Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Mosquito test populations: *Anopheles minimus* species A.

A colony of *An. minimus* species A was received from the Malaria Division, Department of Communicable Disease Control (DCDC), Ministry of Public Health, Nontaburi in 1997. This colony was originally collected from animal shelter, Rong Kwang District, Prae Province, North Thailand in 1993. The colony was raised in the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand in 1998. Females from the colony were completely susceptible to deltamethrin (0.05%) and DDT (4%), based on a standard impregnated paper adult contact assay (WHO, 1998).

2.1.2 Insecticide impregnated papers

Insecticide impregnated papers with different concentrations of deltamethrin and 4% DDT were ordered from the World Health Organization, Vector Control Unit, Penang, Malaysia. All impregnated papers (12 x 15 cm^2) of deltamethrin and DDT were treated at the rate of 2.75 ml of insecticide solution per 180 cm^2 and used before their specified time of expiration.

2.2 Methods

The experiments were conducted at the Department of Entomology, Faculty of Agriculture, Kasetsart University in May, 2000-March, 2002. The experimental strategy for this study was composed of two parts. The first was to establish a deltamethrin resistant colony and to determine the level of physiological resistance in the laboratory reared *An. minimus* species A by using bioassay. The second was to measure the activity of detoxification enzymes involved in resistance mechanism by using biochemical assays.

The experiment started by dividing *An. minimus* species A into two colonies. One was the control, used for the monitoring of pyrethroid susceptibility in a population, and another was used for developing a pyrethroid resistance (Figure 6). Control colony was reared simultaneously in the separated room and handled in the same manner through all manipulations, but were not exposed to insecticide. The control colony was tested to monitor for independent occurrence of resistance. To begin the method, deltamethrin susceptible parent (F_0) was used to conduct susceptibility tests with various doses of deltamethrin, and computed on susceptible levels. After the determination of susceptibile levels, F_0 females were selected with deltamethrin with the dose that caused 50% mortality (LD_{50}). Survivors from the selection were raised to the parent of the next generation (F_1). The susceptibility test of deltamethrin was rechecked in each generation and computed with susceptibility levels for that generation of selection. In addition, resistant status was evaluated according to the WHO protocol.

The second part of this experiment measured the activity of detoxification enzymes involved in resistance mechanisms. Enzyme-based metabolic mechanisms of insecticide resistance were investigated, comparing a deltamethrin susceptible parent stock and resistant colonies of *An. minimus* species A. Expression of levels of elevated esterases, monooxygenases and glutatione S-transferases were measured by using biochemical assays. The total protein content of individual mosquitoes was determined using protein assay reagent kit (Brogdon *et al.*, 1988).

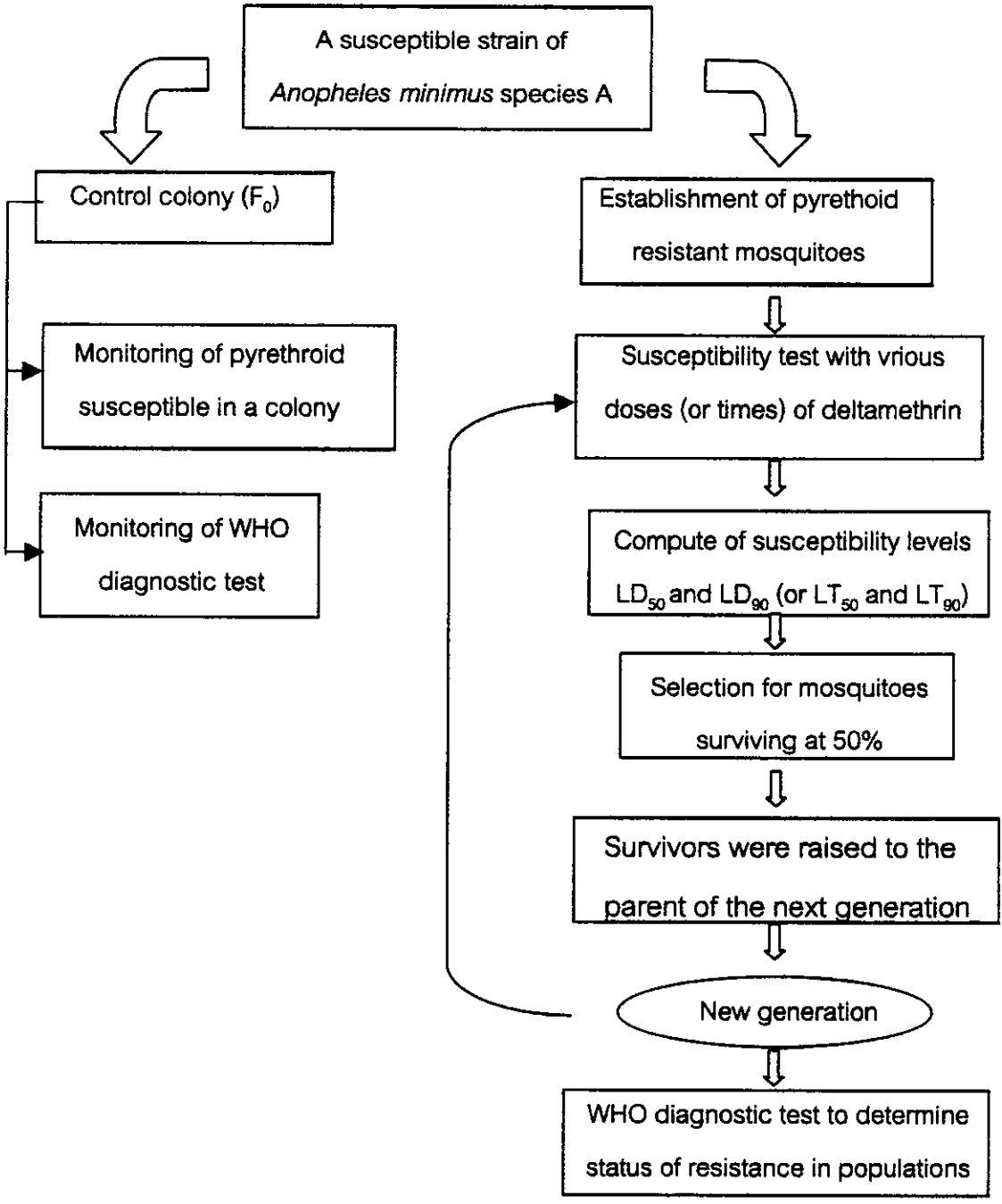
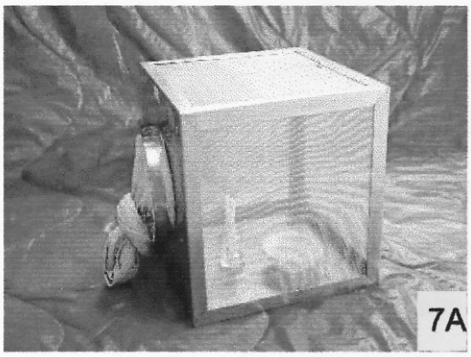
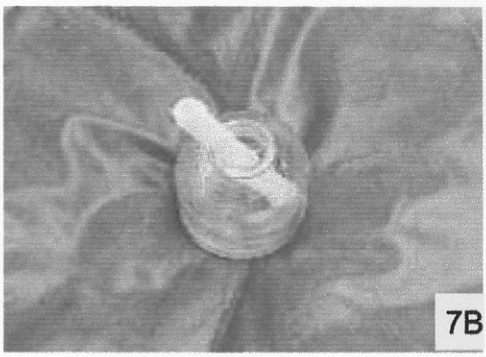


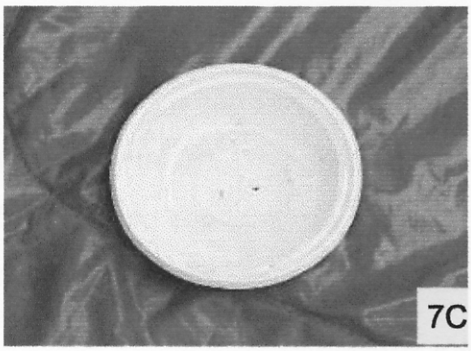
Figure 6 Diagram shows strategy for establisment of pyrethroid resistant mosquitoes.

2.2.1 Mosquito rearing

The standard procedure for rearing *Anopheles* mosquitoes was followed by Ford and Green (1972). All life stages were reared in an environmental controlled insectary (25±3°C, 80±10%RH) at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Adults were maintained in cages (30x30x30cm) with sleeve to serve as the circular door when collecting adults and placing pupae for emergence (Figure 7A). From the day of emergence, cotton is soaked with a 10% sugar solution and was provided as adult mosquitoes food source (Figure 7B). The cotton was changed three times per week. Female mosquitoes were fed on a guinea-pig blood meal on the third day post emergence. Two or three days later, oviposition dish were lined with damp filter paper around the side and filled with a little water (Figure 7C) was placed in the cage containing gravid females. Oviposition dish was then removed and filled water to flood the eggs every morning. Newly hatching larvae were transferred to the rearing trays (12x30cm^2) with approximately one liter of water (Figure 7D). Grind fish food was spread on the surface of water twice daily (3 times daily after reaching the third larval stage). The third larval stage was transferred to the other rearing trays via a wide-mouthed pipette (Figure 7E) at densities of 200-300 larvae per tray. Excess food was removed daily to prevent scum forming by blotting with paper on the water surface and sucking the deposit at the bottom of trays or changing the rearing trays. Pupae were collected daily, and transferred to the small bowls (Figure 7F) containing clean water (approximately 100-150 larvae per bowl).The pupae were placed in cages for adult emergence, and those collected from day 1 to day 3 were placed in the same cage. Then, bowls were removed from the cage after adult emergence.







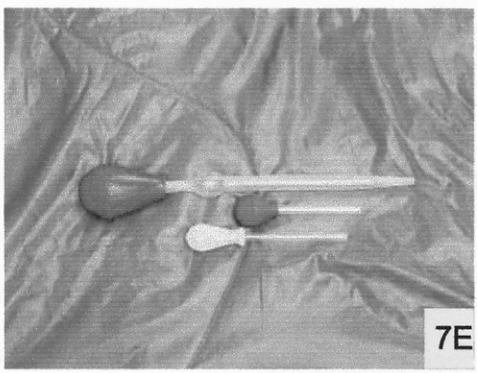


Figure 7 Equipment used to rear mosquitoes.

2.2.2 Bioassays

2.2.2.1 Susceptibility testing

World Health Organization standard test kits for adult mosquitoes (In appendix 1) was modified to conduct susceptibility test with various doses of deltamethrin for F_0– F_{10} and various times of deltamethrin at 0.05% for F_{14}– F_{19}. No susceptibility test was made for F_{11}– F_{13} due to low populations of mosquitoes.

About F_0– F_{10} were exposed to various doses of deltamethrin from 0.00097% to 0.05%, and the each of dose was reduplicated from the lower dose. The control cylinder contained paper impregnated with solvent. The treatment cylinders contained paper impregnated with deltamethrin plus solvent. Two replicates of twenty blood fed females were introduced into each cylinder for one hour. Mosquitoes were then transferred to the holding containers. The holding tubes were kept for 24 hours under temperatures of $25 \pm 3\ ^{0}C$ and a relative humidity of $80 \pm 10\%$. Mosquitoes were provided a 10% sugar solution. Mortality from each dose was recorded after 24 hours.

Due to fineness crisis for purchase survival doses of insecticide impregnated paper, 0.05% deltamethrin was used to test susceptibility level from F_{14}-F_{19} using time-mortality relationship (4-60 min).

2.2.2.2 Selection of deltamethrin resistance

After the determination of susceptibility levels of deltamethrin for each generation, (LD_{50} or LT_{50}) was used for further selection. The LD_{50} dosages were used to select for F_0-F_{10} and LT_{50} were used to select for F_{14}-F_{19}. The progress of selection was monitored by the WHO diagnostic test. Approximately 2,000 females were selected for each generation. Survivals were raised as usual in the insectary for succession to the next generation.

2.2.2.3 WHO diagnostic test

Bioassays were conducted using WHO test kits for adult mosquitoes (Appendix I). Adult females were exposed for 1 h to diagnostic dosages of deltamethrin (0.05%). For each test 5 cylinders, 2 for the controls and 3 for the treatments, were used. Control cylinders contained filter paper impregnated with carrier, whereas the treatments contained paper impregnated with the diagnostic dosage of insecticide and carrier. Twenty mosquitoes were introduced to each cylinder and maintained for 1 h in a normal vertical position. After 1 h, mosquitoes were transferred to holding containers and provided with cotton pads soaked with 10% sucrose solution. Mortalities were recorded at 24 hrs and each test was replicated 3 times. Resistant status was evaluated according to the WHO protocol; a population is considered resistant if more than 20% of population survives the diagnostic dose compared to the susceptible colony (WHO, 1981b). In addition to deltamethrin at the diagnostic dose (0.05%), susceptibility to DDT (4%) was measured in populations under selection pressure to test for cross-resistance to DDT.

2.2.3 Biochemical assays

One susceptible and 3 deltamethrin-selected generations of *An. minimus* were used for comparisons, F_0 (susceptible colony), F_8, F_{12} and F_{18} to measure levels of detoxifying enzymes over the period of increasing selection pressure. The parent F_0 control generation was completely susceptible to deltamethrin and DDT according to recommended diagnostic dosages. The subsequent deltamethrin tolerance or resistant generations of *An. minimus*, F_8, F_{12} and F_{18} test populations were obtained and preserved after each selection period. Based on WHO diagnostic test criteria (WHO, 1998), the F_8 colony was defined as tolerant to deltamethrin and DDT. With increasing selection, F_{12} demonstrated more or less 20% resistance to deltamethrin and DDT, and this increased further in F_{18} to resistance to deltamethrin and DDT.

Freshly killed mosquitoes were placed individually in a 1 ml microtube and were stored in frozen at –70°C. In biochemical assays started with individual mosquitoes were homogenized in 50 µl distilled water and diluted with additional 150 µl distilled

water. In each, homogenates were conducted using two replicated of protein assay and enzyme assays, using a microplate technique.

2.2.3.1 Protein assays

Size variations among mosquitoes are critical for the interpretation of the results. To avoid this problem, a microplate protein assay was performed to measure the protein content of individual mosquito using a BioRad protein assay system (Hercules, California). The fresh set of protein standards was prepared to construct a standard curve (Appendix II). Five µl of each standard or homogenate were transferred to the appropriate microplate wells. Coomassie reagent 250 µl was added to each well. The protein content were measured using the absorbance at 595 nm with microplate reader. The standard curve was used to determine the protein concentration of each unknown sample (Appendix III).

2.2.3.2 Mix Function Oxidases (MFOs)

The procedure described by Vulule *et al.* (1999) was used with some modifications. The fresh set of MFOs standards was prepared for used to standard curve. Twenty µl of each MFOs standard or homogenate were transferred to a microplate well. Eighty µl of 0.0625 M potassium phosphate buffer (pH 7.0) were added to each well. A 0.01 g of 3,3, 5',5'- Tetramethyl benzidine (TMBZ) in 5 ml methanol was prepared and a 0.25 M sodium acetate buffer (pH5.0) was added. One hundred eighty µl of TMBZ solution was added into the 100 µl of mosquito homogenates in each well followed by 25 µl of 3% hydrogen peroxide. The plates were read immediately after 10 minutes interval using microplate reader at 620 nm wavelength. The quantity of the MFOs was calculated from standard curve of the MFOs standard enzyme.

2.2.3.3 Elevated esterase

Two substrates, α - and β- naphthyl propionate were used for the assays, Method of Peiris and Hemingway (1990) was followed with some modifications. The

fresh set of esterase standards was prepared perform to standard curves of α - and β-naphthol. Twenty µl of each standard or homogenates was transferred to appropriate microplate well. Two hundred µl of working solution of α - or β-naphthyl propionate (0.00559 g of α - or β-naphthyl propionate in 1 ml acetone was prepared and 150 µl of that solution was added in 15 ml of 0.02 M phosphate buffer pH 7) was added to each well. Fifty µl of fast blue solution (0.05 g of fast blue BB in 5 ml distilled water and 11.5 ml of SDS 5%) was added in each well after 10 minutes. Working solution for α - or β-naphthyl propionate and fast blue solution was prepared just before use. The plates were read immediately after 10 minutes by using microplate reader at 450 nm. The quantity of naphthol produced from the esterase reactions was calculated from standard curves of α - and β-naphthol.

2.2.3.4 Glutathion S- transferases

The method of Brogden and Barber (1990) was followed with some modifications. Individual mosquito was homogenized in 50 µl distilled water in 1 ml plastic vial. The homogenates were diluted with an additional 150 µl distilled water. Twenty µl of homogenates were transferred to a microplate. Fifty µl of glutathion [0.03 g of glutathion (GST) in 50 ml potassium phosphate buffer] and 50 µl of 1-chloro-2,4-dinitrobenzene (CDNB) (0.01 g of CDNB in 0.5 ml acetone and 50 ml of potassium phosphate buffer) were subsequently added into each well. The plates were read after 30 minutes using microplate reader at 414 nm wavelength.

2.2.4 Data analysis

2.2.4.1 Susceptibility tests

Abbott's formula was used to correct the observed mortality. If mortality in the control colonies is over 5%, but less than 20% a correction of mortality is made by applying the Abbott's formula;

$$\text{corrected \% mortality} = \frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

The computer program based on probit analysis of Finney (1971) was used to analysis mortality data. The LD_{50} and LD_{90} (or LT_{50} and LT_{90}) values were estimated using dosage-mortality (or time-mortality) regression in probit analysis Regression lines show percentage mortality over logarithmic of dosage (or time) of the insecticide. Chi-square distribution that were calculated demonstrate that the response regression completely fit a linear model ($P>0.01$).

2.2.4.2 Biochemical assays

For accurate comparison of results between generations of *An. minimus*, absorbent values for individual mosquitoes should ideally be converted to m-moles of product produced per min (Appendix IV). The quantity of the enzymes produced was calculated from standard curves of each enzyme product. Protein concentrations are used in calculation of absolute values for each enzyme data. This allows different sizes of mosquitoes to be used, while results are directly compared in the same units (m-moles product/min/mg protein).

One-way analysis of variance (ANOVA) was used to compare the protein content and enzyme activity within and among populations. Significance was determined at $P<0.05$.