MATERIAL AND METHODS

Part 1; Risk factors and base line malaria knowledge from different pesticide Land-use systems in malaria endemic area at Kanchanaburi Province,Thailand.

Farming systems survey (August – September, 2003), the selected districts were Sai Yok and Thong Pha Phum district;

Sai Yok District was selected 2 places as follow;

1. Bong Ti Noy village, Wang Krajae sub district, Sai Yok district (N 14° 19', E 98° 59')

2. Pu Tuey village, Sai Yok district (N 14^{o} 20', E 98^{o} 59')

Thong Pha Phum district was selected 4 places as follow**;**

1. Mae Num Noy, only in the part of Rubber forest village, Huay Ka Yeng sub district (N 14° 35', E 98° 36')
2. Huy Bak Kok village, Huay Ka Yeng sub district (N 14° 40', E 98° 31')
3. U-long village, Ta Ka Nun sub district (N 14° 48', E 98° 40')
4. Thung Nang Khruan village, Cha Lae sub district (N 14° 53', E 98°46')

Map of Thailand

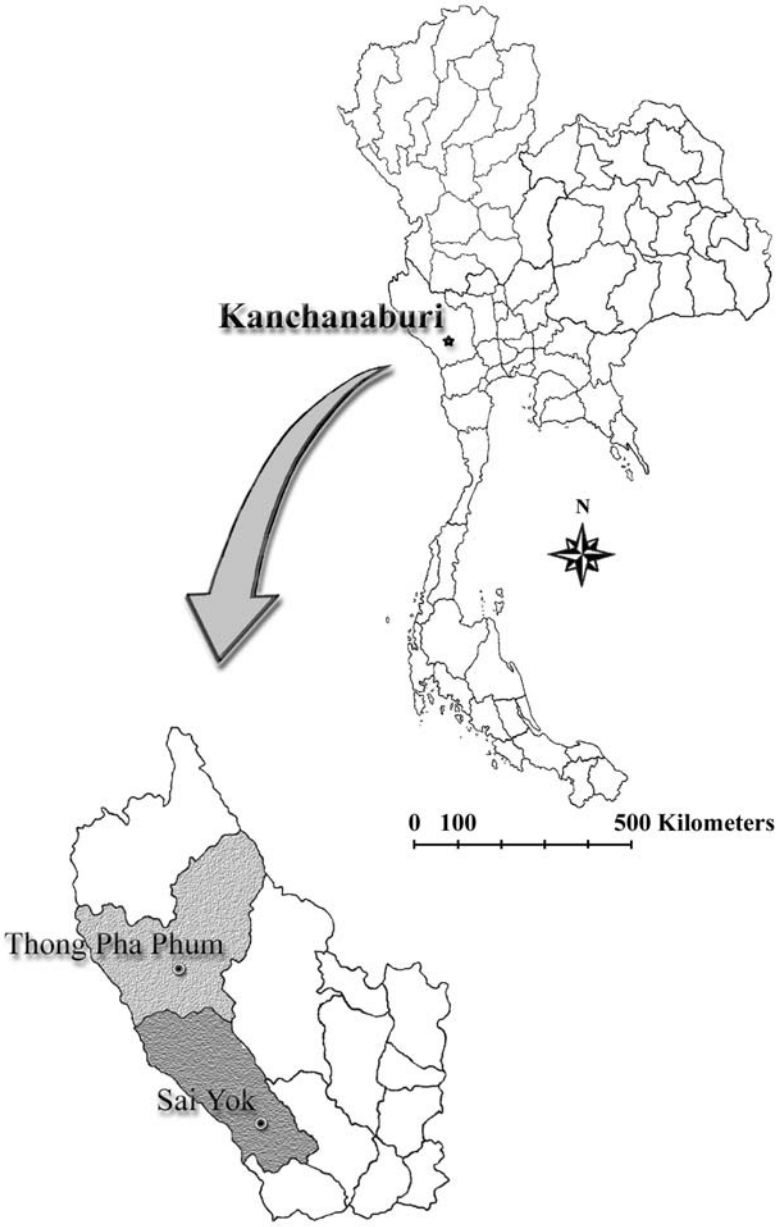


Figure1: Map showing the location for adult and larval of *Anopheles minimus* species complex collection.

Field Survey

Bong Ti Noy

Mae Num Noy

Huy Bak Kok

Thung Nang Khruan

U-long

Pu Toey

Figure 2: Farmer system survey to six villages in Thong Pha Phum and SaiYok District, Kanchanaburi Province, Thailand.

Interviews by questionnaire 1&2

The six villages in Kanchanaburi province were selected, based on information on malaria prevalence, presence of anopheline larvae, land use and agricultural plant protection practices. Once villages were selected a probability sampling design was used to select households. During August – September, 2003, approximately 30% of the households in each village were selected by random sampling, visited, and the head of that household interviewed. The random selection was based on a list of village households from national census data or from data/maps from the Ministry of Public Health's Vector-Borne Disease Units. If household heads were not present, another respondent from that household was interviewed by using questionnaire 1.

Questionnaire 1 (Q1) (see appendix).

This questionnaire provided information on three steps

Step1. General information;

This section contained questions on age, sex, occupation, ethnic group, number of adults and children in the household, time of residence in village, perceptions of the three largest problems and the three most important diseases in village, and patterns of travel outside village.

Step 2. Malaria knowledge;

This section contained questions on knowledge on malaria symptoms, how and in what season malaria is transmitted, mosquito biting times, mosquito larval sites, personal experience of malaria, mosquito preventive measures, and personal assessment of mosquito presence in houses after periods of agricultural insecticide applications.

Step 3. Malaria risk factors.

This section covered occupational and domestic risk factors. Questions were asked on how often and in what season they sleep in a field hut or in the forest; measures of mosquito protection; distances from their house to water, forest, agricultural field, and fruit orchards; house construction; window screening; bed nets; and types of animals kept near houses.

Questionnaire 2 (Q2) (see appendix).

This questionnaire provided information on specific information on agricultural pesticide use, the 20 most important farmers in the village used questioned using Questionnaire 2 (Q2). The ‘most important farmers’ mean those farmers who had the largest area of perennial (e.g. fruits) and annual crops (e.g. rice, maize, etc.). At least 10 farmers were selected from this group. Q2 also provided information on insecticide use, concentration, amount, application date, how often in the past applied, etc.

The statistic analysis

The results from a six section undertaken simultaneously, containing information on farmer’s pesticide use, will be reported elsewhere. Data from questionnaires were entered, manipulated, and analyzed using SPSS software.

Field Observation and Interviewing with Questionnaires

Figure 3: Contacted to local peoples by interviewing with questionnaire 1 and questionnaire 2.

Part 2; **Biting peak and population dynamics of *Anopheles minimus* species A, from high and low agricultural insecticide area in the two villages at Kanchanaburi Province, Thailand.**

Study Area

The study sites are located in an endemic malarious area with both *Plasmodium falciparum* and *Plasmodium vivax* infections occurring (A1 area; see appendix). The principal malaria vectors in this region are *Anopheles minimus* A and species of the *Anopheles dirus* complex. The study sites were selected on the basis of agrochemical use. A questionnaire (Q2, see Part 1) was used to collect information on farmers' pesticide use. Based on the results obtained from this questionnaire the following villages were selected for further insect collections:

Low pesticide village

The village selected as having low pesticide agricultural use was Ban Mae Num Noy village (MNN), located in Huay Ka Yeng sub-district, Thong Pha Phum district, Kanchanaburi province, near the western border of Thailand. The study site is situated at latitude 14° 35' and longitude 98° 36', approximately 100 km from the High pesticide village. The village is surrounded by rubber plantations and hills. There are two large clean pools in the village formed by spring water damming up near the village. There is a small permanent stream nearby (Figure 4**).** The two selected locations where anopheline larvae were collected in this site were:

1. Pool (HP)

This is a pool which is located beside a road in the village. The central parts of the pool were absent of emergent aquatic vegetation. The depth of the pool was in general more than 1.0 m. Almost all of the pool area was covered with green algae and floating weeds in the sun-exposed sites. Grasses were growing around the pool and the vegetation sometimes entered the water close to the edge. A shallow waterway connected this pool with the second habitat (Figure 5).

2. Stream (ST)

This is a small stream running along a citrus garden in the village. The stream was narrow (usually less than 0.5m wide) and 0.1-0.2 m or less in depth. The stream connected to the end of HP pool and water flowed slowly in the dry season. Grasses and some emergent vegetation were growing along the margins of the stream. The water velocity was between 1.5-3.0 m/minute (Figure 6).

Figure 4: Map of Mae Num Noy village (MNN) (only part of Rubber forest village) showing 3 stations for adult collection and 10 points on 2 breeding sites for larvae collection.

Figure 5: Right picture is a pool **(HP)** which is located beside a road in the village. Left picture is a shallow waterway connected this pool with the ST habitat.

Figure 6: A small stream **(ST)** running along a garden in the village.

High pesticide village

The village selected as having high pesticide agricultural use was Bong Ti Noy village (BTN), located in Wang Krajae sub-district, Sai Yok district, Kanchanaburi province, near the western border of Thailand. The study site is situated at latitude 14^{o} 19' and longitude 98^{o} 59'. The village is located near the forest fringe, surrounded by

hills, and with the presence of clean water bodies. There is one large stream running through the area and many small temporary streams are present that are water-filled only during the rainy season (Figure 7) The two selected locations where anopheline larvae were collected in this site were:

1. Big stream (Bst)

This stream is a large perennial stream which runs along the village. The larval collection site was a 20 m long stretch close to the village temple. The width of the stream varied between 5-10 m across and the depth was 0.2-0.5 m in the dry season. In the rainy season the width was more than 10 m and the depth more than 3.0 m. Emergent vegetation were growing near the edge with some green algae along sun-exposed areas. Grasses often grew along the margin of the stream. Stream water velocity was between 1.5-30.0 m/minute (Figure 8).

2. Small stream (Sst)

This stream is a relatively small perennial stream running along a road. The width varied between 0.3-1.0 m across and 0.05-0.5 m in depth. There was some emergent vegetation and grasses growing near the edge. The water volume and velocity was quite low in the dry season during the dry season (Figure 9).

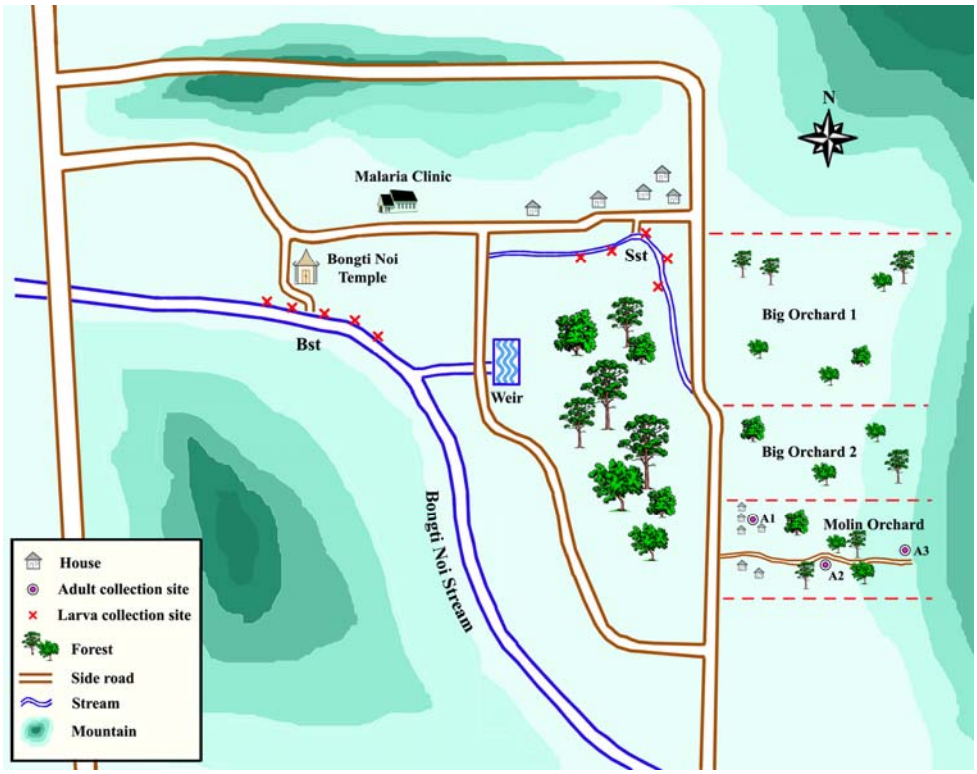


Figure 7: Map of Bong Ti Noy village (BTN) showing three stations for adult collection and 10 points on two breeding sites for larvae collection

Figure 8: A large perennial stream **(Bst)** which runs along the BTN village.

Figure 9: A small perennial stream **(Sst)** running along a road in BTN village.

2. Mosquito collections

Adult sampling

Wild adult females of *An. minimus* A were collected monthly between October 2003 and September 2004 during three seasons by outdoor human bait catches (Figure 10) in each village. Adult collections were undertaken at three sites, approximately 200 m apart from each other, in each village. The three sites were a) inside village, b) in an orchard (for BTN) or in a rubber plantation (for MNN), and c) in the forest (Figure 11). Mosquito collections were undertaken during the night by two teams of two persons each at each site. The first team collected mosquitoes from 18.00 to 24.00 hours and the second team collected from 00.00-06.00 hours. Torches were used to observe mosquitoes and aspirators for collecting them. Collections were made two consecutive nights each month at the same sites. Collected mosquitoes were placed in separate marked plastic cups, one for each hour, and covered with netting material. All live mosquito specimens were provided with 10% sugar solution and transported to the field laboratory for morphological identification the following morning (Figure 12). During transport and storage mosquitoes were kept in larger containers covered with damp cotton towels to avoid desiccation. The humidity and temperature were recorded each hour.

Figure 10: Mosquitoes collecting by outdoor human bait catches

In the village site

In the rubber forest site (MNN)

In the orchard site (BTN)

In the forest site

Figure 11: The three sites were undertaken during the night by two teams of two persons each at each site.

Figure 12: Collected mosquitoes were placed in separate marked plastic cups, all alive mosquito specimens were provided with 10% sugar solution and transported to the field laboratory for morphological identification

Figure 13: Species identification was carried out by stereo-microscope.

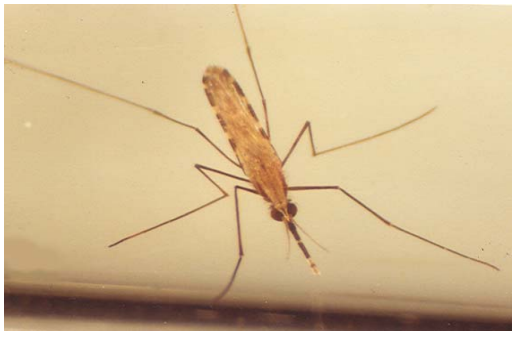


Figure 14: *Anopheles minimus* species A

Larval sampling

At each breeding site, stream or pool, a section of about 20 m long and 0.05-0.20 m wide from the edge was selected and visited monthly (October 2003-September 2004). All breeding sites were closer than 3 kilometers away from the each village. Larval and pupal stages of anopheline mosquitoes were collected using the dipping method. Larvae were collected at four points at each selected habitat. The distance between each point was 5 m. Larval sampling was undertaken during daytime between 10.00-13.00 hrs with 30 dips per point (approximatly 120 dips per habitat) (Figure 15). Larvae were kept in a plastic bag half-filled with water from the respective habitat. The frequency of larval instars and pupae were recorded for one year.

3. Identification of adult and larval mosquitoes

Adults and larvae were brought to the field laboratory and the laboratory at the Department of Entomology, Kasetsart University for identification. Species identification was carried out by stereo-microscope, using morphological characters and keys by Peyton and Scanlon (1966), Rattanarithikul and Panthusiri (1994) and Harrison (1980). Larvae were identified alive and preserved (if dead) in the laboratory by the method described by Rattanarithikul and Panthusiri (1994).

4. Data analysis

Differences in mean numbers of *An. minimus* A mosquitoes between two villages, three sites, and three season were compared using analysis of variance (ANOVA) (SPSS Base 11, 2001, SPSS Inc.)

Larva sampling at MNN village

HP site ST site

Larva sampling at BTN village

Bst site Sst site

Figure 15: Larval and pupal stages of anopheline mosquitoes were collected using the dipping method

Part 3; **Behavioral responses by *Anopheles minimus* species A and C to three agrochemicals.**

Test population

Anopheles minimus species A was collected by human bait in Mae Nam Noi Village, Thong Pha-Phoom District, Kanchanaburi province Province (N 14° 35', E 98° 36') and *An. minimus* species C was collected by cow bait in Pu Teuy Village, Sai Yok District, Kanchanaburi (N 14^{o} 20', E 98^{o} 59')The province is located in western Thailand and borders Myanmar. The collected mosquitoes were kept in mosquito plastic cups, provided with 10% sugar solution and transported to the field laboratory for morphological identification the following morning. During transport and storage mosquitoes were kept in larger containers covered with damp cotton towels to avoid desiccation.

Insecticide-treated papers

Papers were impregnated using formulation grade insecticides at the operational field concentrations as recommended on the label. The concentrations used were 0.40 g/m^2 of carbaryl, 0.19 g/m^2 of malathion, and 0.04 g/m^2 of cypermethrin. All papers were treated at the rate of 12.5 ml of the insecticide solution per 0.0928 m^2 (26.5 x 35 cm).

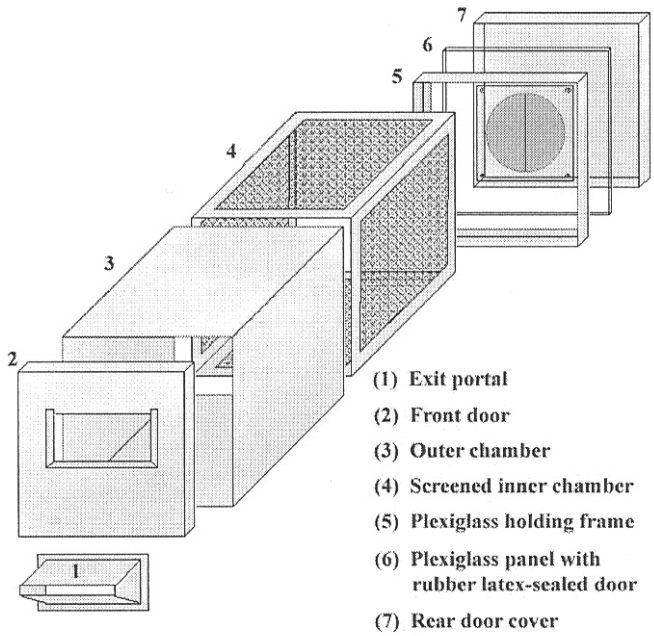
Excito-Repellency tests

In this study, we used the improved test chamber for all tests as described in a recent publication (Chareonviriyaphap and Aum-Aong, 2000). Figure shows the stainless steel, collapsible excito-repellency escape chamber (34 X 32 X 32 cm), facing the front panel and escape runnel. The box comprises 4 side walls, a rear Plexiglas inner door, a rear outer door cover and a front door, and a removable exit portal (an escape funnel). Each wall is constructed of stainless steel sheet (0.7-mm thickness), which has an aluminum sliding rib on each end and a socket, providing a

surface for the test paper holder in the middle. The test paper holder has 2 sides; a sheet of fine mesh iron screen net is permanently attached on side, and a panel to hold test papers to secure the panel on top is on the opposite side. A 0.8-cm gap between the test papers and screen prevents mosquitoes from making physical contact with the surface of test paper in the exposure windows during the non contact repellency trials. The test paper holder is convenient and functions similarly under contact and non contact conditions, depending on the purpose of the test. The holder simply has to be inverted to provide the proper conditions. A spring mechanism on side of the test paper holder secures it tightly when putting the holder into the socket. The front door is constructed of a stainless steel frame with stainless steel sheet affixed on the front side. The steel sheet has a trough for sliding the exit funnel into place. Two screws at end secure the funnel to the front panel. The inner rear door is constructed of a stainless steel frame and a transparent Plexiglas door that is attached to the frame. The Plexiglas door serves to seal the chamber and at the same time allow the investigator to look inside the exposure chamber before and after a test is conducted. A self-sealing 6-in. (15.5 cm)-diameter portal made of dental dam is used for placing test specimens inside the chamber and for removing the specimens from the chamber after each test. The outer rear door is constructed of stainless steel and is used to shut off all light inside the chamber when the test is being conducted. The last part is a removable exit runnel attached to the outside of the chamber. The escape runnel gap is a 20.5-cm-long and 1.5-cm-wide opening (Chareonviriyaphap *et al*., 2001) (Figure 16).

Tests were designed to compare two field populations of the two collected species in contact and non-contact exposures using insecticide treated papers and excito-repellency test chambers as described above. The tests were undertaken within 48 hours of capture of mosquitoes. Only female specimens were used in the tests. Mosquitoes were deprived of all nutrition supplies, except water for a minimum of 12 hours before exposure. All tests were performed in the field laboratory during daylight hours and each test was replicated three times. Temperatures and relative humidity were recorded during tests. Observations of escaping mosquitoes were made at 1 min intervals for 30 min. The number of dead or knockdown specimens was recorded separately for each exposure chamber, external holding cage, and control chambers

(without insecticide). Escaped specimens and those remaining inside chambers, for both treatments and controls, were held separately in small holding containers, provided with 10% sugar solution, and mortalities were recorded after 24 hours.



Chareonviriyaphap, *et al*., 2001

Figure 16: Exito-repellency test chamber model.

Figure 17: Exito-repellency test chamber were designed to compare two field populations of the wild caught species in contact and non-contact exposures using insecticide treated papers**.**

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 between two field populations and two insecticides (Kleinbaum, 1995). In the analysis, mosquitoes that escaped were treated as "deaths" and those remaining in the test chamber were considered as "survivals" as previously described (Roberts *et al.*, 1997). The escape times (ET) for 30, 50, and 70 percent (ET_{30}, ET_{50}, and ET_{70}) of the test populations to escape were estimated from data collected at 1-min intervals. Patterns of escape response between treatment groups were determined using the log-rank method (Mantel and Haenzel, 1959). Statistical software (STATA®, city, state) was used in the analysis (Roberts *et al.*, 1997).

Part 4; Behavioral responses by *Anopheles minimus* species A and species C to DDT and pyrethroids.

Mosquito collection.

Anopheles minimus complex mosquitoes were identified based on morphologic keys (Harison,1980 and Peyton and Scanlon, 1966). Species were differentiated by the presence or absence of the humeral pale spot on the costal wing vein. *Anopheles minimus* A has a wing costa without the humeral pale spot whereas An. minirnus C has the humeral pale spot. A diagnostic enzyme, octanal dehydrogenase, indicated 95% concurrence with species A, which does not have the humeral pale spot. This spot is lacking in 73% of species C (Green *et al*., 1990). *Anopheles minimus* A and C adult females were collected off human volunteer baits during the evening hours (6:00 PM to 6:00 AM). These volunteers (collectors) worked for the Ministry of Public Health. Behavioral tests were performed within 24 hours of capture. All mosquitoes were starved of blood and sugar 24 hours before the tests (Sungvornyothin *et al*., 2001). Temperatures and relative humidity were recorded during the tests. Both populations were physiologically susceptible to DDT, deltamethrin, and lambdacyhalothrin (Chareonviriyaphap, T and others, unpublished data).

Insecticide-treated papers.

Analytical grade insecticide was impregnated on papers at operational field concentrations of 2 g/m^2 of DDT, 0.02 g/m^2 of deltamethrin, and 0.03 g/m^2 of lambda-cyhalothrin and prepared using diluent according to World Health Organization protocol (Busvine, 1958).

Behavioral tests

Tests were designed to compare two wild caught populations in contact versus non-contact exposures using three different insecticides. Identical, specially designed test chambers (four per test trial) were used for all bioassays as previously described (Chareonviriyaphap *et al*., 2001). The stainless steel outer chamber of excito-repeIlency testing device measures 34 cm 32 cm 32 cm (Figure 18), and faces the front panel with the single escape portal. The box is composed of a rear door cover, an inner Plexiglas glass panel with a rubber latex-sealed door, a Plexiglas holding frame, a screened inner chamber, an outer chamber, a front door, and an exit portal slot. Only female *An. minimus* specimens were used in excito-repellency tests. Mosquitoes were deprived of all nutrition and water for a minimum of 24 hours before exposure. Laboratory tests were performed during daylight hours only and each test was replicated four times. Observations were taken at one-minute intervals for 30 minutes. After each test was completed, the number of dead or knockdown specimens was recorded separately for each exposure chamber, external holding cage, and paired control chamber (without insecticide). Escaped specimens and those remaining inside the chamber, for both controls and treatments, were held separately in small holding containers with food and water and 24-hour mortalities were recorded.

Data analysis

A Kaplan-Meier survival analysis method was used to analyze and interpret the behavioral response data (Robert *et al*., 1997; Chareonviriyaphap *et al*., 1997; Sungvornyothin *et al*., 2001 and Chareonviriyaphap *et al*., 2002). Survival analysis was used to estimate the probability of escape time (ET) and compare differences in mosquito response among the two populations and three insecticides. Mosquitoes that escape were treated as deaths and those remaining in the test chamber were considered survivals (Chareonviriyaphap *et al*., 1997). The ET_{50}, ET_{75} and ET_{90} time in minutes for 50%, 75% and 90% of the test population to escape, respectidy, were estimated from data collected at one-minute intervals. Patterns of escape response were determined using the log – rank Method (Mantel and Haenzel, 1959). Stata statistical

software (Stata Corp., College Station, TX) was used in the analysis (Robert *et al*., 1997).

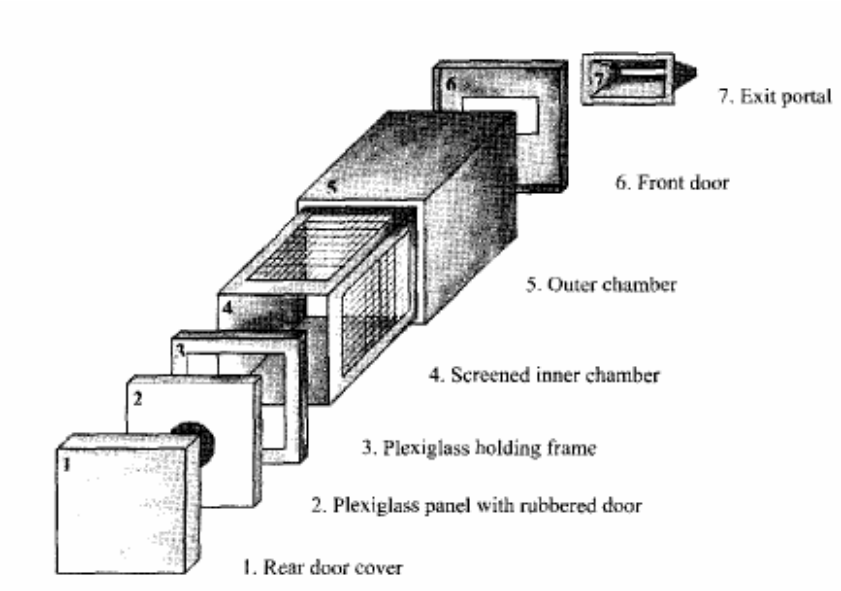


Figure 18: Excito-repellency test chamber used to study insecticide behavioral responses.