectors in Asia and Africa (Harbach, 2013). This species complex has long been recognized as malaria vectors in coastal areas and on islands in Southeast Asia (Sukowati *et al.*, 1996; Sukowati *et al*., 1999; Dusfour *et al*., 2004b; Linton *et al*., 2005) however, their relative importance as either major or secondary malaria vectors varies by locality and epidemiological factors influencing transmission (Schaefer and Kirnowardayo, 1983; Meek, 1995; Dusfour *et al*., 2007a). Of the 4 known allopatric species in the complex, only *An. epiroticus* has been identified in Thailand and has a known distribution in the eastern and southern coastal areas (Rattanarithikul *et al*., 2006; Dusfour *et al*., 2004b; Linton *et al*., 2005;

Dusfour *et al*., 2007b). The more recent development and wider application of PCR assays allowing for the identification of individual sibling species, a growing number of studies have described the trophic behavior, biting activity and seasonal abundance of several inland sibling vector species in Thailand (Chareonviriyaphap *et al.*, 2003; Sungvornyothin *et al*., 2006; Muenworn *et al*., 2009; Tananchai *et al*., 2012a; Tisgratog *et al*., 2012; Kongmee *et al*., 2012a) whereas similar investigations on coastal and island species, like *An. epiroticus*, has been limited.

Although, *An. epiroticus* is typically regarded a secondary vector of malaria in mainland Southeast Asia (Harinasuta *et al.*, 1974; Meek 1995; Trung *et al*., 2004) its potential as an efficient, albeit possibly incidental, vector remains a prime concern near tourist areas, as well as local coastal settlements on Chang Island. The information gathered in this study indicates two primary vectors on the island; *An. epiroticus* along the coastal zone and *An. dirus* in the interior parts. Although malaria parasite were not detected in any of the anophelines captured on humans or in buffalo-baited traps, these 2 species were the predominant vectors in their respective localities throughout much of the study period (albeit adult densities varied by season); therefore from an epidemiological perspective, all available evidence implicates both anophelines as the most likely vector candidates. As only 2 sites were longitudinally sampled, this does preclude other potential vector species playing a role in transmission on the island. Further investigations in other localities will better define and map vector distribution in relation to malaria transmission. Moreover, a far better understanding of the full range and preferred *An. epiroticus* larval habitats is also required and a pre-requisite to any meaningful attempt to control this species via source reduction and transmission of malaria.

Recent malaria statistics revealed that malaria transmission has been lower than in past. Since 2006, which reported a high number of 113 cases, between 2007 and 2013, an average of less than 13 cases of malaria per year has been reported from Chang Island. For this reason, it may be feasible to attempt an island-wide integrated campaign to eliminate malaria entirely from the island by identifying all residual foci and treat all human reservoirs of malaria. For those areas with evidence of recent or

high risk for renewed transmission, a time-limited vector control strategy could also complement the elimination effort when appropriate. As importation of malaria into Chang Island remains a threat, especially the risk of malaria is high among the workers who came from Cambodia, where is considered malaria endemic, any successful elimination effort will still require a robust surveillance system be in place to quickly identify cases and prevent secondary transmission. To advance the most appropriate vector control on Chang Island, additional investigations are needed on vector biology and transmission potential of local anopheline populations.

2. Detection of *Plasmodium* parasites in *Anopheles* mosquitoes

Determination of malaria sporozoite rates in mosquito vectors is an important component of malaria control programs, allowing the assessment of transmission risk and the impact of control interventions (Bass *et al*., 2008). Detection of sporozoites in *Anopheles* have been recorded in Thailand. The sporozoite rates of *An. minimus* were found to range between 0.3-1%, 6% for *An. dirus* s.l, 1-2% for *An. sawadwongporni* (Manguin *et al.*, 2008a). There are different methods available to analyze malaria parasites in mosquitoes, such as microscopy, ELISA and PCR; however, PCR method is more useful for detecting malaria parasites due to its high accuracy, with greater sensitivity and specificity (Tassanakajon *et al*., 1993; Snounou *et al*., 1993). In this study, 86.1% of *An. epiroticus* and 68.2% of *An. dirus* collected from human bait were used. The PCR method developed by Cunha *et al.* (2009) for detecting of *P. falciparum* and *P. vivax* by amplification of parasite mtDNA was used. In the final analysis, the PCR did not detect malaria parasites in the 640 *An. epiroticus* tested.

The efficiency of *Anopheles* mosquitoes for transmitting *Plasmodium* depends on the competence of the species and epidemiological circumstances. Although *An. epiroticus* was the most abundant anopheline detected in Chang Island during this study, however it is considered a secondary vector in Thailand. The same conclusion was drawn by Trung *et al.* (2004) who found that this species in the Mekong Delta, southern Vietnam, demonstrated very low vectorial capacity due to low survival and parous rates and the absence of sporozoite-positive mosquitoes during their surveys

(none of the 11 002 specimens was positive for *Plasmodium* circumsporozoite protein). On the other hand, the high parity rate (74%) and presence of infective mosquitoes (sporozoite rate of 0.97% with both *P. falciparum* and *P. vivax* detected by nested PCR and real-time PCR of 926 specimens) in Rayong Province would indicate this species is an important vector in some localities (Sumruayphol *et al*., 2010).

In this study, *An. dirus* initially gave apparent false positive results for *P. vivax* 36 positive of 90 tested). Obsomer *et al*. (2007) did a literature review on *An. dirus* complex, sporozoite rates of this species vary with season and location, with the highest rates 7.8% recorded in India and rates up to 14% in Burma (Myanmar). In addition, sporozoite rates in *An. dirus* of between1.45% - 2.56% have been recorded in Lao PDR (Vythilingam *et al*., 2005). Consequently, we sent PCR products for sequencing to confirm amplification for *P. vivax*, unfortunately the PCR products was unable to be sequenced because of low concentration of DNA. In addition, we also tested a nested-PCR, which failed to detect parasites. Therefore, we concluded that no malaria infection was seen in *An. dirus* samples from Chang Island. False positive results from PCR may be the result of contamination with previously amplified DNA. Rubio *et al*. (2002) noted that false positives, although relatively rare (0.3 %) can occur because of cross-contamination. Any PCR, especially consecutive rounds of amplification, may result in false-positives because of cross-contamination of samples. Foley *et al*. (2012) provided recommendations to minimize this risk by: 1) reduce DNA degradation in the field; 2) mosquito abdomens be separated anterior to the junction of the thorax and abdomen; and 3) DNA sequencing of a sub-sample of positive results should be undertaken if possible.

3. Insecticide susceptibility and behavioral avoidance in *Anopheles epiroticus*

Four field-collected populations of *An. epiroticus* displayed complete susceptibility to deltamethrin, permethrin and alpha-cypermethrin, even though deltamethrin and permethrin in various forms have been used in different locations in Thailand to control malaria since 1990 and 1992, respectively (Chareonviriyaphap *et*

al., 1999). Deltamethrin has been used primary for indoor residual spraying (IRS) of human dwellings and either deltamethrin or permethrin for individual and factory treatment of bednets to combat malaria transmission in Thailand (MOPH, 2012). Two of the study locations, Trat and Phang Nga Provinces are areas that experience 'periodic' malaria transmission and have had pyrethroids used in anti-malarial vector control campaigns (TR= permethrin, deltamethrin and alpha-cypermethrin, and PN= bifenthrin). By MOPH criteria, both SK and ST areas are regarded as 'low risk' malaria locations and have had no insecticide-based malaria control activities in the areas where specimens were collected for this study. To date, insecticide resistance has not been reported in *An. epiroticus* (or formerly when called *An. sundaicus*) in Thailand (Chareonviriyaphap *et al*., 1999; Dusfour *et al*., 2004a). However, Van Bortel *et al*. (2008) reported that *An. epiroticus* that occurs in southern Vietnam only was resistant to 4 different pyrethroids (permethrin, deltamethrin, alpha-cypermethrin and lambda-cyhalothrin).

This study is the first report to distinguish two types of behavioral responses, contact irritancy and noncontact repellency of *An. epiroticus* to insecticides, namely deltamethrin, permethrin and alpha-cypermethrin. We found that two wild-caught populations of *An. epiroticus* rapidly escaped from direct contact with surfaces treated with each compound compared to paired untreated controls. Alpha-cypermethrin exhibited the strongest irritant effect on *An. epiroticus* followed by deltamethrin and permethrin. These findings indicate that contact irritancy is the primary behavioral response of all three compounds. Similar findings of strong contact excitatory action of pyrethroids have been observed in other *Anopheles* species tested in Thailand (*An. dirus*, *An. minimus*, *An. harrisoni*, *An. maculatus* and *An. sawadwongporni*) (Chareonviriyaphap *et al*., 2004; Tisgratog *et al*., 2011; Tananchai *et al*., 2012b).

The most striking escape response following physical contact with each of three pyrethroids was observed in the Chang Island population, Trat Province (TR). More moderate or intermediate escape responses were observed with the population from Phang Nga Province (PN) but remained significantly different from control responses. Post-exposure mortality was low in mosquitoes escaping the treated

chambers in virtually all contact and noncontact trials, suggesting that behavioral avoidance rather than toxicity may play a more significant role in control of transmission and contact with humans near treated surfaces.

Interestingly, TR produced a strong overall combined 'excito-repellency' to all three chemicals, while PN reactions were more modest in excitation and relatively low for spatial repellency. On the other hand, no or very minimal escape responses to both contact and noncontact exposures to the 3 pyrethroids were observed in the two populations from the Surat Thani and Songkhla Provinces (ST and SK) collected on the eastern coast of southernmost Thailand. These two populations were collected from cattle near rice fields. It is unclear why there were very poor escape responses in these two populations, especially in the contact tests as both were found equally susceptible to the insecticides in toxicity tests as the TR and PN mosquitoes. This runs counter to years of similar investigations with other anopheline species in Thailand and elsewhere. All tests took place during daylight hours. All 4 populations were wild-caught and predominately freshly bloodfed at time of testing. Age-grading (e.g., parity) was not examined so the relative age of each population is not known. Therefore, differences between these 2 excito-refractory populations and TR and PN may have possibly been due to age or influenced by some other aspect of physiological status of the female mosquitoes at the time of testing (Sungvornyothin *et al*., 2001; Chareonviriyaphap *et al*., 2006). Dates of experimentation for each population may have played a role but a plausible association is not readily apparent. TR was tested in March 2012, SK in November 2012, and both ST and PN in February 2013. Moreover, testing of populations was done independently by 2 persons (TR & SK and ST & PN, respectively) thus making it unlikely to obtain observations due to some form of systematic experimental error. However, previous history of exposure to residual pyrethroids used in the national malaria control program does showed a difference between responsive (TR and PN) and non-responsive (SK and ST) populations. Both refractory populations came from non-control areas, while both populations showing either strong or moderate escape responses had been presumably exposed to pyrethroids (without measurable selection for resistance) in the recent past.

It is also possible there might be some unique, natural genetic pre-disposition in the ST and SK populations regard relative insusceptibility to the excitatory and repellency effects of the pyrethroids or concentrations used in this study. Interestingly, it has been shown that an insecticide susceptible population maintained as an inbred colony in a laboratory setting for over 20 years demonstrated much less avoidance behavior to insecticides compared to newly wild-caught susceptible populations of the same species (Chareonviriyaphap *et al*., 1997, 2004). Moreover, observations on anopheline response to insecticides have found that very closely related, but different species or different populations of the same species can produce markedly different behavioral responses to the same active ingredients and test systems. For example, relatively strong repellency response to bifenthrin was present in a laboratory colony of *Anopheles minimus*, whereas a field population provided only a weak escape reaction (Tisgratog *et al.*, 2011). Similarly, relatively weak repellency responses in *Anopheles harrisoni* were seen compared to its sibling species, *An. minimus*, showing marked repellency to pyrethroids (Pothikasikorn *et al*., 2005, 2007).

The use of wild-caught mosquitoes carries both advantages and liabilities for interpretation of behavioral data. One advantage with using natural populations is for indicating changes in insecticide susceptibility that more closely reflect the changes in intervention efficacy in the field. Moreover, the age distribution of the vectors should be representative of the wild vector population at a given time and location as well as being more genetically representative of the population at large when compared with F1 progeny in the laboratory that may be more restricted genetically because of inadvertent selective rearing in the process. The primary disadvantage with using wild-caught specimens is varying and sometimes unknown age distribution and physiological condition of vectors that can reduce the comparability of results from one sampling period to the next. Use of laboratory-reared F1 progeny allows the age of vectors to be controlled and uniform between tests, thus allowing more meaningful comparisons between samples from different times and locations.

Previous studies have clearly demonstrated that behavioral responses to DDT and other chemicals more commonly in use for IRS can occur in many malaria vectors thus raising the concern of the true impact of avoidance behavior in transmission control. As synthetic pyrethroids have dominated as the control chemicals against malaria vectors in Thailand for over two decades, detailed monitoring and understanding of behavioral responses of mosquitoes to pyrethroids is of operational significance for determining the most likely impact on vector populations and transmission control. To our knowledge there has been no published information on behavioral avoidance of *An. epiroticus* (or formerly reported as either *Anopheles sundaicus* A or *An. sundaicus* s.l.) to insecticidal compounds in Thailand and only limited information on physiological responses to chemical exposure. Van Bortel *et al.* (2008) reported two Thai populations of *An. epiroticus* as susceptible to DDT and other data collected in 2003-2005 provided strong indications of permethrin susceptibility. Therefore, this study represents the first comprehensive attempt for characterizing the physiological and behavioral responses of *An. epiroticus* to deltamethrin, permethrin and alpha-cypermethrin.

Behavioral responses by mosquitoes to insecticides have been recognized for many decades (Kennedy, 1947; Davidson, 1953). In the past, chemicals that produced avoidance responses in mosquitoes were often regarded as inferior attributes when making an informed decision on selecting compounds for vector control programs. Virtually all work of the past focused on the direct insecticidal (toxic) action on mosquito populations as the primary, if not only, means to control vectors and transmission. Until recently, relatively few investigations concentrated on behavioral responses, specifically avoidance or deterrence of mosquitoes to sub-lethal exposure to toxic chemicals. In addition to toxicity, at least two different forms of behavioral responses can be broadly defined as 'excito-repellency': contact excitation (irritancy) and noncontact spatial repellency (Roberts *et al*., 2000). Irritant escape responses follow direct physical contact with an active ingredient (e.g., a chemically-treated surface); whereas repellency results when an insect spatially detects and avoids a space containing an active ingredient therefore without making physical contact (Roberts and Andre, 1994; Chareonviriyaphap *et al*., 1997). Both types of behavioral

responses can be experimentally differentiated by using an excito-repellency (ER) test system (Chareonviriyaphap *et al*., 1997; Tanasinchayakul *et al*., 2006). Additionally, a modular, high-throughput laboratory-based assay system for screening of irritancy, repellency and toxicity has been developed (Grieco *et al*., 2007). Since the development of these two independent test systems and a quantified mathematical framework for analysis, reports on mosquito behavioral responses to public health insecticides and topical repellent compounds have progressively increased (Sungvornyothin *et al*., 2001; Kongmee *et al*., 2004; Chareonviriyaphap *et al*., 2001, 2004; Grieco *et al*., 2005, 2007; Pothikasikorn *et al*., 2005, 2007; Muenvorn *et al*., 2006; Polsomboon *et al*., 2008; Monkalangoon *et al*., 2009; Thanispong *et al*., 2010; Tisgratog *et al*., 2011; Tananchai *et al*., 2012b).

The World Health Organization (2013b) has recently issued a document on guidelines for efficacy testing of spatial repellents. However, there remains a need for the optimization and standardization of ER test systems to assess and compare behavioral responses of mosquitoes to current and new public health insecticides under varying conditions. Despite the progress in insecticide avoidance studies, there remains much to understand about insecticidal influence on the short- and long-term effects on the biology and behavior of mosquitoes that impacts the probability of malaria transmission. Clarification on the innate behavioral responses assists in the rationale for selecting the most appropriate mosquito control activities and justification of expenditures. Together with toxicity assays, ER assays should be an integral component of any evaluation of an insecticide's full traits, capabilities and potential to suppress disease transmission.

CONCLUSION AND RECOMMENDATIONS

The bionomics and vector incrimination of *Anopheles* species in two village locations on Chang Island, Thailand, Khlong Yuan located near the coast and Khlong Jao Lueam in the low hills of the central interior is described. This information serves as a basis for designing vector control programs that target specific species of public health importance. With enhanced vector control, integrated with other interventions, the possibility of elimination of endemic malaria transmission on the island might be possible.

A total of 15 species were collected on Chang Island from January 2011 to November 2012. Five important putative malaria vectors were molecularly identified, including *An. epiroticus, An. dirus, An. sawadwongporni, An. maculatus,* and *An. minimus.* Of those, *An. epiroticus* was the most commonly collected species in the coastal site, whereas *An. dirus* was found to be most abundant in the forest-hill site. From both locations, a greater number of mosquitoes were collected during the dry season compared to the wet months. *Anopheles epiroticus* showed greater exophagic and zoophilic behavior with the highest blood feeding densities occurring between 18:00 and 19:00 h. In contrast, *An. dirus* demonstrated an activity peak between midnight and 01:00 h.

A total of 640 *An. epiroticus* and 90 *An. dirus* collected by HLC were tested for presence of *Plasmodium* infection using two PCR methods. Neither species was found to harbor malaria parasite infection.

Insecticide susceptibility and behavioral responses of wild-caught populations of female *Anopheles epiroticus* to three synthetic pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin) were assessed. Test populations were collected from three localities along the eastern coast, Trat (TR), Songkhla (SK), and Surat Thani (ST) and one population from the western coast, Phang Nga (PN). Results showed that all four populations of *An. epiroticus* were completely susceptible to all

three synthetic pyrethroids tested at the W.H.O. recommended concentration of each. Behavioral (escape) responses to test compounds were characterized for all four populations using an excito-repellency test system. TR displayed the strongest contact excitation ('irritancy') escape response (76.8% exposed to deltamethrin, 74.1% permethrin, and 78.4% alpha-cypermethrin), followed by the PN population (24.4% deltamethrin, 35% permethrin, and 34.4% for alpha-cypermethrin) during direct contact with surfaces treated with each active ingredient compared with match-paired untreated controls. Moderate noncontact (spatial) repellency responses to all three compounds was observed in the TR population but was comparatively weaker than paired contact tests. Few mosquitoes from the SK and ST populations escaped from test chambers, regardless of insecticide tested or type of trial design (irritancy or repellency). We conclude that contact excitation was a major behavioral response in two populations of *An. epiroticus*, whereas 2 other populations showed virtually no escape response following exposure to pyrethroids. The explanation for these large unexpected differences in avoidance responses between pyrethroid-susceptible populations of the same species is unclear and warrants further investigation.

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APPENDIX

1 MTris HCl pH 8.0 (volume 100 ml)

Reagents:

Tris base (MW= 121.14)	12.11	g
Distilled H_2O	70	ml
Concentrated HCl		

Preparation:

1) Mix 12.11 g of Tris base with 70 ml of ddH_2O

2) Fine adjust to the desired pH 8.0 with concentrated HCl

4) Add distilled H_2O until final volume is 100 ml

5) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

5 mM Potassium acetate, pH 9.0 (volume 100 ml)

Reagents:

Potassium acetate (MW= 98.142)	0.0491	g
Distilled H_2O	70	ml
NaOH (0.1 M)		

Preparation:

1) Mix 0.0491 g of Potassium acetate with 70 ml of ddH_2O

2) Fine adjust to the desired pH 9.0 with NaOH (0.1 M)

4) Add distilled H_2O until final volume is 100 ml

5) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

0.5 M EDTA, pH 8.0 (volume 100 ml)

Reagents:

EDTA (Ethylenediamine tetraacetic acid (MW= 372.24)	18.61	g
Distilled H_2O	80	ml
NaOH		

Preparation:

1) Mix 186.1 g EDTA with to 80 ml of Distilled H_2O

2) Add about 2g of NaOH pellets while stirring to bring the pH to 8.0

(EDTA won't completely dissolve until the pH is around 8.0)

3) Add distilled H_2O until final volume is 100 ml

4) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

TE Buffer (volume 100ml)

Reagents:

1M Tris-HCl	1 ml
0.5M EDTA	0.2 ml
Distilled H_2O	

Preparation:

1) Mix 1 ml of 1M Tris-HCl and 0.2 ml of 0.5M EDTA solution with distilled H_2O and make up the volume to 100ml

2) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

50X TAE buffer (volume 1,000 ml)

Reagents:

Tris base ($C_4H_{11}NO_3$, Molecular Weight: 121.14)	242.28 g
Glacial acetic acid (H_3BO_3, Molecular Weight: 61.83)	57.1 ml
0.5 M EDTA stock solution (pH 8.0)	
Distilled H_2O	800 ml

Preparation:

1) Mix 242.28 g of Tris base with to 800 ml of distilled H_2O

2) Add 100 ml of 0.5 M EDTA and 57.1 ml glacial acetic acid.

3) Add ddH_2O until final volume is 1,000 ml

4) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

Extraction buffer (volume 200 ml)

Reagents:

0.2 M Sucross (MW 342.3) No.104	13.692	g
0.5% SDS	1	g
0.5 M EDTA stock solution (pH 8.0)	20	ml
1 M Tris-HCl pH 8	20	ml

Distilled H_2O

Preparation:

1) Mix 13.692 g of 0.2 M Sucross and 1 g of 0.5% SDS with to 130 ml of distilled H_2O

2) Add 20 ml of 0.5 M EDTA pH 8 and 20 ml 1 M Tris-HCl pH 8

3) Add distilled H_2O until final volume is 200 ml

4) Autoclave to sterilize (20 minutes at 15 lb/sq.in. (psi) from 121-124°C)

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