BEHAVIORAL RESPONSES OF *ANOPHELES MACULATUS* GROUP IN RELATION TO REPELLENCY EFFECTS OF SELECTED INSECTICIDES

INTRODUCTION

Malaria is currently one of the most important vector borne diseases in Thailand [Ministry of Public Health (MOPH), 2005]. The disease is transmitted by Anopheline mosquitoes. From a total of 74 Anopheline species, three species are major vectors of malaria i.e. *Anopheles dirus, Anopheles minimus,* and *Anopheles maculatus.* All the species represent complex of which can not be morphological distinguished (Baimai, 1988 and Rattanarithikul and Panthusiri, 1994). Therefore, the identification must depend on various techniques such as chromosomal analysis (Baimai, 1988), allozyme typing (Green *et al*., 1992), and allele-specific polymerase chain reaction (AS-PCR) technique (Walton *et al*., 1999). Field observation on behavioral variations, breeding habitats and mating system, is another important tool used for the study of species complex. *Anopheles dirus* is a primary vector of malaria, followed by the other two species, *An. minimus* and *An. maculatus* (Chareonviriyaphap *et al*., 2000).

Anopheles (Cellia) maculatus group is widely recognized as an important vector of malaria throughout the Oriental realm, including Thailand, Indonesia, Malaysia and the Philippines (Reid, 1968). Those formally described include at least eight biologically related species based on variability in morphological, behavioral and genetic characters (Green *et al*., 1985, Rattanarithikul and Green, 1986, Chiang *et al*., 1991, Kittiyapong *et al*., 1992 and Bangs *et al*., 2002). In Thailand, six species were identified i.e. *An. maculatus* Theobald *sensu stricto, Anopheles sawadwongporni* Rattanarithikul and Green, *Anopheles dravidicus* Christophers, *Anopheles notanandai* Rattanarithikul and Green, *Anopheles willmori* (James), and *Anopheles psuedowillmori* (Theobald) (Green *et al*., 1985, Rattanarithikul and Green, 1986, Rattanarithikul and Harbach, 1990, Kittayapong *et al*., 1990 and Green *et al*., 1992).

Among this group, three play a major role in human malaria transmission in Southeast Asia i.e. *An. maculatus s.s.* (Reid, 1968), *An. willmori* (James) (Pradhan *et al*., 1970) and *An. pseudowillmori* (Theobald) (Green *et al*., 1991). *Anopheles sawadwongporni* is a common species and found in high density throughout Thailand, especially in the provinces along the Thai-Myanmar and Thai-Malaysia borders (MOPH, 2005). This species has been reported an important vector of *Plasmodium falciparum* in Thailand (Rattanarithikul *et al*., 1996).

For 50 years, DDT was used for malaria control as an indoor residual spray (IRS) in Thailand. DDT was completely stopped from public health use in 2001 although phase-out period was planned from 1995 to 1999 (Chareonviriyaphap *et al*., 2000). The reasons for removal of DDT from the control of malaria were mainly due to perceived adverse impact on environment and poor community compliance as well as undesirable compound from the MOPH (Chareonviriyaphap *et al*., 1999). DDT was replaced by two potential synthetic pyrethroids, deltamethrin and permethrin (Chareonviriyaphap *et al*., 2000, Sungvornyothin *et al*., 2001, Chareonviriyaphap *et al*., 2004, Kongmee *et al*., 2004, Potikasikorn *et al*., 2005, and Chareonviriyaphap *et al*., 2006). The first has been primary used as the IRS whereas the latter been applied as impregnated-treated net (ITN) (Chareonviriyaphap *et al*., 2004 and MOPH, 2005). Although DDT was completely withdrawn from malaria control program in Thailand in 2001, real impact of this compound on vector populations in terms of behavioral aspects remains unclear. Behavioral responses of mosquito vectors to insecticides definitely reflect their vector control status and remain significant components for insecticide-malaria control program. The effects of insecticides on altering normal behavioral activity, therefore, are significant in the understanding and control the disease (Spark *et al*., 1989, Klowden, 1996 and Costantini *et al*., 1999). More field research is needed to verify the responses of insecticides by many known vector populations from different geographical areas (Chareonviriyaphap *et al*., 1997, Bortel *et al*., 2004 and Potikasikorn *et al*., 2005).

Behavioral responses or known as “insecticide avoidance” can be separated into two important and distinct categories, contact irritancy and noncontact repellency

(Roberts *et al*., 1997). Irritant responses result from physical contact with chemical-treated surfaces, whereas repellency is an avoidance response to devoid of making actual contact with insecticides (Lockwood *et al*., 1984, Chareonviriyaphap *et al*.,1997, Roberts *et al*., 1997 and Potikasikorn *et al*., 2005). Although, behavioral responses have been recorded with various mosquito species and populations of *Anopheles* from Thailand using the excito-repellency test box, none so far has been performed to compare the behavioral responses between wild caught *An. maculatus and An. sawadwongporni*, important vectors of malaria in Thailand, to DDT and permethrin. Besides IRS by DDT that was completely withdrawn from Thailand, fabric impregnated with permethrin has been widely introduced in some malarious areas of Thailand. Although this compound is used in a small scale, true impact of this compound on *An. maculatus* and *An. sawadwongporni* should be carefully monitored. Described herein are the observations on using the excito-repellency test system to quantitatively measure behavioral responses between wild-caught populations of *An. maculatus and An. sawadwongporni* exposed to recommended field doses of DDT and permethrin. In addition, biting cycle and feeding preference of *An. maculatus* group and other related species along with geographic distribution were investigated. The *An. maculatus* group was characterized for insecticide susceptibilities and molecular variations.

LITERATURE REVIEWS

1. Malaria situation

Malaria remains one of the most serious infectious diseases in the tropical and subtropical zones of the world (WHO, 2005). Most of malaria endemic areas include zones where severe malaria problems have originated from major ecological and sociological changes. These areas include several countries in Africa, the Americas and Asia (WHO, 2005). In Asia, the number of malaria cases remains unacceptably high, except for a few countries, such as India, Sri Lanka and Bangladesh (WHO, 2005). In Thailand, malaria cases have been significantly reduced. The apparent reduction in malaria cases has been attributed partly to implementing new strategies and strengthen existing disease control programs and to a certain extent as a result of effective, well organized vector control program using indoor residual spray (IRS) and impregnated-treated net (ITN). The current distribution of malaria in Thailand is given in Table 1.

Table 1 Number of malaria cases in Thailand from 1998 to 2005

Year	No. of populations	No. of blood examinations	No. of malaria cases		API** /1000	ABER (%)
			Thai	Non-Thai		
1998	56,581,759	4,212,794	125,013	67,029	2.21	7.45
1999	56,706,163	4,455,315	128,833	79,490	2.27	7.86
2000	57,356,571	4,403,739	91,703	57,883	1.6	7.68
2001	57,823,000	4,353,655	67,749	58,846	1.17	7.53
2002	58,681,371	3,936,014	47,948	33,983	0.82	6.71
2003	59,884,424	3,339,072	38,902	32,385	0.63	5.58
2004	60,452,157	3,069,490	30,482	27,110	0.5	5.08
2005	60,846,656	2,524,788	11,416*	11,193*	0.19	4.15

Source : Ministry of Public Health (2005)

* : Preliminary report (October 2004-June 2005)

**: Thai malaria cases only

Department of Disease Control (DDC) is the main government institute that is responsible for gathering and collecting all malaria cases in the country. The DDC has been recording malaria since 1949 in the form of the Annual Parasite Incidence (API) and the Annual Blood Examination Rate (ABER) (Chareonviriyaphap *et al*., 2000). The API is the number of positive case per 1000 populations derived from both passive and active case survey and the ABER is the number of blood slides examined per 1000 populations as a measure of public population per year. Malaria cases were documented by Vector Borne Disease Center (VBDC), DDC, MOPH (Table 1). Based on the activities on malaria surveillance from 1998 to 2005 (Table 1), reported cases of malaria were peaked at 1999 with 128,833 and 79,490 cases in Thai and Non-Thai populations, respectively. Most malaria cases have been reported from the undeveloped borders of eastern Myanmar, western Cambodia and northern Malaysia (MOPH, 2005). Recent surveillance data indicate that malaria has returned as a consequence of the political problems along the international boundaries, especially between Thailand-Malaysia and Thailand-Myanmar.

2. Malaria vectors

There are approximately 74 *Anopheles* species in Thailand. Of these 3 species are considered to be important malaria vectors including *An. dirus*, *An. minimus* and *An. maculatus*. All 3 taxa represent individual complexes which are not easily separated from one another (Rattanarithikul and Panthusiri, 1994). *Anopheles dirus* is a forest and forest-fringe mosquito whereas *An. minimus* and *An. maculatus* are more likely to associate with low hilled-forest areas and seem to have close contact with human along the margin of the village (Chareonviriyaphap *et al*., 2000).

Anopheles (Cellia) maculatus Theobald was identified in the Theobaldi group of Neocellia series. This group includes *Anopheles karwari* (James) and *Anopheles theobaldi* Giles (Subbaroa, 1998). The *An. maculatus* complex is deemed a main vector of malaria in all Oriental region, including southern Thailand, western Indonesia, Malaysia and the Philippines (Bangs *et al*., 2002). Eight biological species have been previously recognized, based on techniques such as morphological

character and cytogenetic of polylene chromosomes (Rattanarithikul and Green, 1986 and Rattanarithikul and Harbach, 1990). These include *An. maculatus*, *An. pseudowillmori*, *An. willmori*, *An. notanandai*, *An. sawadwongporni* and *An. dravidicus* (Rattanarithikul and Green, 1986 and Bangs *et al*., 2002) and two species in the Philippines, *An. dispar* and *An. greeni* (Rattanarithikul and Harbach, 1990). Within *An. maculatus*, two forms were chromosomally identified and designated as B and E. Form B has been found northward from 13^{o} north latitude and form E has been found southward from12^{o} north latitude (Rongnoparut *et al*., 1996). This study indicated that natural gene flow between B and E populations seems to be restricted, mainly due to geographical barriers.

Bionomics of *An. maculatus* complex have been observed from various areas in Thailand. Study in Pakchong District, Nakhon Ratchasrima Province, northeastern Thailand and Sadao District, Songkhla Province, southern Thailand provided valuable results on seasonal density and prevalence of *An. maculatus* complex (Upathum *et al*., 1988). Results showed that the density of *An. maculatus* complex in Pakchong was approximately 4.5 times greater than those in Sadao during the wet season, with their total numbers of 6,090 and 1,344, respectively. In Pakchong District, the numbers of *An. sawadwongporni*, *An. maculatus* and *An. dravidicus* were 4,994 (82%), 1,067 (17.5%) and 29 (0.5%), respectively. *Anopheles maculatus* and *An. sawadwongporni* were observed in high density during the rainy season and gradually decreased in the end of the season. The peak density of *An. dravidicus* also occurred during the rainy season. In contrast, only one species of *An. maculatus* group, *An. maculatus s.s.*, was found in Sadao District and the densities were comparatively high during two years of observations (Upathum *et al*., 1988). Observation on Anopheline abundance was also conducted in Tak Province, northwestern Thailand using human bait collections from March to July 1986 (Harbach *et al*., 1987). Results revealed that *An. maculatus* was found in high density (55%) and obvious biting peak was seen at 1930 hrs. Rattanarithikul *et al*. (1996) observed the seasonal abundance of *An. maculatus* group from three Provinces, Phetchaburi, Prachuap Khiri Khan and Chumphon in southern Thailand. Only two species, *An. maculatus* and *An. sawadwongporni* were found in high densities and mostly were associated with the rainy season.

Host preference of *An. maculatus* complex in Pakchong and Sadao Districts indicated that the females of the *An. maculatus* complex preferred to feed on cattle rather than on human. Hassan *et al*. (2001) conducted the composition and biting activity of *Anopheles* attracted to human bait in a malaria endemic village in peninsular Malaysia near the Thailand border. This study found that *An. maculatus* was more likely to feed on humans outside rather than inside, exhibiting significant zoophilic and exophagic behaviors.

Because *An. maculatus* is a species complex, especially form B and E, morphological identification alone can not be used as a standard tool. Molecular techniques for species identification have provided useful information and received great attention in recent years. The methods have been applied to all organisms, including important groups of mosquito species complex. Precise identification of target species has proved to be one of the successful vector control. Previous works indicated that mosquito taxonomy has been successfully identified by morphological characters, chromosomal characters and isozyme markers. There are, however, a number of molecular techniques such as DNA sequencing in which has greatly improved the accuracy of species identification.

The nuclear ribosomal DNA (rDNA) consists of tandemly repeated transcriptional units. The repeated transcriptional unit composes of a leader promoter region (external transcribed spacer), 18s rRNA, first internal transcribed spacer (ITS1), 5.8s rRNA, second internal transcribed spacer (ITS2), 28s rRNA and intergenic nontranscribed spacer (IGS). The nucleotide sequence variation in noncoding regions of rDNA such as ITS2 is dramatically useful for species identification (Collins and Paskewitz, 1996). Torres *et al*. (2000) investigated the usefulness of ITS2 and D3 (third domain of 28s) sequence variations in identifying *An. dispar* and *An. greeni* in the Philippines. The ITS2 sequences of *An. dispar* and *An. greeni* at 320 and 318 bp in length, respectively, were obtained. Within ITS2, 13 interspecific base differences were noted (2 indels, 6 transitions and 5 transversions). Sequencing of D3 resulted 367 bp in length, 9 interspecific base differences were noted (7 transitions and 2 transversions). No intraspecific variation

was detected in both ITS2 and D3 sequences. The GC content of D3 sequences (60 and 59%) is similar to those of ITS2 (59.4 and 58.5%) for *An. dispar* and *An. greeni*, respectively. Of the restriction enzymes used for distinguishing the two ITS2 sequence types, *Hae* II was the best based on cost and predicted separation of bands, with two bands in both *An. dispar* (173 and 134 bp) and *An. greeni* (263 and 173 bp) (Torres *et al*., 2000).

3. Vector control

By far, vector control is considered as one of the most successful methods in control malaria. Several strategies for vector control have been proposed including, indoor residual spray (IRS), impregnated-treated net (ITN), and Ultra Low Volume (ULV) application. Of these, IRS by DDT was proved to be a successful technique to control malaria during the global malaria eradication program in 1950s to 1970s (Prasittisuk, 1985 and WHO, 2002). Thousands of tones of DDT were used for vector control during the last 40 years, but gradually declined and completely stopped from malaria control in 2001. The reasons for this are unclear but could be due to a poor compliance from human community and negative impact to environment as well as the evidence of development insecticide resistance in mosquito populations (Chareonviriyaphap *et al*., 1999). High ratio resistance to DDT was observed from Sri Lanka, India and Mexico (Walker, 2000). This forced to shift the IRS by DDT from malaria control to use the alternative insecticides, primarily pyrethroids. However, the Stockholm Convention on Persistant Organic Pollutants (POPs) granted exemption for malaria endemic countries to continue using DDT to control malaria vectors (Corin and Weaver, 2005). In addition to IRS by DDT, a number of small trials demonstrated significant reductions in malaria morbidity associated with ITN use in Africa, Asian and Latin American populations during the 1980s (Muller and Jahn, 2003).

The impact of permethrin impregnated bednets was investigated on *An. maculatus* in Malaysia (Vythilingam *et al*., 1995). From this study, the biting densities and the parous rate of *An. maculatus* in the treated villages were

significantly reduced after launching the ITN technology whereas the untreated villages remain unacceptably high before and after intervention. Irritant effect of permethrin was also observed in *An. maculatus* from Malaysia (Ree and Loong, 1989). Results revealed that irritability level of *An. maculatus* to permethrin was 2.7 times higher than the control. Chareonviriyaphap *et al*. (2004) observed the dramatic escape responses of *An. maculatus* and *An. sawadwongporni* from Thailand to deltamethrin.

4. Behavioral response to insecticide

The term "avoidance behavior" has been used to describe behavior that is stimulated by some combination of irritancy and repellency. Irritancy is that the insects leave the treated surfaces after physical contact with treated surfaces whereas repellency is that the insects are stimulated before making physical contact with treated surfaces. Excito-repellency is defined as a broad classification of behavioral responses including both irritancy and repellency (Chareonviriyaphap *et al*., 1997).

Behavioral responses of mosquitoes to insecticides must be carefully evaluated. A number of test methods has been previously described. Initial test was developed by World Health Organization (WHO) (1970) using the demountable plywood to construct the excito-repellency test box for investigating the irritant effect of the insecticides to mosquitoes. Several studies were subsequently carried out following the modified WHO test boxes (Bondareva *et al*., 1986, Ree and Loong, 1989, Pell *et al*., 1989 and Quinones and Suarez, 1989). In 1963, Rachou *et al*. developed the plywood experimental box for testing the escape responses of *Anopheles albimanus* population to DDT. Similar test system was used to test behavioral responses to DDT in *Anopheles darlingi* (Charlwood and Paraluppi, 1978). Roberts *et al*. (1984) constructed a collapsible excito-repellency test box and tested the responses of *An. darlingi* against DDT. Subsequently, light proof test chambers were developed in order to study the behavioral responses of a laboratory colony of *Anopheles gambiae* to several test compounds (Evans, 1993). Due to the complexities of excito-repellency testing, no test method for the assessment of mosquito behavioral

responses has been widely accepted as a standard protocol for conducting excito-repellency testing, data gathering, data analysis and interpretation (Brown, 1964 and Roberts *et al*., 1984).

Recently, an experimental escape chamber system was developed and provided information on both contact irritancy and noncontact repellency (Roberts *et al*., 1997). This test system was first used to study the pesticide avoidance behavior of *An. albimanus* to DDT and some synthetic pyrethroids in Central America by Chareonviriyaphap *et al*. (1997). Unfortunately, this prototype test system was cumbersome and required extended time to place the test papers onto the interior walls. Chareonviriyaphap and Aum-Aong (2000) and Chareonviriyaphap *et al*., (2002) proposed an improved version of excito-repellency test chamber for evaluate the behavioral responses of mosquitoes.

Knowledge on true behavioral responses by mosquito vectors to test compounds began after Chareonviriyaphap *et al*. (2002) published an improved excito-repellency test system to measure the behavioral responses of several species of mosquitoes. This test system was used to evaluate the behavioral responses to insecticides by several laboratory and field test populations of mosquito species in Thailand and Indonesia (Chareonviriyaphap *et al*., 2001, 2004, Sungvornyothin *et al*., 2001, Kongme *et al*., 2004, Potikasikorn *et al*., 2005 and Chareonviriyaphap *et al*., 2006). Among several test populations, *An. maculatus* and *An. sawadwongporni* were also subjected to so-call repellent compounds such as deltamethrin at 0.02 g/m^2 using an improved version of excito-repellency test system. Significant differences in escape responses were observed when contact trial was compared to the control, contact trial was compared to noncontact trial and noncontact trial was compared to control ($P< 0.05$). Percentage of escape response in contact trials was higher than those in noncontact trials (Chareonviriyaphap *et al*., 2004).

MATERIALS AND METHODS

1. Survey of *Anopheles maculatus* group in Thailand

The *An. maculatus* group was surveyed to provide an update information the status of species from the five leading malaria endemic provinces of Thailand, northern Thailand from Samoeng District, Chiang Mai Province (18° 51′N, 98° 43′E), northwestern Thailand from Mae Sot District, Tak Province (16° 43′N, 98° 34′E), eastern Thailand from Soi Dao District, Chanthaburi Province (12° 35′N, 102° 9′E), southern Thailand from Phanom District, Surat Thani Province (8° 49′N, 98° 49′E) as well as western Thailand from Sai Yok District, Kanchanaburi Province (14° 17′N, 99° 11′E). In Tak and Chantaburi Provinces, mosquitoes were collected during 1800 to 2400 hrs using human baits for three consecutive nights. Cow was served as animal baits for mosquito collections in Chiang Mai and Surat Thani Provinces. In the same area in Chiang Mai and Surat Thani, adult collections were additionally done by four human hosts, two inside the house and two outside the house. In Kanchanaburi, whole night collection (1800-0600 hrs) was performed to observe an entire biting cycle and host preference. More details on biting cycle and host preference of *An. maculatus* group from Kanchanaburi were provided in the next section.

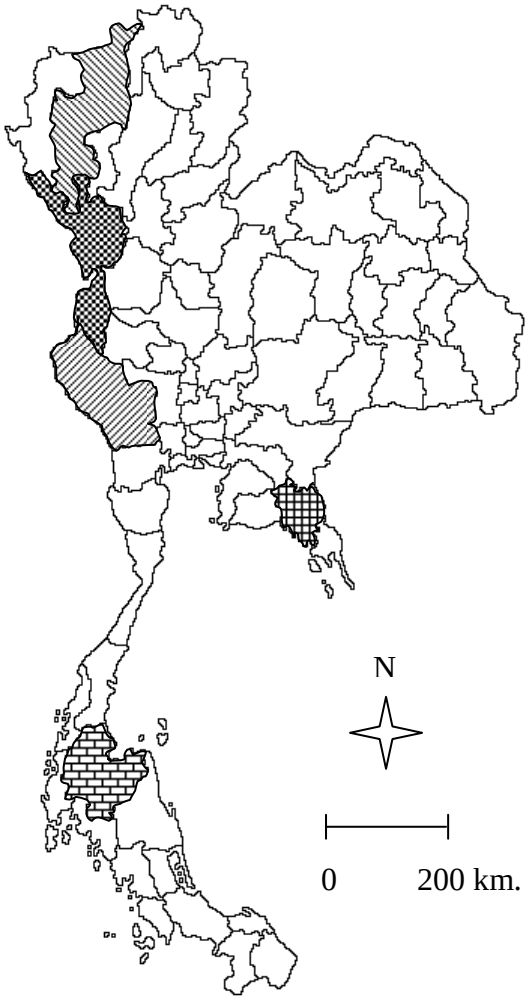


Figure 1 Map of mosquito collection sites in Thailand

= Samoeng District, Chiang Mai Province

= Mae Sot District, Tak Province

= Sai Yok District, Kanchanaburi Province

= Soi Dao District, Chanthaburi Province

= Phanom District, Surat Thani Province

2. Feeding patterns and biting prevalences

2.1 Mosquito collections

The monthly collections of mosquitoes were conducted in Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand. The mosquitoes were collected from human and cow baits from August 2004 to July 2005 for three consecutive nights of each months. Eight human hosts were divided into two groups, each group comprised four people, two indoors and two outdoors. Outdoor collectors were set at 10 m away from house. First group of indoor and outdoor was scheduled as human bait from 1800 to 2400 hrs and the second was from 2400 to 0600 hrs with 15 min rest for each hour. One cow was used as an animal bait, 5 m away from the collection house. The captured mosquitoes were kept in mosquito cups, identified by sites and hours of collection and then separated by species in the following morning. Hourly ambient temperature and relative humidity were recorded during the period of mosquito collections.

2.2 Data analysis

Three factors concerning in landing collection are chosen for analysis, including seasons, time periods, and collection methods. Seasons include wet (June to October), dry (November to February) and hot (March to May), time periods are divided into 12 hrs and collection methods are identified as indoor and outdoor human baits and cow bait. The differences in number of mosquitoes among groups were analyzed by ANOVA, using the GLM procedure in SPSS for windows, version 11.5. Probability values at 95% were considered as a cut off significant level. Multiple regression analysis was used to investigate the interaction between the number of mosquitoes and environmental data.

3. Insecticide susceptibility assay

3.1 Test method

The susceptibility test of *An. maculatus* and *An. sawadwongporni* wild-caught populations at diagnostic concentration of DDT (4%) and permethrin (0.75%) were assessed by the standard testing procedure of WHO (1998). The tests were performed with natural populations of *An. maculatus* and *An. sawadwongporni* from Pu Teuy Village, Sai Yok District, Kanchanaburi Province. For each test, five cylinders, two controls and three treatments, were used. Control cylinders included filter paper impregnated with acetone and silicon only; whereas treatments contained papers impregnated with the diagnostic concentration of insecticide and solvent. Twenty-five female mosquitoes were introduced into each cylinder and were then transferred back to the holding tubes and recorded for the knockdown. Ten-percent sucrose solution was provided. Mortalities were recorded at 24 hrs. If control mortality was between 5% and 20%, the percentage mortalities were corrected by Abbott′s formula as followed;

$$\%\text{Correct mortality} = \frac{\%\text{Test mortality} - \%\text{Control mortality}}{100 - \%\text{Control mortality}} \times 100$$

and If control mortalities excess 20%, tests were discarded.

3.2 Insecticide-treated papers

The filter papers were impregnated with DDT (4%) and permethrin (0.75%) according to WHO protocol using acetone solutions of insecticide and silicone oil as the carrier (WHO, 1998). The impregnation of insecticide was done by dropping onto the filter papers (Hougard *et al*., 2002). The papers were then dried for 24 hrs before tested in the susceptibility assay.

3.3 Data analysis

Based on WHO criteria on discriminating diagnostic dose (WHO, 1998), insecticide susceptibility status is interpreted as followed;

If mortality is between 98-100%, the test population is defined as “a susceptible population”.

If mortality is between 80-97%, the test population is defined as “a verification required population”.

If mortality is less than 80%, the test population is defined as “a resistant population”.

4. Responses of *An. maculatus* and *An. sawadwongporni* to test chemicals

4.1 Mosquito collections

The test populations of *An. maculatus* and *An. sawadwongporni* were obtained from cattle shelter at Pu Teuy Village, Sai Yok District, Kanchanaburi Province. This wild population was collected during the first half of the night (1800-2400 hrs) by two collectors. Collections were performed during the rainy period (June to October 2005). To increase the number of test specimens, *An. maculatus* and *An. sawadwongporni* collected from human and cow baits were supplementary used for excito-repellency tests.

4.2 Insecticide-treated papers

Analytical grade insecticides were impregnated on papers at 2 g/m^2 of DDT and 0.5 g/m^2 of permethrin. The filter papers were treated according to WHO protocol using acetone solutions of insecticide and silicone oil as the carrier (WHO, 1998). The impregnation of insecticide was done by dropping onto the filter papers (Hougard *et al*., 2002). The papers were dried for 24 hrs before using in the excito-repellency test chambers. The treated papers were expired after times of testing.

4.3 Behavioral tests

The wild-caught populations of *An. maculatus* and *An. sawadwongporni* were tested in the excito-repellency test chambers (Figure 2). Each test comprised 2 controls (contact and noncontact) and 2 treatments (contact and noncontact) (Figure 3). The tests were performed during daylight hours only and each test was replicated four times (Sungvornyothin *et al*., 2001). The observations were taken at one-min intervals for 30 min. After completion, the number of dead or knockdown mosquitoes was recorded for each exposure chamber and paired control chamber. The escaped mosquitoes and the remained mosquitoes were held separately in mosquito cups with cotton soaked in 10% sugar solution for mortality recorded after 24 hrs post-exposure.

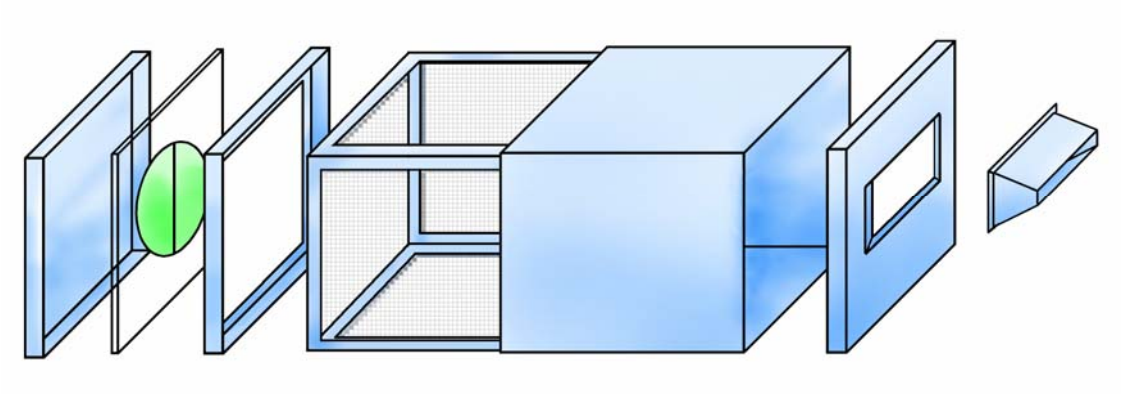


Figure 2 Excito-repellency test chamber for behavioral studies (Chareonviriyaphap *et al*., 2002)

1 = Rear door

2 = Plexiglass panel with rubbered door

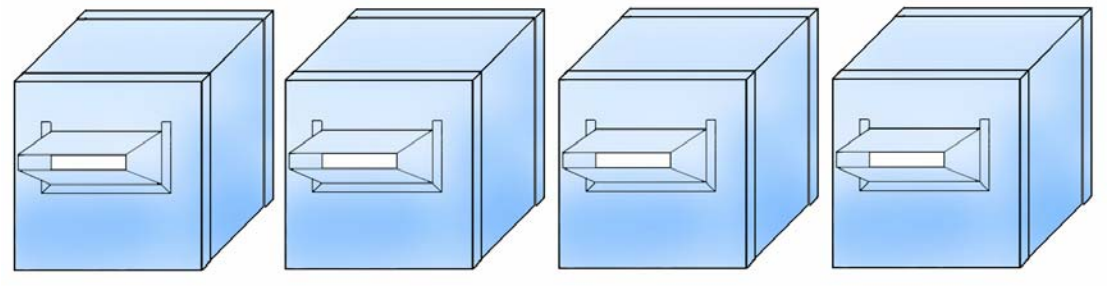
3 = Plexiglass holding frame

4 = Screened inner chamber

5 = Outer chamber

6 = Front door

7 = Exit window



1 2 3 4

Figure 3 Composition of excito-repellency test chambers

1 = Control noncontact chamber

2 = Treatment noncontact chamber

3 = Control contact chamber

4 = Treatment contact chamber

4.4 Data analysis

A Kaplan-Meier survival analysis method was used to analyze and interpret the behavioral response data (Roberts *et al*., 1997). Survival analysis was used to estimate the probability of escape time (ET) and compare differences in mosquito response among the two populations and two insecticides. ET_{25} and ET_{50}, time in minutes for 25% and 50% of the mosquitoes to escape, respectively, were estimated from data collected at one-min intervals. Patterns of escape response were determined using the log-rank method (Mantel and Haenzel, 1959). Stata statistical software was used in the analysis (Roberts *et al*., 1997).

5. Molecular variation of *An. maculatus*

5.1 Mosquito populations

The specimens of *An. maculatus* from Indonesia and Timor-Leste were used for the study of the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) sequence variation. Two populations of *An. maculatus* were collected from Pelabuhan Ratu and Purworejo in Indonesia. The third population was obtained from Timor timur selatan in Timor-Leste. In addition, the specimens of *An. maculatus* that were collected from Samoeng District, Chiang Mai Province were used to compare with Indonesia and Timor-Leste populations.

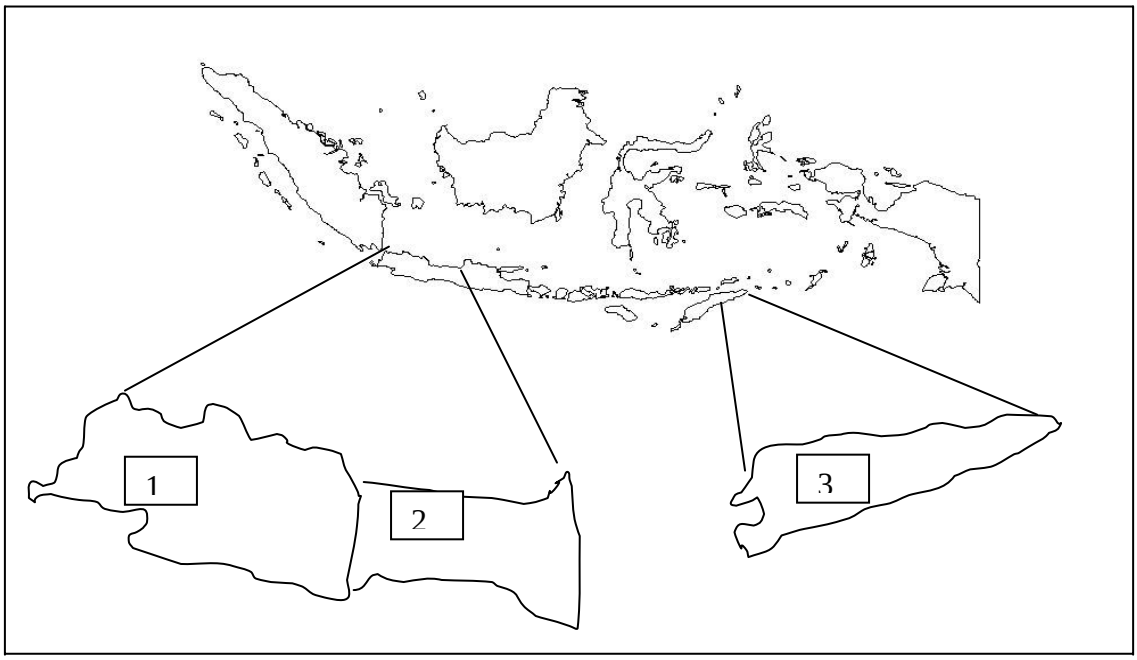


Figure 4 Map of mosquito collection sites in Indonesia and Timor-Leste

1 = Pelabuhan Ratu, western Java, Indonesia

2 = Purworejo, central Java, Indonesia

3 = Timor timur selatan, Timor-Leste

5.2 Mosquito DNA extraction

Genomic DNA was extracted according to the procedure of Ballinger-Crabtree *et al*. (1992). A single mosquito was crushed in 1.5 ml microcentrifuge tube using lysis buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 1% SDS) mixed with proteinase K and incubated at 65 ^{o}C for 2 hrs. Phenol-chloroform was added to the extraction process. The supernatant was transferred to a new tube and DNA was precipitated using sodium acetate and ice-cold 100% ethanol and kept at -20 ^{o}C overnight. After centrifugation at 10,000g under 4 ^{o}C for 10 min, the pellet was washed with 70% ethanol. Subsequently, centrifugation at 12,000g under 4 ^{o}C for 5 min was performed to allow pellet to dry. DNA was resuspended in 20 μl of sterile water. Finally, 0.5 μl of 10 mg/ml RNase was added and was incubated at 37 ^{o}C for 30 min.

5.3 Amplification and sequencing of rDNA ITS2

The rDNA ITS2 regions were amplified by the Polymerase Chain Reaction (PCR) using primers based on conserved sequences of the 5.8s and 28s coding regions for forward and reverse primers, respectively. The primers were modified from the previous study of Torres *et al*. (2000) as followed;

forward primer (5′ TGTGAACT GCAGGACACATG 3′) and

reverse primer (5′ TATGCTTAAATTCAGGGGGT 3′)

Each 25 μl PCR reaction mixture contained 2.5 μl of 10x buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of each 10 mM dNTPs, 1.2 μl of forward primer, 1.2 μl of reverse primer, 0.5 μl of Ampli Taq, 1.5 μl of DNA template and 12.1 μl of sterile water. The conditions of PCR were an initial denaturation for 2 mins at 94 ^{o}C, followed by 30 cycles (94 ^{o}C for 1 min, 45 ^{o}C for 1 min and 72 ^{o}C for 30 secs) and ending with an extension cycle (72 ^{o}C for 5 mins). Amplification product was visualized on 1.2% agarose gel. Subsequently, PCR product was purified and sequenced.

5.4 Sequence analysis

The ITS2 sequencing data were edited manually and adjusted if necessary. The ITS2 sequences of *An. maculatus* group from GENBANK were compared with the sequencing data. The species and the accession numbers of *An. maculatus* group from GENBANK were designated as followed; *An. maculatus* accession number AY549313, *An. dispar* accession number AF234778, *An. greeni* accession number AF234779, *An. pseudowillmori* accession number AF512550, *An. sawadwongporni* accession number AF512551, *An. willmori* accession number AF512552 and *An. dravidicus* accession number DQ002906. The ITS2 sequences were aligned using CLUSTALW program (EBI, 2006). Percent sequence identity and GC content were calculated (MBCF, 2006).

RESULTS

A survey of *An. maculatus* group was conducted in five different geographic Provinces, Chiang Mai, Chanthaburi, Tak, Surat Thani and Kanchanaburi (Figure 1). Adult survey results were summarized in Table 2. Among all collection sites, five species of *An. maculatus* group were found, including *An. maculatus*, *An. sawadwongporni*, *An. notanandai*, *An. dravidicus* and *An. willmori*. Of these, *An. maculatus* and *An. sawadwongporni* were comparatively common. *Anopheles notanandai*, *An. dravidicus* and *An. willmori* were not often found as compared to the others and presented exclusively in Kanchanaburi, Tak and Chiang Mai Provinces, respectively. Three species in the Maculatus group were found in Kanchanaburi with seventy-two percent (72%) of *An. sawadwongporni*, twenty-seven percent (27%) of *An. maculatus* and one percent (1%) of *An. notanandai*.

Number of *An. maculatus*, *An. sawadwongporni* and *An. notanandai* mosquito was recorded from indoor and outdoor human collections and cow bait collection over one year period. Significant differences in number of mosquitoes collected from three different methods were not obtained ($P>0.05$). In addition, there is no statistically significant in number of mosquito species collected from different seasons ($P>0.05$). Generally, outdoor collection exceeded indoor collection and mostly found during the rainy period (June-October). The number of *An. maculatus* and *An. sawadwongporni* began to increase in May, reaching a peak in June before dramatically decreasing in October. No significant interaction between the number of *An. maculatus* group and environmental data was found, suggesting that the number of *An. maculatus* group was independent of environmental data. However, a positive linear relationship with the number of *An. maculatus* group was observed on average minimum temperature ($r^2 = 0.398$; $P<0.05$).

Indoor and outdoor human feeding cycles of *An. maculatus*, *An. sawadwongporni* and *An. notanandai* were observed during the period of one year. Although small numbers of *An. maculatus* and *An. sawadwongporni* were captured outdoor, peak feeding activity varies from 1800-2400 hrs, with a maximum on 1800-

2000 hrs. No specimens of *An. maculatus* group was collected indoor whereas one specimen of *An. sawadwongporni* was captured during 2000-2100 hrs. Cow bait feeding cycles of these three species were statistically significant differences between each hour ($P< 0.05$). Peak biting activities of all three species on cow bait were similar to those of human, with a maximum at 2100-2400 hrs. In generally, number of mosquitoes from cow bait collections exceeded from those human collections (Table 4 and Figure 6).

The susceptibility level of *An. maculatus* and *An. sawadwongporni* wild-caught populations at diagnostic concentration of DDT (4%) and permethrin (0.75%) were summarized in Table 6. *Anopheles maculatus* population was physiologically susceptible to DDT and permethrin as indicated by high percent mortality (98-100%). Development of physiological resistance to DDT (76%) and slight tolerance to permethrin (96%) were detected in *An. sawadwongporni*. No experiment has been performed on *An. notanandai* due to the shortages of test samples.

The behavioral responses of *An. maculatus* and *An. sawadwongporni* wild-caught populations exposed to operational field concentrations of DDT (2 g/m^2) and permethrin (0.5 g/m^2) were characterized (Table 7). This study was designed to compare behavioral responses of two wild-caught populations of *An. maculatus* and *An. sawadwongporni* females. In general, two types of behavioral responses, contact irritancy and noncontact repellency, were observed in both test populations with exposure to DDT and permethrin. Percent mortalities of escape and nonescape mosquitoes from control and treated chambers were also assessed. In contact trial, patterns and rate of escape were significantly stronger in two test populations when exposed to permethrin than those to DDT (Table 7). After 30 min exposure, the number of escape responses from DDT was rather similar in both test populations (38% in *An. maculatus* and 37% in *An. sawadwongporni*). In contrast, strong escape responses were observed in both test populations when tested against permethrin (76% for *An. maculatus* and 64% for *An. sawadwongporni*). In noncontact trial, repellency function of DDT was pronounced (37%) in *An. sawadwongporni* whereas it was moderate in *An. maculatus* (12%). For permethrin, obvious repellency function

was also observed in both test populations (27% for *An. maculatus* and 26% for *An. sawadwongporni*). Overall, fewer females escaped from treated chamber without direct contact with insecticides but the response was statistically different from that of the controls ($P < 0.05$) (Tables 7 and 10).

Percent mortalities of test specimens after a 24-h holding period from contact and noncontact are given in Table 7. In contact trial, percent mortalities of escaped specimens were comparatively low, ranging between 9 and 18% for DDT and between 7 and 8% for permethrin. The percent mortalities were somewhat high from those remained in the test chambers, ranging between 20 and 31% for DDT and between 6 and 22% for permethrin. In noncontact trial, the percent mortalities of escaped and nonescaped specimens were generally low (0-9%), except those escaped (20%) and nonescaped (13%) specimens of *An. maculatus* from permethrin treated chamber (Table 7).

The escape patterns obtained from insecticide-treated chambers are expressed in 1 minute intervals for 25 and 50% (ET_{25} and ET_{50}) of the test population to escape from exposure chambers (Table 8). In contact trial, the ET_{25} values for *An. maculatus* and *An. sawadwongporni* were 8 and 3 for DDT and were 4 and 5 for permethrin, respectively. The ET_{50} values for *An. maculatus* and *An. sawadwongporni* were 9 and 13 for permethrin, respectively. The ET_{50} values for DDT against *An. maculatus* and *An. sawadwongporni* could not be estimated because of insufficient numbers of mosquitoes escaping. In noncontact trial, the ET_{25} values for DDT and permethrin against *An. sawadwongporni* was 12 and 26, respectively (Table 8).

Comparisons of escape responses between any two test populations of contact and noncontact trials against operational field doses of DDT and permethrin are given in Table 9. In contact and noncontact trials with DDT and permethrin, no statistical differences in escape responses were observed in all pairs ($P > 0.05$), except when *An. maculatus* and *An. sawadwongporni* were compared against DDT in noncontact trial ($P < 0.05$).

Escape patterns were statistically significant when contact was compared to noncontact, contact was compared to control and noncontact was compared to control in tests with DDT and permethrin against two test populations ($P < 0.05$), except when contact was compared to noncontact with DDT against *An. sawadwongporni* ($P > 0.05$) (Table 10). Escape rate in contact trials with DDT and permethrin was significantly higher than in the controls for both test populations ($P < 0.05$). In addition, statistically escape responses were found in all pairs when noncontact was compared to the controls ($P < 0.05$), except when *An. maculatus* was tested against DDT (Table 10). Figures 10-15 demonstrated the proportions of mosquitoes remaining in the exposure and control chambers under different test conditions and chemical exposure. These proportions are used to develop escape rate patterns and show probabilities for escaping from exposure chambers in contact versus noncontact against DDT (Figure 10), contact versus noncontact against permethrin (Figure 11), contact versus control against DDT (Figure 12), contact versus control against permethrin (Figure 13), noncontact versus control against DDT (Figure 14) and noncontact versus control against permethrin (Figure 15). These were significant differences in escape responses seen in all contact compared with paired control trials ($P < 0.05$) (Figures 12 and 13). Strong repellency action of DDT was observed in *An. sawadwongporni* whereas significantly less reaction was observed with *An. maculatus* (Figure 14). In noncontact trial with DDT, no significant differences in escape patterns of *An. maculatus* were observed between treatment and control ($P > 0.05$) (Table 10 and Figure 14). However, higher number of test specimens departed the treated chamber than those from the control (Table 7 and Figure 14).

The ITS2 sequencing data of *An. maculatus* from four collection sites (Pelabuhan ratu in Indonesia; Purworejo in Indonesia; Timor timur selatan in Timor-Leste and Chiang Mai Province in Thailand) were sequenced. The ITS2 regions of the sequencing data were indicated by the ITS2 sequences of *An. dispar* and *An. greeni* in the previous study of Torres *et al*. (2000). The lengths of the ITS2 sequences of *An. maculatus* from Pelabuhan ratu, Purworejo, Timor timur selatan and Chiang Mai were 327, 332, 322 and 322 base pairs, respectively. The ITS2 sequences of *An. maculatus* from Timor timur selatan and Chiang Mai were completely similar

to the ITS2 sequence of *An. maculatus* accession number AY549313 from GENBANK. Five base pairs insertions were found from the ITS2 sequence of *An. maculatus* in Pelabuhan ratu. Fifty base differences (13 insertions, 3 deletions, 17 transitions and 17 transversions) were detected in the ITS2 sequence of *An. maculatus* from Purworejo (Figure 16). The ITS2 sequence of *Anopheles maculatus* from Purworejo was 88% identity when compared to the other collection sites. The ITS2 sequences of *Anopheles maculatus* from four collection sites were similar GC content with 57%. Additionally, the ITS2 sequences of *An. maculatus* group from GENBANK were aligned with the ITS2 sequence of *An. maculatus* in Purworejo (Figure 17).

Table 2 Number of *Anopheles maculatus* group in five different geographic Provinces

Province	No. of MAC	No. of SAW	No. of NOT	No. of DRA	No. of WIL	Collection dates	Latitude, longitude
Chiang Mai	19	19	0	0	1	Sep 04	18° 51′ N, 98° 43′ E
Tak	36	26	0	2	0	Sep 04	16° 43′ N, 98° 34′ E
Surat Thani	15	0	0	0	0	Aug 04	8° 49′ N, 98° 49′ E
Chanthaburi	0	3	0	0	0	Aug 05	12° 35′ N, 102° 9′ E
Kanchanaburi	221	584	6	0	0	Aug 04 to Jul 05	14° 17′ N, 99° 11′ E

Remark : MAC = *An. maculatus*, SAW = *An. sawadwongporni*, NOT = *An. notanandai*, DRA = *An. dravidicus* and WIL = *An. willmori*

Table 3 Monthly number of *Anopheles maculatus* group in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

Month	Number of *An. maculatus*			Number of *An. sawadwongporni*			Number of *An. notanandai*		
	HI	HO	C	HI	HO	C	HI	HO	C
Aug	0	2	6	0	0	7	0	0	0
Sep	0	0	10	0	1	2	0	0	0
Oct	0	1	5	0	2	3	0	0	0
Nov	0	1	12	0	0	14	0	0	0
Dec	0	0	1	0	1	10	0	0	0
Jan	0	0	0	0	0	0	0	0	2
Feb	0	0	0	0	1	0	0	0	0
Mar	0	0	4	0	0	1	0	0	0
Apr	0	0	2	0	1	32	0	0	2
May	0	1	17	0	0	81	0	0	1
Jun	0	2	121	0	2	360	0	0	0
Jul	0	1	35	1	0	65	0	0	1
Total	0	8	213	1	8	575	0	0	6

Remark : HI = human indoor, HO = human outdoor and C = cattle

Table 4 Hourly number of *Anopheles maculatus* group in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

Hour	Number of *An. maculatus*			Number of *An. sawadwongporni*			Number of *An. notanandai*		
	HI	HO	C	HI	HO	C	HI	HO	C
18-19	0	1	1	0	3	3	0	0	0
19-20	0	3	13	0	1	26	0	0	1
20-21	0	1	24	1	1	93	0	0	1
21-22	0	1	36	0	1	88	0	0	0
22-23	0	1	42	0	1	112	0	0	0
23-24	0	0	26	0	0	63	0	0	2
00-01	0	1	15	0	1	43	0	0	0
01-02	0	0	22	0	0	64	0	0	1
02-03	0	0	20	0	0	49	0	0	1
03-04	0	0	6	0	0	8	0	0	0
04-05	0	0	4	0	0	16	0	0	0
05-06	0	0	4	0	0	10	0	0	0
Total	0	8	213	1	8	575	0	0	6

Remark : HI = human indoor, HO = human outdoor and C = cattle

Table 5 Environmental data in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

Month	Average Rainfall (mm)	Average Temperature		Average Humidity (%)
		Max.	Min.	
Aug	3.5	31.5	19	96
Sep	5.6	32.7	18.7	96
Oct	1	33.6	16.7	96
Nov	0	35.4	15.7	93
Dec	0	34.8	11.2	91
Jan	0	35.7	13.5	89
Feb	0	39.2	16.5	87
Mar	1.7	38.5	17.5	89
Apr	4.2	38.9	19.5	90
May	4	36	24.7	92
Jun	3.3	33.2	24.7	96
Jul	8.9	32	23.4	97

Source : Meteorological and hydrological station, Electricity Generating Authority of Thailand at Pu Teuy Village, Sai Yok District, Kanchanaburi Province

Table 6 Susceptibility test of *Anopheles maculatus* and *Anopheles sawadwongporni* at diagnostic concentrations of DDT (4%) and permethrin (0.75%)

Population	DDT		Permethrin	
	No. Test	% Mortality	No. Test	% Mortality
An. maculatus	60	98	45	100
An. sawadwongporni	45	76	45	96

Table 7 Percentage escape responses and mortalities of *Anopheles maculatus* (MAC) and *Anopheles sawadwongporni* (SAW) to DDT and permethrin in contact and noncontact trials

Test condition	Insecticide	Population	Treatment No. Tested	Treatment % Esc	Control No. Tested	Control % Esc	% Mortality Treatment Esc	% Mortality Treatment Not esc	% Mortality Control Esc	% Mortality Control Not esc
Contact										
	DDT	MAC	93	38	97	5	9	31	0	1
		SAW	93	37	97	7	18	20	0	1
	PER	MAC	96	76	99	17	7	22	0	0
		SAW	94	64	89	15	8	6	0	1
Noncontact										
	DDT	MAC	95	12	94	8	9	0	0	1
		SAW	94	37	94	12	3	3	0	0
	PER	MAC	92	27	88	14	20	13	0	0
		SAW	91	26	91	18	4	0	0	0

Remark : PER = permethrin, Esc = Escaped and Not esc = Not escaped

Table 8 Escape time (ET) in minutes for 25% and 50% of *Anopheles maculatus* (MAC) and *Anopheles sawadwongporni* (SAW) to escape from insecticide-treated chambers

Test condition	Population	DDT		Permethrin	
		ET_{25}	ET_{50}	ET_{25}	ET_{50}
Contact	MAC	8	-	4	9
	SAW	3	-	5	13
Non- contact	MAC	-	-	27	-
	SAW	12	-	26	-

Table 9 Comparison of escape responses between *Anopheles maculatus* and *Anopheles sawadwongporni* to insecticide in contact and noncontact trials

Chemicals	Contact exposure	Noncontact exposure
DDT	0.9729	0.0001*
PER	0.1100	0.9066

*: Log rank tests with statistically significant ($P < 0.05$) differences in escape patterns

Table 10 Comparison of escape responses between contact vs. noncontact, contact vs. control and noncontact vs. control for *Anopheles maculatus*(MAC) and *Anopheles sawadwongporni* (SAW) by insecticides

Species	Chemicals	Treatment pairs		
		Contact vs.Control	Contact vs. Noncontact	Noncontact vs Control
MAC				
	DDT	0.0001	0.0001	0.4875*
	PER	0.0001	0.0001	0.0001
SAW				
	DDT	0.0001	0.8296*	0.0001
	PER	0.0001	0.0001	0.0180

* : Log rank tests showing no statistically significant ($P > 0.05$) differences in escape patterns

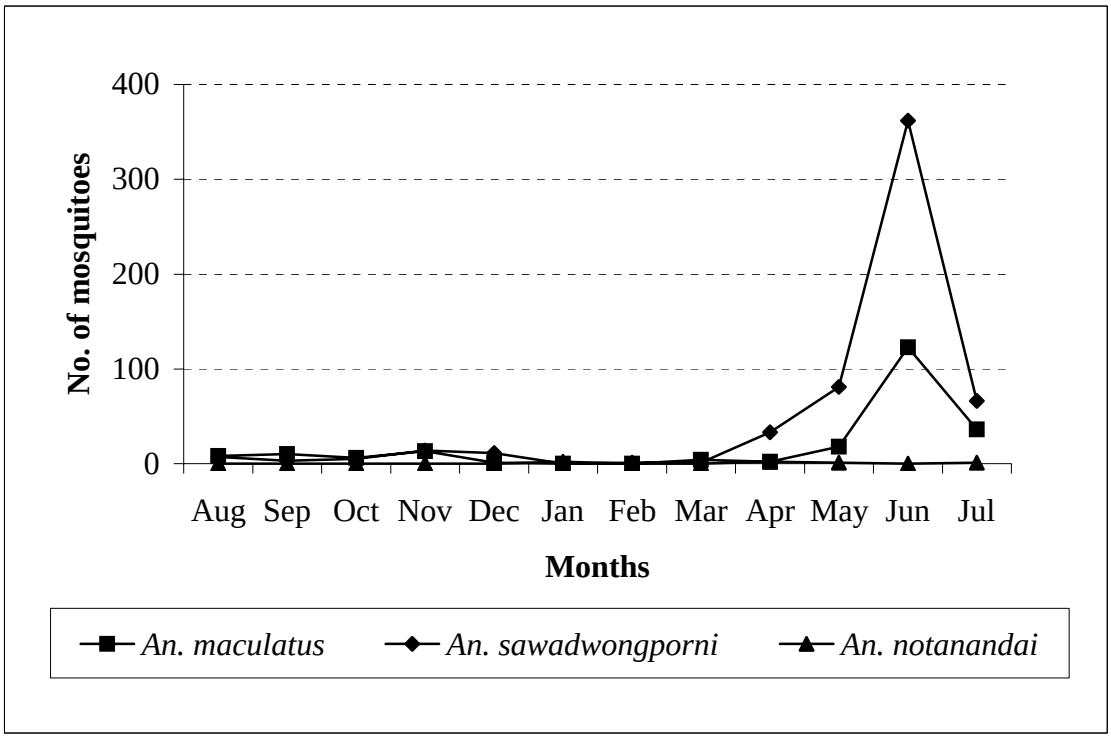


Figure 5 Monthly number of *Anopheles maculatus* group in Pu Teuy Village, Sai Yok District, Kanchanaburi Province

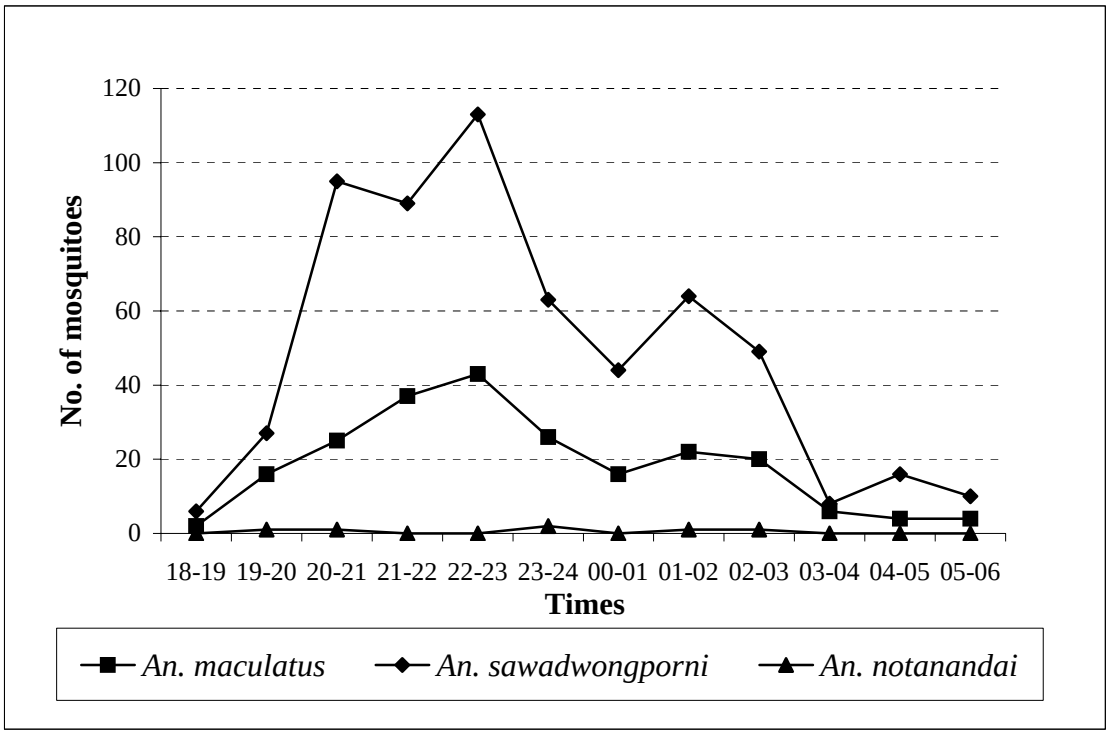


Figure 6 Hourly number of *Anopheles maculatus* group in Pu Teuy Village, Sai Yok District, Kanchanaburi Province

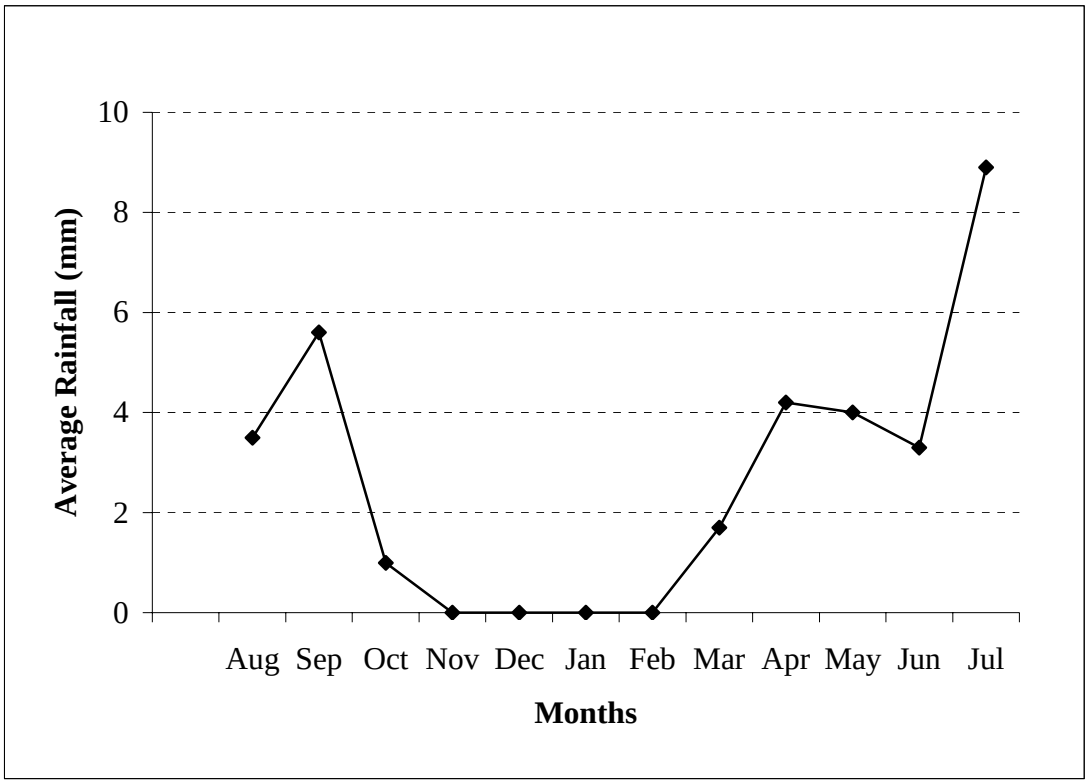


Figure 7 Monthly average rainfall in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

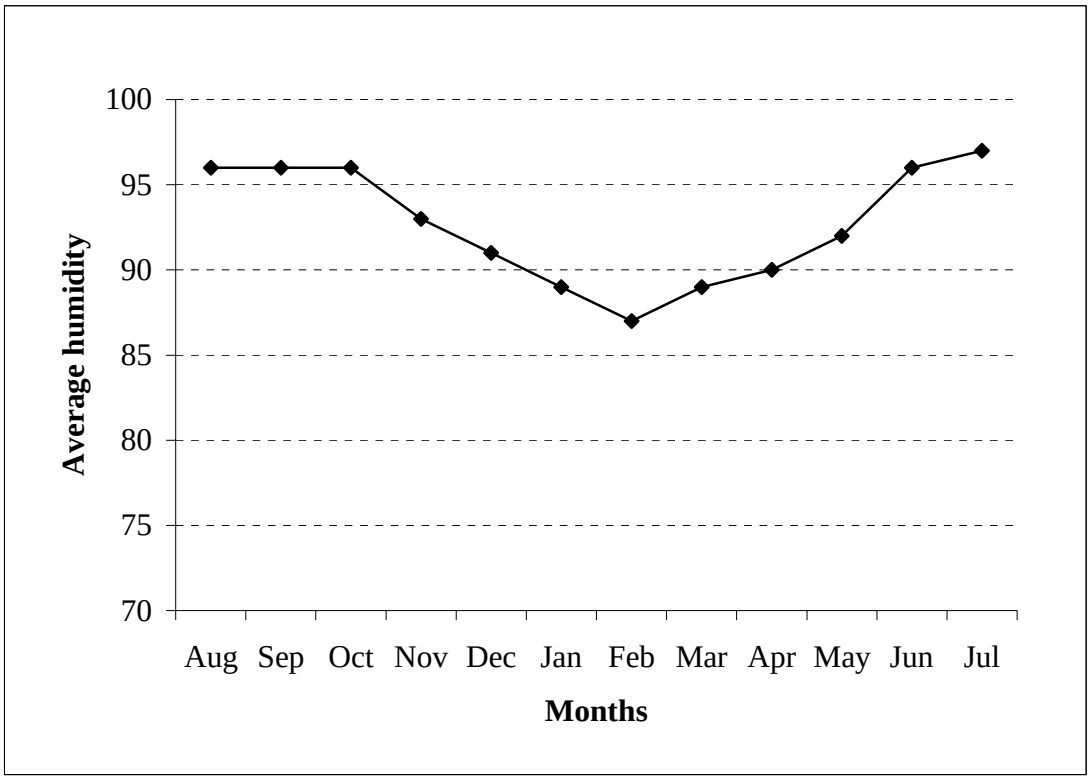


Figure 8 Monthly average humidity (%) in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

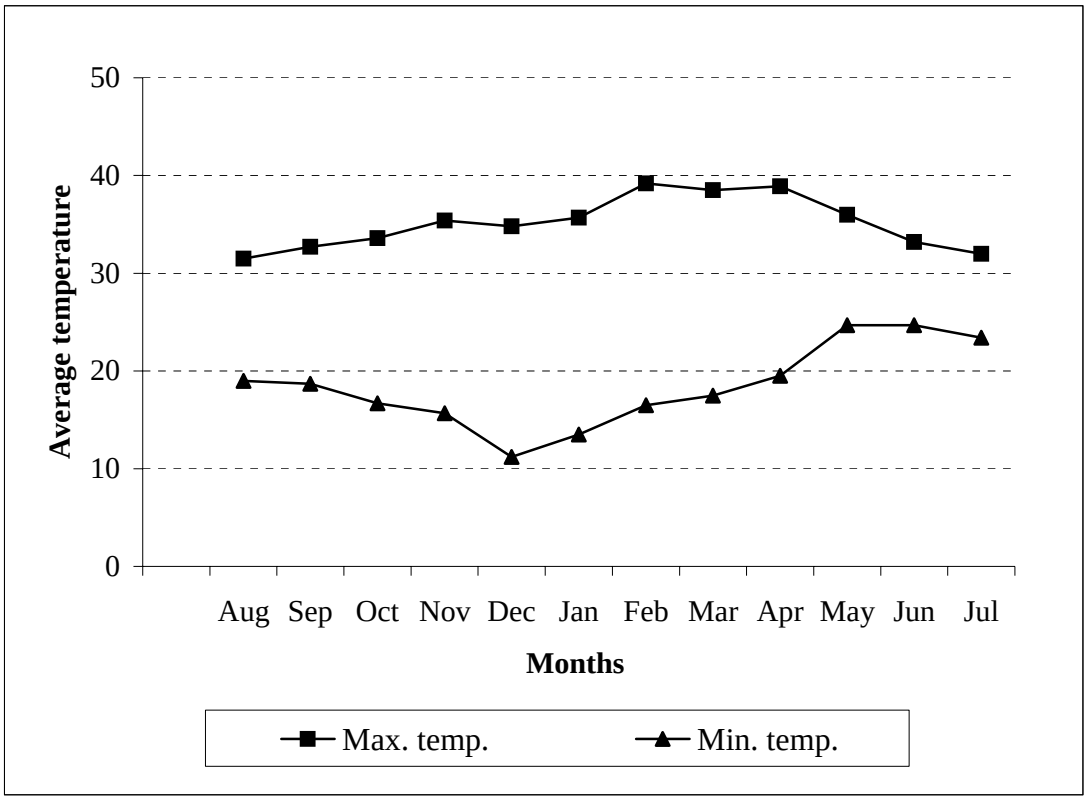


Figure 9 Monthly average maximum and average minimum temperature (°C) in Pu Teuy Village, Sai Yok District, Kanchanaburi Province from August 2004 to July 2005

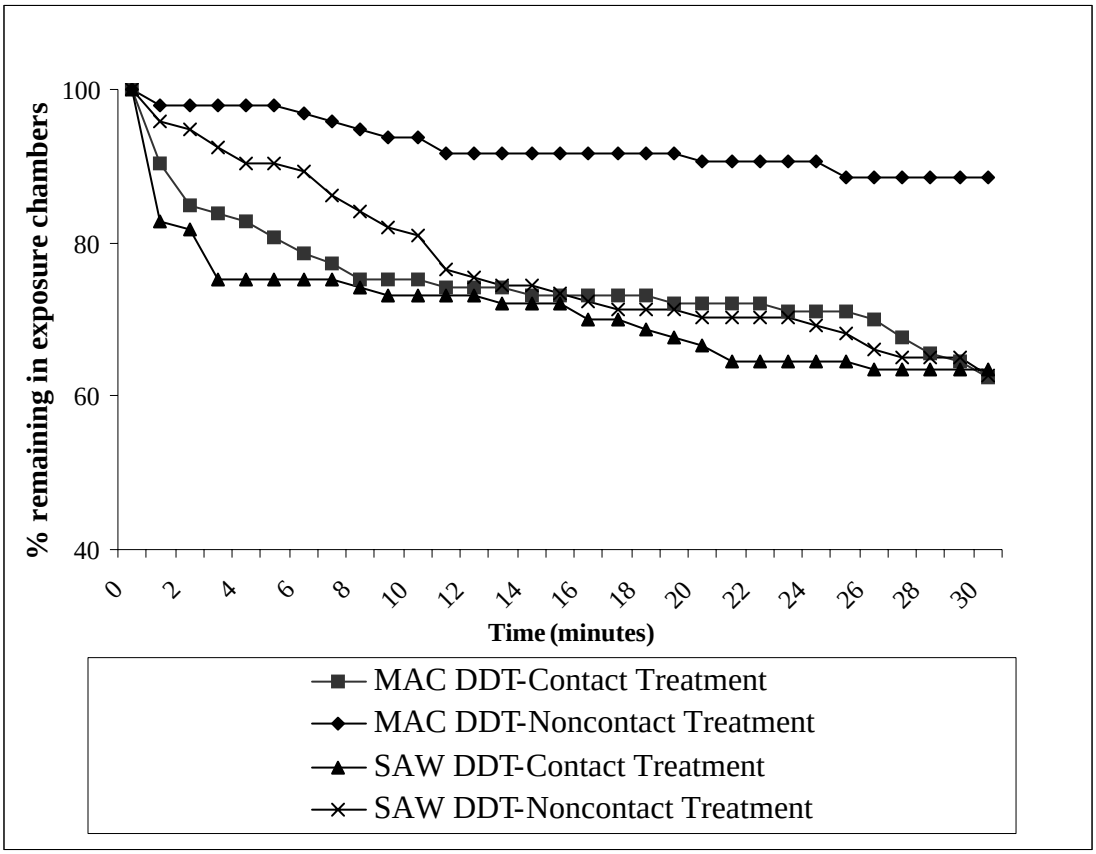


Figure 10 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in contact and noncontact trials with DDT

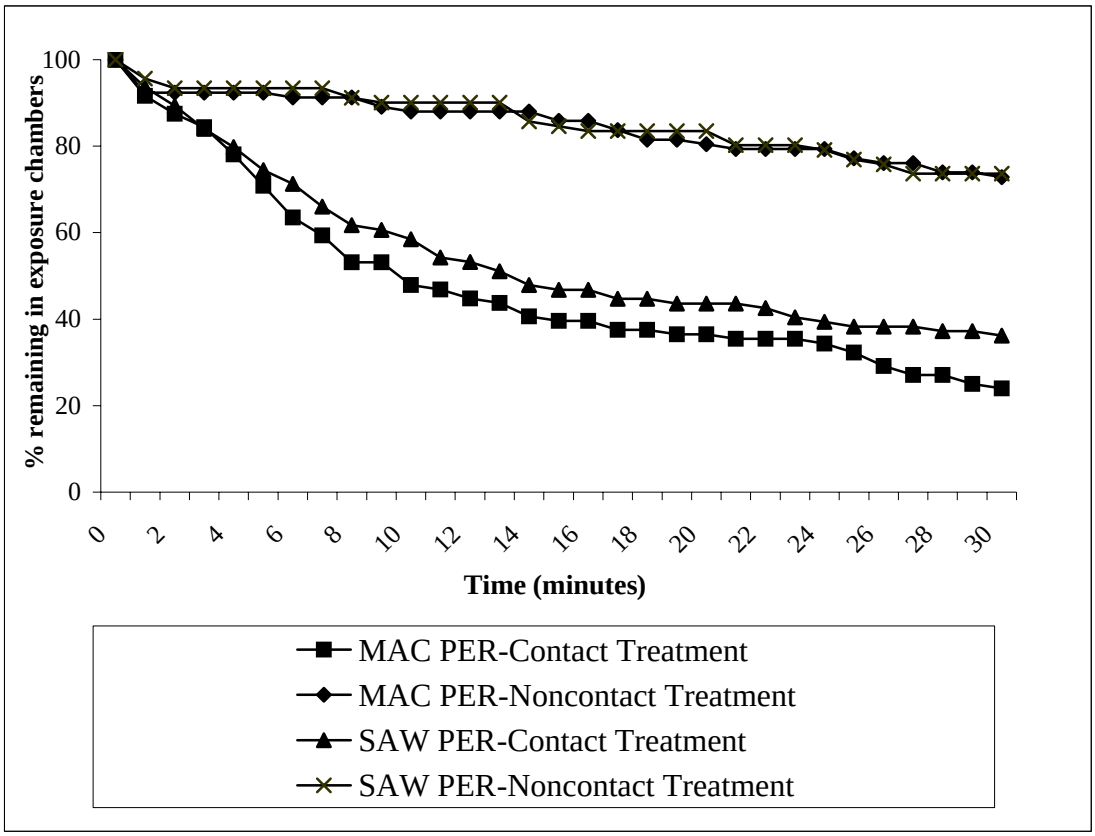


Figure 11 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in contact and noncontact trials with permethrin

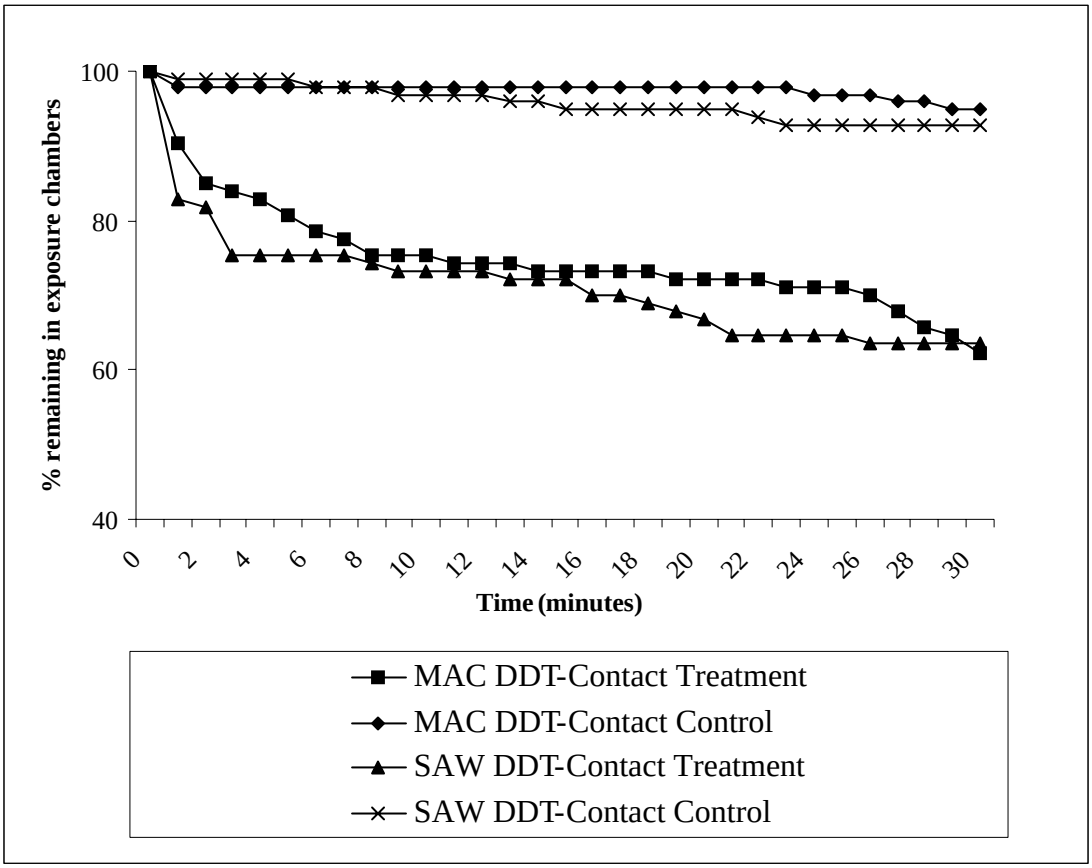


Figure 12 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in contact and control trials with DDT

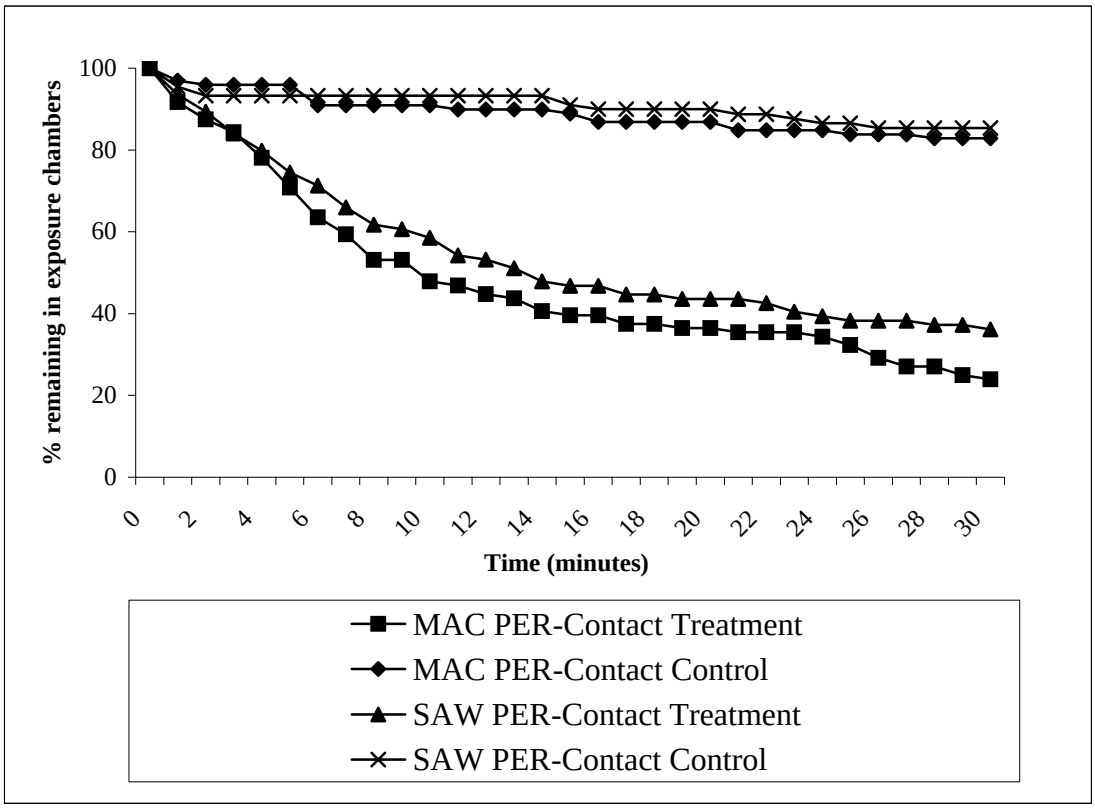


Figure 13 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in contact and control trials with permethrin

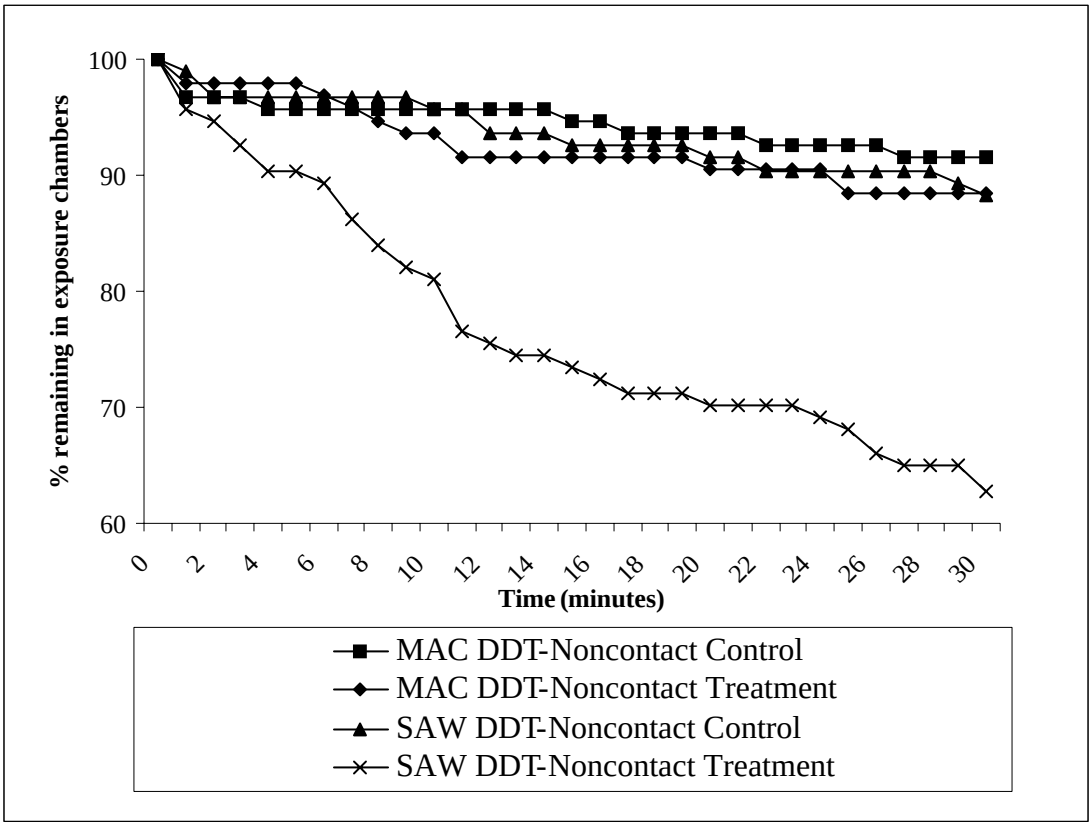


Figure 14 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in noncontact and control trials with DDT

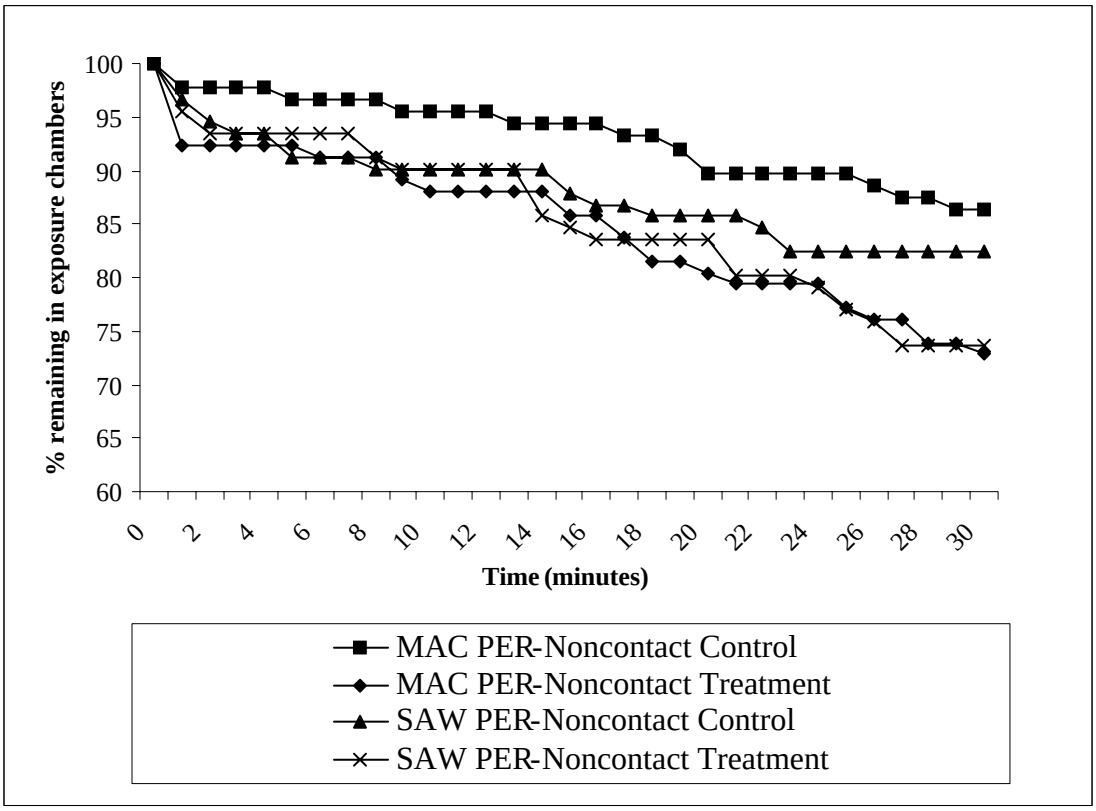


Figure 15 Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in the exposure chambers in noncontact and control trials with permethrin

```
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MAC             CAAGTTATGTATACACTT-TACCAGACTGACCGTCCCATCCCCGTGATGGGCTGTCGCAG 59
CM              CAAGTTATGTATACACTT-TACCAGACTGACCGTCCCATCCCCGTGATGGGCTGTCGCAG 59
PR              CAAGTTATGTATACACTT-TACCAGACTGACCGTCCCATCCCCGTGATGGGCTGTCGCAG 59
PW              CAAGTTATCGATATACTCCTACCAGACTGACCGTCCCATCCTCGCGATGGGCTGTCGCAG 60
                ********  *** ***  ********************** ** ***************

TT              AATGGCGTGCTCGGACCCCGCTAGTGGGCCCGTGGGCGCTGAAAGTGAGAGTGCTATTGT 119
MAC             AATGGCGTGCTCGGACCCCGCTAGTGGGCCCGTGGGCGCTGAAAGTGAGAGTGCTATTGT 119
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                ************** ******* ***** ****************************

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MAC             AATAGGATGGTACGCTAGGTGAGAGATGAACAGGCGCGCGTCAAGTCGCACGGTTCGACC 179
CM              AATAGGATGGTACGCTAGGTGAGAGATGAACGGGCGCGCGTCAAGTCGCACGGTTCGACC 179
PR              AATAGGATGGTACGCTAGGTGAGAGATGAACGGGCGCGCGTCAAGTCGCACGGTTCGACC 179
PW              GACAGG-TGGTACGCAAGACGAGAGATGAACGGGCGCGCGTCAAGTCGCACGGTTCGACC 178
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MAC             TCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAACAGATTAACA-CCAGGCG-CT 235
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PW              TCCAGTATCAAACTAGGGATGAAACCCCCGCAGCACTAACAGATTAACAGCCAGGCGTCT 238
                *********** ********************** ************** ******* **

TT              AGCAAAGGGG---T-CCCCGGTTGGCTCGGGTCGAGTAACA-CTTGCGGCCCAA-CGCG- 288
MAC             AGCAAAGGGG---T-CCCCGGTTGGCTCGGGTCGAGTAACA-CTTGCGGCCCAA-CGCG- 288
CM              AGCAAAGGGG---T-CCCCGGTTGGCTCGGGTCGAGTAACA-CTTGCGGCCCAA-CGCG- 288
PR              AGCAAAGGGG---TGCCCCGGTTGGCTCGGGTCGAGTAACAGCTTGCGGCCCAAGCGCGG 293
PW              AGTCAAGAGGAGGTACCCCGGTGGGCACGGGTGGAGTAACATCTTGCGGATTAAGCGCGA 298
                **  *** **   * ******* *** ***** ******** *******   ** ****

TT              CCCGT-CACCATCTGCTCTGCCTTACTCTCTCATA 322
MAC             CCCGT-CACCATCTGCTCTGCCTTACTCTCTCATG 322
CM              CCCGT-CACCATCTGCTCTGCCTTACTCTCTCATA 322
PR              CCCGT-CACCATCTGCTCTGCCTTACTCTCTCATA 327
PW              GCCGTACACCATGC-TTGCGCCTAGCTCTCTGAAA 332
                 **** ******    *  ****  ****** *
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Figure 16 Multiple sequence alignment of ITS2 sequences of *Anopheles maculatus* from four collection sites

Remark : PR = Pelabuhan ratu, west Java Island, Indonesia; PW = Purworejo, central Java Island, Indonesia; TT = Timor timur selatan, Timor-Leste, CM = Chiang Mai Province, Thailand and MAC = *Anopheles maculatus* from GENBANK

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MAC             CAAGTTATGT-ATACACTT-TACCAGACTGACCGTCCCATCCCCG-----TGATGGGCTG 53
PW              CAAGTTATCG-ATATACTCCTACCAGACTGACCGTCCCATCCTCG-----CGATGGGCTG 54
DIS             CAAGTTATGT-ATATGCTCCTACCAGACTGACCGTCCCATCCCCG-----CGATGGGCTG 54
GRE             CAAGTTAAGC-A-ATGCTCCTACCAGACTGACTGTCCCATCCCCG-----CGATGGGCTG 53
SAW             CAAGTTATCA-ATAAGCTCATACCAAACTGACTGTCCCATC-TCG-----CGATGGGCTG 53
DRA             CAAGTTATCT-ATATGCTCCTACCAGACTGACCGTCCCATCCCCG-----TGATGGGCTG 54
WIL             CAAGTTATCT-ATTTTCTCCTACCAAACTGACCGTCCCATCCCCG-----TGATGGGCTG 54
PSE             TCAGTTATCTTATATGCCCATACCAGACTGACCTGTCCCTGTTTGACACCCGGGGGCGGG 60
                  *****    *    *   ***** ******    ** *    *      *  **   *

MAC             TCGCAGAATGGCGTGCTCGGACCC-CGCTAG---TGGGCCCGTGGGCGCTGAAAGTGAGA 109
PW              TCGCAGAATGGCGTGCTCGGTCCC-CGCTTG---TGGGACCGTGGGCGCTGAAAGTGAGA 110
DIS             TCGCAGAATGGCGTGCTCGGTCCC-CGCTCG---TGGGACCGTGGGCGCTGAAAGTGAGA 110
GRE             TCGCAGAATGGCGTGCTCGGACCC-CGTTCG---TGGGACCGTGGGCGCTGAAAGTGAGA 109
SAW             TCGCAGCATGGCGTGCTCGGACCCGCACCTGATGCGGGACCGTGGGCGCTGAAAGTGAGA 113
DRA             TCGCAGAATGGCGTGCTCGGACCC--GCTTG-CGCGGGACCGTGGGCGCTGAAAGTGAGA 111
WIL             TCGCAGCATGGCGTGCTCGGACCCGCATCTG--TCGGGACCGTGGGCGTTGAAAGTGAGA 112
PSE             TCGCAAAATGGCGTGCTCGGGCCC-TGTATA---TGGGCCCGTGGGCGCTGAAAGCGAGA 116
                *****  ************* ***           *** ********* ****** ****

MAC             GTGCTATTGTAATAGGA--TGGTACGC---TAGGTGAGAGATGAACAGGCGCGCGTCAAG 164
PW              GTGCTATTG-GACAGG---TGGTACGC---AAGACGAGAGATGAACGGGCGCGCGTCAAG 163
DIS             GTGCTATTA-CACAGG---TGGTACGC---AAGGCGAGAGATGAACGGGCGCGCGTCAAG 163
GRE             GTGCTATTA-CAAAGA---TGGTACGC---AAGGCGAGAGATGAACGGGCGCGCGTCAAG 162
SAW             GTGCTATTATGACAGG---TGGTACATGCAAGGGCGAGCGATGAACGGGCGCGCGACAAG 170
DRA             GTGCTATTA-GACAGGTA-TGGTACACGC-AAGGCGAGAGATGAACGGGCGCGCGTCAAG 168
WIL             GTGCTATTATAACGAATGGTGGTACACTATGGGGCGAGAGATGGCCGGGCGCGCGTCAAG 172
PSE             GTGCTA-----ACACA---TGAAACAG----TGGTGGGTG-CGTACGGGCGCGCGTCAAG 163
                ******     *       **  **       *  * * *  *  * ******** ****

MAC             TCGCA-CGGTTCGACCTCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAACAGAT 221
PW              TCGCA-CGGTTCGACCTCCAGTATCAAACTAGGGATGAAACCCCCGCAGCACTAACAGAT 222
DIS             TCGCA-CGGTTCGACCTCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAACAGAT 220
GRE             TCGCA-CGGTTCGACCTCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAACAGAT 219
SAW             CCGCA-CGGTTCGACCTCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAACAGAT 227
DRA             TCGCA-CGGTTCGACCTCCAGTATCAA-CTAGGGATGAAACCCCCGCAGC-CTAATGTAT 225
WIL             TCGCA-AGGGTCGACCTCCAGTATCAA-CCAGGGATGAAACCCCCGCAGC-CTAACAGAT 229
PSE             TCGCAACGGTTCGACCTCCAGTATCAA-CCAGGGATGAAACCCCCGCAGC-CTAACAGAT 221
                 ****  ** ***************** * ******************** ****   **

MAC             TAACA-CCAGGCG-CTAGCAAAGGGGT----CCCCGGTTGGCTCGGGTCGAGTAACA--- 272
PW              TAACAGCCAGGCGTCTAGTCAAGAGGAGGTACCCCGGTGGGCACGGGTGGAGTAACAT-- 280
DIS             TAACA-CCAGGCG-CTAGCAAAGGGGT----CCCCGGTTGGCTCGGGTCGAGTAACA--- 271
GRE             TAGCA-CCTGGCG--TAGCAAAGGGGT----CCCCGGTTGGCTCGGGTCGAGTAACA--- 269
SAW             -AACA-CCAGGCG-CTAGCAAAGGGGT----CCCCGGTTGGCTCGGGTCGAGTAACA--- 277
DRA             TAACA-CCGGGCG-CTAGCAAAGGGGT----CCTTGGTTGGCTCGGGTCGAGTAGTAACA 279
WIL             TAGCA-CCAGGCG-CTAGCAAAGGGGT----CCCCGGTTGGCTCGGGTCGAGTAACA--- 280
PSE             TAACA-CCAGGCG-CTAGCAAAGGGGT----CCCAGGTTGGCTCGGGTCGTGTAACA--- 272
                 * ** ** ****  ***  *** **     **  *** *** ***** * ***  *

MAC             CTTGCGGCCCAA-CGCGCCCGTCACCATCTGCTC-TGC-CTTACTCTCTCATG 322
PW              CTTGCGGATTAAGCGCGAGCCGTACACCATGCTTGCGC-CTAGCTCTCTGAAA 332
DIS             CTTGCGGCCCAA-CGCGCCCGTCACCATCTGCTC--GC-CTTTCTCTCTCAAA 320
GRE             CTTGCGGCCCAA-CGCGCTCGTCACCATCTGCTC--GC-CATTCTCTCTCAAA 318
SAW             CTTGCGGCCCAA-CGCGCCCGTTAAATCATCGATTCGCGCCTTCTCTCTCAAA 329
DRA             CTTGCGGCCCAA-CGCGCCCGTCTTCGTCTGCTC--GC-TGGTCTCGCTCAAA 328
WIL             CTTGCGGCCCAA-CGCGCCCGATACCGTCTGCTC--GT-CCTGCTCTCTCGAG 329
PSE             CTTGCGGCCCAA-CGCGCCCAT--ACGTCCGCCACCGT---ATTTGTAGCAAA 319
                *******   ** ****  *               *       *
```

Figure 17 Multiple sequence alignment of ITS2 sequences of *Anopheles maculatus* group from GENBANK and *Anopheles maculatus* from Purworejo

Remark : MAC = *An. maculatus*, DIS = *An. dispar*, GRE = *An. greeni*, PSE = *An. pseudowillmori*, SAW = *An. sawadwongporni*, WIL = *An. willmori*, DRA = *An. dravidicus* and PW = *An. maculatus* from Purworejo

DISCUSSION

Three important findings of *Anopheles maculatus* complex and *Anopheles sawadwongporni* were observed in this study. First, possibility of physiological resistances to DDT and have tolerance to permethrin in both species were detected for the first time in Pu Teuy area, Kanchaburi Province. Secondly, true behavioral responses to DDT and permethrin by both species were characterized. Lastly, possibility of a local group of *An. maculatus s.s.* may exist based on molecular information.

Although behavioral responses of insecticides by malaria vectors remain enigma for years, several reports strongly supported the existence of behavioral responses in malaria vectors (Sparks *et al*., 1989, Chareonviriyaphap *et al*., 1997, 2001, 2004, Sungvornyothin *et al*., 2001 and Potikasikorn *et al*., 2005). In the past, behavioral responses have been normally overlooked in national malaria control programs, with focusing solely on biochemical (toxicological) response to insecticides. Today, the development of insecticide resistance in insect pests and disease vectors occurs in some countries, but it has been very limited in many areas in spite of an extensive use of chemicals to control insect pests and disease vectors (Roberts and Andre, 1994 and Chareonviriyaphap *et al*., 1997). This event suggests that behavioral avoidance could be critical in effective reduction of human-vector contact, not toxicology.

Assay for evaluating behavioral responses of insecticides by malaria vectors have been progressively reviewed (Roberts *et al*., 1997 and Rutledge *et al*., 1999). Most tests in the past were done using modified WHO excito-repellency test box (Bondareva *et al*., 1986, Quinones and Suarez, 1989 and Ree and Loong, 1989) and do not discriminate between contact irritancy and noncontact repellency, irritancy occurring after physical contact and repellency occurring without physical contact with insecticide (Potikasikorn *et al*., 2005). All tests rely exclusively on a prejudicial and unrealistic concept (Roberts *et al*., 1997). Furthermore, a qualified and accepted method for behavioral responses by mosquitoes has never been available. One of the

reasons of this unavailability is because there is no conceptual knowledge of behavioral responses, indicating the past difficulties of conducting excito-repellency testing, data gathering, data analysis, and interpretation (Roberts *et al*., 1984, Evans, 1993, Chareonviriyaphap *et al*., 1997, Rutledge *et al*., 1999, Sungvornyothin *et al*., 2001 and Potikasikorn *et al*., 2005). Remarkable works have begun after true excito-repellency test box was developed by Roberts *et al*. (1997). This test system allows observing two different types of behavioral avoidance, irritancy and repellency. The test system was first used by Chareonviriyaphap *et al*. (1997). Unfortunately, this prototype was found some difficulties to handle and required extended time to attach the test papers onto the inner wall surfaces. Chareonviriyaphap *et al*. (2002) provided the improved version of excito-repellency test chamber and the system has been successfully used to evaluate the behavioral responses of several mosquito vectors in Thailand (Sungvornyothin *et al*., 2001, Chareonviriyaphap *et al*., 2001, 2004, Kongmee *et al*., 2004, Potikasikorn *et al*., 2005 and Chareonviriyaphap *et al*., 2006). More recently, a modular and noval high-throughput assay system for rapid mass screening of test compounds in the study of behavioral responses of adult mosquitoes has been developed (Grieco *et al*., 2005). Although, this novel system can differentiate three different functions of test chemicals, including contact irritancy, spatial repellency, and toxicity assays, this system was not designed for field application.

Although excito-repellency to insecticides has been investigated in several mosquito species in Thailand (Potikasikorn *et al*., 2005), none has been reported on wild-caught populations of *An. maculatus* and *An. sawadwongporni*, important vectors of malaria in Thailand, to DDT and the most commonly used pyrethroids, permethrin. For years, DDT was an insecticide of choice in Thailand as extensive intradomicillarly use for chemical control once or twice a year (Prasittisuk, 1995, Chareonviriyaphap *et al*., 1999 and Potikasikorn *et al*., 2005). Despite the widespread use of DDT for malaria control in the past, true impact in terms of behavioral responses of this compound remains unclear. Government of Thailand stopped using DDT in 2001, this compound, however, is still effective and widely used in several poor countries in Africa to prevent malaria transmission (UNDP,

2001). The final acceptance of DDT by the Stockholm Convention on Persistent Organic Pollutants for the continued use in the benefit of public health is clear testament to its unique effectiveness to combat malaria and the realization that the relatively small amount required for indoor spraying have very limited effect on the environment while sparing countless lives from malaria in endemic countries (UNDP, 2001 and Roberts *et al*., 2004).

Roberts *et al*. (2000) examined the property of DDT use in malaria control and demonstrate the powerful evidence of the combine effect of repellency and irritancy exerted the dominant actions on mosquitoes in reducing indoor man-vector contact. This works as well as other related entomological studies has proposed that the excito-repellent and toxicological actions must be carefully reevaluated and accurately assessed by using vector populations from different geographic locations to various chemical insecticides (Sungvornyothin *et al*., 2001, Chareonviriyaphap *et al*., 2004 and Potikasikorn *et al*., 2005). Besides DDT, pyrethroids also elicit behavioral responses in insects (Threlkeld, 1986). Mosquito control through the use of ITN with permethrin has been initiated in Thailand since 1997 (Chareonviriyaphap *et al*., 1999). The increased use of permethrin should be a major stimulus for extensive tests and field studies on pyrethoid avoidance behavior in malaria vectors in Thailand. This study observed the behavioral responses of two important malaria vectors from Thailand to operational field doses of DDT (2 g/m^2) for the IRS and permthrin (0.5 g/m^2) for the ITN, presently used for malaria control in Thailand. This investigation supports the ongoing research on the optimization and standardization of an excito-repellency test system that is considered to be a significant component for assessing public health insecticides and their mode of action in disease control.

Significant behavioral avoidance responses were observed in contact trials, compared to the control trials. The greatest behavioral response after physical contact with permethrin was observed in *An. maculatus*, followed by *An. sawadwongporni* test specimens. Moderate behavioral responses after physical contact with DDT were found in both test populations and significantly different from the control. Noncontact repellency plays a significant role in the escape response of *An.*

sawadwongporni against DDT. This observation on repellency action of DDT is in agreement with the results from previous investigations (Chareonviryaphap *et al*., 1997, 2000, 2004 and Sungvornyothin *et al*., 2001). Mortality was comparatively low in mosquitoes escaping the treated chambers in contact and noncontact trials, suggesting the overcome of behavioral avoidance of mosquitoes to test compounds, not toxicity.

Avoidance responses of both malaria vectors to insecticides are similar to those of previous works (Ree and Long, 1989; Evan, 1993; Chareonviriyaphap *et al*., 1997, 2001, 2004, Bangs, 1999 and Potikasikorn *et al*., 2005). Strong repellency to DDT by *An. sawadwongporni* could be partly a consequence of previous use of DDT or an innate characteristic of test population. Wild caught mosquitoes are generally heterogenous in age and nutritional conditions. Previous work demonstrated that physiological and nutritional conditions influenced avoidance behavior, therefore careful interpretation of avoidance insecticide responses should be undertaken with serious caution (Roberts *et al*., 1984 and Sungvornyothin *et al*., 2001).

Anopheles maculatus and *An. sawadwongporni* are considered to be the important vectors of malaria in the southern part of Thailand and some areas along the Thai-Myanmar border (Baimai, 1989, Rattanarithikul *et al*., 1996 and Chareonviriyaphap *et al*., 2003). Thailand is currently the world's third largest producer of natural rubber, most of which comes from plantations in the south. Rubber plantation is considered as one of the most common breeding and resting habitats of *An. maculatus* and *An. sawadwongporni*. Local people in the area usually protect them from biting mosquitoes by wearing a long sleeve cloth during the night work in the rubber plantation area. Government of Thailand has launched a new disease control program of using ITN technology with permethrin and distributed the impregnated bednets to local people who live in the areas (Chareonviriyaphap *et al*., 2004 and MOPH, 2005). Many synthetic pyrethroids cause mosquitoes to escape sprayed surfaces (Miller, 1990, and Lindsay *et al*., 1991). Results showed that permethrin produces strong behavioral escape responses from *An. maculatus* and *An. sawadwongporni* females. Knowing of behavioral responses by mosquitoes to

insecticide that can disrupt or interfere with vector feeding must be considered when assessing the true impact of insecticides on the national disease control program. Thus, response to insecticides in malaria vectors should be evaluated before initiating a large scale insecticide use.

The ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) was proved as a useful subject for species identification (Walton *et al*., 1999, Torres *et al*., 2000, Garros *et al*., 2004 and Wilkerson *et al*., 2004). The specimens of *An. maculatus* from Indonesia, Timor-Leste and Thailand were used to compare variations at the molecular level. From the ITS2 sequence among *An. maculatus* populations, sequencing data from Purworejo, central Java Island, Indonesia have been separated from other collection sites. The ITS2 sequences of *An. maculatus* from Timor timur selatan in Timor-Leste and from Chiang Mai Province in Thailand, were similar to the ITS2 sequence of *An. maculatus* from Pelabuhan ratu, west Java Island, Indonesia. On the other hand, the ITS2 sequence of *An. maculatus* from Purworejo was observed many base differences from Pelabuhan ratu in spite of the same collection island. The evidence of variation showed that *An. maculatus* in Purworejo was restricted the distribution by natural barrier until could not changed the genetic information with other populations. If *An. maculatus* population in Purworejo was completely adaptive the reproductive isolated mechanism to protect the genetic recombination with other populations, the speciation would be occurred and evolved into the new species.

CONCLUSION

Five species of *An. maculatus* group, *An. maculatus*, *An. sawadwongporni*, *An. notanandai*, *An. dravidicus* and *An. willmori* were collected from Chiang Mai, Chantaburi, Tak, Surat Thani and Kanchanaburi Provinces. Number of *An. maculatus* group was recorded from indoor and outdoor human collections and cow bait collection over one year period at Pu Teuy Village, Sai Yok District, Kanchanaburi Province. The results showed that three species of *An. maculatus* group, *An. sawadwongporni* (72%), *An. maculatus* (27%), and *An. notanandai* (1%) were collected by cow bait collection higher than human collections with outdoor exceeded indoor collections. *An. maculatus* and *An. sawadwongporni* mosquitoes were collected in the highest number in June with the prominent peak at 2100-2400 hrs.

Behavioral responses of *An. maculatus* and *An. sawadwongporni* wild-caught populations exposed to DDT and permethrin were observed. Numbers of escape responses to DDT and permethrin were significantly stronger in contact irritancy than noncontact repellency in both test populations ($P < 0.05$), except when contact was compared to noncontact with DDT against *An. sawadwongporni* ($P < 0.05$). In contact trial, the numbers of escape responses were significantly higher in two test populations when exposed to permethrin than those to DDT. Strong repellency action of DDT was observed in *An. sawadwongporni* whereas significantly less reaction was observed with *An. maculatus*.

The ITS2 sequencing data of *An. maculatus* from Pelabuhan ratu in Indonesia, Purworejo in Indonesia, Timor timur selatan in Timor-Leste and Chiang Mai Province in Thailand, were 327, 332, 322 and 322 base pairs in lengths. The ITS2 sequences of *An. maculatus* from Timor timur selatan and Chiang Mai were completely similar to the ITS2 sequence of *An. maculatus* accession number AY549313 from GENBANK whereas 88% identity was detected in the ITS2 sequence of *An. maculatus* from Purworejo. The ITS2 sequences of *An. maculatus* from four collection sites were similar GC content with 57%.

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