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Laboratory resistance induction of *Anopheles minimus* and *Anopheles dirus* by the exposure of adult females to permethrin treated mosquito nettings

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Abstract

A laboratory resistance induction study was conducted to determine the effects of permethrin impregnated nettings on the two major vectors of malaria in Thailand, *Anopheles minimus* and *Anopheles dirus*. To simulate the field use of bed nets, only females of the two species were exposed. *An. minimus* and *An. dirus* were selected using WHO bioassay tubes and cones, respectively, both using permethrin treated netting materials for three minutes. Changes in the phenotypic expression of resistance were monitored through the baseline and subsequent assessments of susceptibilities from the reference strains and within the generations of selected populations. The effects of permethrin selection on adult size were observed between the reference and last selected generation of both vectors. The activity of detoxifying enzymes (mixed function oxidases and non-specific esterases) were also compared between the reference and selected groups of both species for possible resistance mechanisms. Results showed a decrease in susceptibility level of 1.56 folds from the ninth selected generation (S_9) of *An. minimus*. *An. dirus* developed low level resistance of 4.88 folds at S_{12}. No significant effect was observed on adult sizes of the progenies of selected *An. minimus* and its reference strain. Varying results however were observed from *An. dirus*, with body weight significantly increased at S_{12} while no significant difference was observed between the wing lengths of the selected (S_{12}) and reference strains. Significant increase in the levels of mixed function oxidases were detected from both selected generations of the two species. Further studies are needed to directly determine the presence of the resistance genes and the magnitude by which they are expressed in successive generations of vectors.

Keywords: *Anopheles*; Resistance; Permethrin; Bed Nets; Thailand

Abbreviations: WHO: World Health Organization; PIBNs: pyrethroid impregnated bed nets; LLINs: long lasting insecticidal nets; API: annual parasite index; MFOs: Mixed Function Oxidases; NSEs: Non-Specific Esterases; RR: resistance ratio; OD: optical density.

Introduction

During those days when malaria was one of the leading causes of mortality and morbidity worldwide, the novel idea of using insecticide treated bed nets to reduce transmission came up. This strategy was envisioned to be the answer

to an urgent need for an inexpensive self-help control strategy that would reduce risk of malaria transmission. The World Health Organization (WHO) promoted the idea by including the use of insecticide impregnated bed nets as a part of the personal protection measure in primary health care programs. For several decades now, pyrethroid impregnated bed nets (PIBNs) have been the vector control method of choice due to their long and lasting effects and low mammalian toxicity [1-3]. Early documented reports attested to PIBNs effectiveness in preventing malaria. Preventive effects decreased the malaria incidence ratio [4] and the number of deaths among children <10 years old [5]. The large-scale trials conducted in China showed PIBNs to be as equally effective as DDT spraying [6]. PIBNs are still considered the most effective method of controlling malaria until now [7,8]. The wide scale use of factory insecticide pre-treated bed nets now known as long lasting insecticidal nets (LLINs) has resulted in major reduction in malaria transmission. As a result, most countries in Southeast Asia have shifted from control to elimination.

In 2018, the WHO South-East Asian Region estimated 8 million malaria cases [9]. Global cases estimate reached 241million in 2020 with Thailand reporting 2,836 indigenous malaria cases [10]. This record propelled Thailand towards malaria elimination by 2025 [11,12]. In 1998, Thailand reported 120,000 malaria cases giving an annual parasite index (API) of 2.2 per 1000 population. *Anopheles dirus* and *Anopheles minimus* are the two major malaria vectors not only in Thailand but in the greater Mekong region. *An. dirus* ranked first in terms of vectorial capacity followed by *An. minimus* [13]. These two species complemented one another to maintain the transmission from forest reservoirs to communities living in forest fringes. *An. dirus* is sylvatic, mainly exophilic and exophagic but it enters the house to feed on man and leaves soon after [14]. On the other hand, *An. minimus* is anthropophilic, endophilic and endophagic forest fringe species. Previous vector control measures relied mainly on insecticide residual spraying using DDT [13]. In early 2000, DDT was employed in remote areas at the dosage of 2 g/m^2 twice a year while deltamethrin 5% WP was used for the house residual sprays, twice a year in perennial transmission areas. PIBNs were then introduced as a supplementary measure in areas where residual house spraying acceptance was low. In high malaria transmission areas, free nets were then and now being provided. Early on, bed nets used were impregnated with 0.3 g/ m^2 of permethrin, twice a year. The use of PIBNs spread all over malarious areas of Thailand as the major vector control measure. With observed continuous reduction of cases, use of the PIBNs was scaled up. Currently, LLINs are commercially available and have replaced conventional treated bed nets. These LLINs exhibit safety and long residual efficacy of 2-3 years or for about 20 washes [15].

It has been theoretically believed that a steep increase in the number of people protected with any insecticide-based vector control measure will likely result in increased selection pressure due to insecticides. Pyrethroid resistance genes (in *Anopheles* vectors) of various protective capacities can be expected to ascend, thus the main concern then is to detect them at an early stage [16]. Minimal selection pressure is expected with the use of PIBNs. Only the female mosquitoes with endophagic and endophilic behavior are expected to be in contact with the insecticide treated mosquito nets, halving the degree of the selection pressure compared when the selection is directed towards the larval or adult stages that may kill or select for both sexes. This study therefore was designed to evaluate in the laboratory probable development of resistance and its expression (susceptibility/resistance levels) after the intergenerational selection exposures to permethrin treated nettings. Back then, this study was expected to provide insights in the scaled-up and prolonged use of PIBNs on the resistance development in the two malaria vectors. Ideally, the study should reflect the intergenerational period for the resistance to develop while possible resistance mechanism(s) could be detected. Identification of the biochemical mechanisms will allow early detection of resistance using advanced techniques (biochemical and molecular), thereby providing information on appropriate choice of alternative vector control compounds for future use. The identification of resistance mechanisms indicator will help determine the cross-resistance spectrum and will allow mapping of areas with resistant populations.

The objectives of the study were:

- To determine whether the selection with permethrin impregnated bed net will result in tolerance or resistance in the *Anopheles* mosquito populations,
- To determine the possible after exposure effects on the adult size and
- To screen for the mechanisms of pyrethroid resistance in the two mosquito species; *Anopheles minimus* and *Anopheles dirus*.

Materials and Methods

Collection and Rearing of *Anopheles minimus* and *Anopheles dirus*

Using cattle-baited traps [17], fully engorged *Anopheles* females were collected, segregated and reared at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. Pure and established strains of *Anopheles minimus* and *Anopheles dirus* collected from Kanchanaburi, Ratchaburi, and Phrae provinces were separated for resistance induction purposes of this study. The populations were mass-produced in separate insectary

compartments to select for resistance. Mosquito colonies were maintained in the insectary under 80-90% relative humidity at 27°C. Adults were kept in cages (30 x 30 x 30 cm) with circular openings securely tied with sleeves to serve as the door in collecting adults and placing pupae for emergence. Cotton pads soaked in 10% sugar solution and 10% multivitamin solution were provided for the newly emerged mosquitoes.

Matured females (3-6 days old) were collected and transferred to separate cups for blood feeding. Fully engorged females were mated following the artificial mating technique described by Ow-Yang CK, *et al.* [18]. The mass reared populations of *An. dirus* and *An. minimus* were divided into halves, one part to be used for susceptibility testing and the other half for resistance induction. The activities and general procedures were presented in the flow charts (Figures 1 & 2). The study was conducted from June 1998 to June 2000 at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, and Bangkok, Thailand.

Test Insecticide

The insecticide used in the bed net impregnation programs in Thailand at early use of PIBNs was permethrin [13]. Permethrin belongs to the group of pyrethroids which is classified under moderately hazardous category. Permethrin has no unpleasant side effects [19,20] and has been approved and recommended by the WHO Expert Committee on bed net use [21]. Permethrin used in this study has the following specification: [(3- phenoxy phenyl) methyl 3- (2,2 dichloroethenyl)-2 dimethyl cyclopropane carboxylate] cis: trans isomeric ratio 25:75 w/v emulsifiable concentrate. Welcome Singapore Pte. Ltd.

Net Impregnation

Synthetic nylon netting material (Thai Bednets Manufacture Co. Ltd., 162 Moo 5, Tumbon Pantainorasingha, Amphur Muang, Samutsakorn, Thailand) was used based on the earlier reports that nylon nets produced high mortalities compared to its cotton alternatives and retained insecticides on the surface for a longer duration [22-24]. To determine the absorption rate of the nylon nets, a measured piece (12 x 15 cm) was dipped into a cylinder filled with a specific volume of water. After complete saturation, the net was removed and the new volume of water was recorded. The difference between the two readings was the amount of water used to dissolve the insecticide and impregnate individual pieces.

The amount/volume of insecticide formulation was calculated using the formula:

$$\text{Amount of Insecticide} = \frac{\text{Target dose}\left(\text{mg of active ingredient (ai)} / \text{m}^2\right) \text{ x area m}^2}{\text{Insecticide concentration}\left(\text{mg ai} / \text{ml}\right)}$$

Insecticide solutions were prepared by adding the calculated amount of the formulation with the required volume of water diluent. Pieces of net were soaked individually in a prescribed volume of insecticide solution in a non-absorbent plastic bag. The individual pieces were rubbed and squeezed to obtain uniform distribution of the insecticide in the nylon net. Impregnated pieces were spread over a plastic mantle indoors to dry overnight. After drying, the impregnated nets were grouped according to insecticide concentration, and were placed individually in plastic bags. The impregnated nets were labeled and were stored in opaque envelopes at room temperature to prevent insecticide degradation.

Susceptibility Testing

Baseline and regular assessments of susceptibilities were done to monitor development of resistance from the reference strain and within the generations of selected populations (Figure 1). World Health Organization (WHO) Standard Susceptibility Procedure [25] was followed to conduct susceptibility tests. Three to four replicates of 15-25 sugar fed females of each test mosquito species (*An. minimus* and *An. dirus*) were collected in individual holding tubes and were kept there for one hour prior to exposure. Afterwards mosquitoes were transferred to exposure tubes containing papers impregnated with a predetermined range of concentrations; 5 concentrations each for *An. dirus* and *An. minimus*. The mosquitoes were kept for an hour in the exposure tubes covered with black cloth. Afterwards, the mosquitoes were blown back to the holding tubes and were given sugar solution. Mortalities from each concentration were then recorded after 24 hours. There was no susceptibility test made at S_4 and S_5 of *An. minimus*. No susceptibility test was conducted at S_3, S_5, S_7, S_9 S_{10} and S_{11} due to low population turnout on *An. dirus.* Table 1 summarizes the susceptibility tests conducted throughout the laboratory inductions done for both of the species.

Selection for Resistance to PIBNs

After the determination of the baseline susceptibility levels, pieces of nylon nets were impregnated with the LC_{10} to LC_{50} of the test insecticides in preparation for resistance induction. Selection of *An. minimus* was done using WHO tube [25] method while *An. dirus* was done using WHO cone [26]. The initial plan was to determine the possible effects of the two methods in the selection. Due to difficulty in reaching the numbers enough to replicate for both methods, only the use of the above methods were performed. Insufficient numbers did not allow comparison between the two selection methods (tube vs cone). Both species were mass reared to reach population numbers sufficient to start laboratory induction (selection) and conduct baseline and

successive susceptibility tests (Figure 1 & Table 1). It took several generations (3-4) to have the induction process started. The generations (*An. minimus* and *An. dirus*) first exposed to permethrin treated nettings were considered "parent (P)" while the progenies were considered the first selected generations (S_1). Reference strains were grown parallel to the selected generations of *An. minimus* and *An. dirus*.

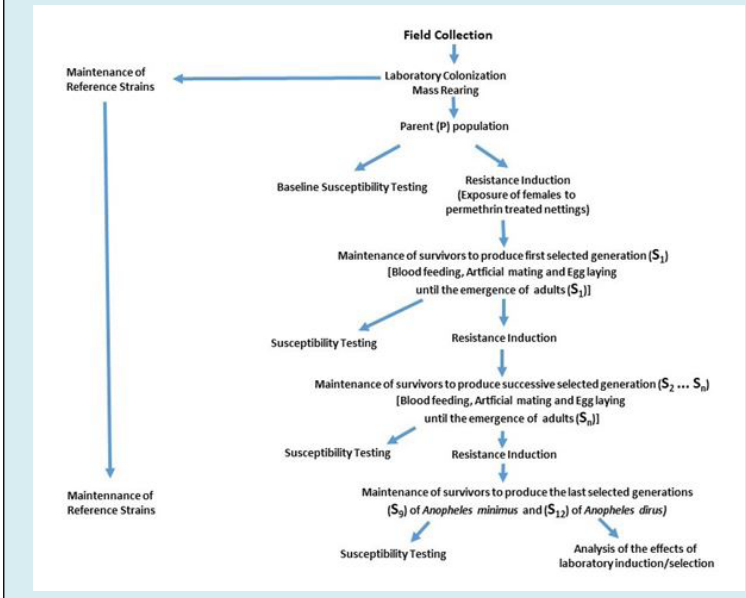

Figure 1: Laboratory induction (selection) procedures flow chart.

Laboratory Grown Generations	*Anopheles minimus* Phrae strain		*Anopheles dirus* Ratchburi strain	
	Selection Conducted	Susceptibility Tests Conducted	Selection Conducted	Susceptibility Tests Conducted
	(Yes - √)	(Yes - √)	(Yes - √)	(Yes - √)
	(No - X)	(No - X)	(No - X)	(No - X)
Parent (P)	√	√	√	√
First Selected (S_1)	√	√	√	√
S_2	√	√	√	√
S_3	√	√	X	X
S_4	X	X	√	√
S_5	X	X	√	X
S_6	√	√	√	√
S_7	√	√	√	X
S_8	√	√	√	√
S_9	X	√	√	X
S_{10}	--	--	√	X
S_{11}	--	--	√	X
S_{12}	--	--	X	√

Table 1: Summary of laboratory inductions (selections) and susceptibility tests conducted for *Anopheles minimus* and *Anopheles dirus*

For *An. minimus*, LC_{10} (20 mg-ai/m^2) was used to induce resistance for the first four generations (P, S_1 to S_3). Replicates of 25 females were exposed for 3 minutes inside WHO susceptibility tubes with treated net pieces on top of clean filter papers [25]. Survivors were grown continuously. At the start of selection, female mosquitoes were exposed to treated bed nets before giving blood meal and followed by artificial mating. However, direct exposure (selection before blood feeding) reduced the population drastically specially *An. minimus* Kanchanaburi strain. The colony was wiped out eventually after a few generations probably due to selection pressure that caused feeding inhibition (summarized in the results) and laboratory induction procedure proceeded with only *An. minimus* Phrae strain. No selection was made at the fourth (S_4) and fifth (S_5) generations due to low population of test mosquitoes. After the *An. minimus* population gained sufficient numbers at S_6, selection continued with Phrae strain. Modification to the methodology was made by feeding females first with blood and only those that were fully engorged were exposed or selected. Blood meals were given earlier to increase the strength of the population and recover more survivors after exposure, using LC_{50} (29 mg-ai/m^2) this time to increase pressure to see if it would likely result in the development of resistance. Only *An. minimus* Phrae strain was successfully maintained and selected for seven times within nine generations that was described above (Table 1).

For *An. dirus* Ratchaburi strain, blood fed females were selected using WHO bioassay test cones [26]. The cones were fastened to a piece of nylon netting material impregnated with 6.25 mg-ai/m^2 of permethrin. Replicates of 25 female three to seven days old mosquitoes were aspirated into the test cones. After three minutes, the mosquitoes were placed back on their respective cups used to initially collect each batch. After 24 hours, surviving females were given a blood meal and mated using the artificial mating technique described earlier. The same procedure was followed for each generation of selection. Continuous selection was done except at S_3 due to insufficient number of mosquitoes produced from the previous selection (Table 1) thereby completing 11 selections throughout the 12 continuous generations of *An. dirus*. Susceptibility tests were carried out starting with the parent, and succeeding selected generations: S_1, S_2, S_4, S_6, S_8 and S_{12} (Table 1).

Effects of Selection on Adult Size

A minimum of 10 females were randomly selected to compare adult size. Female mosquitoes were fasted for 12 hours and body weights were measured individually using analytical balance. After the body weight was measured, mosquitoes were anaesthetized. Wings were removed and temporarily mounted on a slide using distilled water. After the water had dried and the wing had adhered to the slide, wing lengths were measured from the axillary incision to the apical margin excluding the fringe [27].

Effects of Selection on the Activity of Detoxifying Enzymes

Progenies from the last selected generations (S_{12} for *An. dirus* and S_9 for *An. minimus*) and reference (unselected) strains were used in all the biochemical assays (mixed function oxidase and esterase assays) (Figure 2).

Mixed Function Oxidases (MFOs) Enzyme Assay

To measure the activity of monooxygenases from individual insect samples, the procedure described by Vulule J, *et al.* [28] was adopted. Individual mosquitoes were homogenized in 100ul of potassium phosphate buffer (90 mg Na_2HPO_4 and 34 mg KH_2PO_4 in 10 ml of distilled water) adjusted to pH 7.0. The homogenates were diluted with an additional 400 ul of this buffer. Activity of the homogenates from the selected strains and unexposed strain were compared.

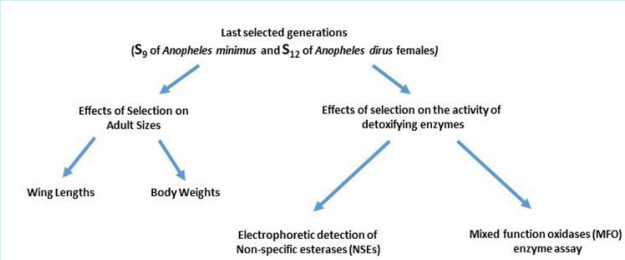

Figure 2: Diagram to determine the effects of laboratory selection on adult sizes and activities of detoxifying enzymes.

A 0.16-mol/liter solution of 3, 3', 5, 5'- tetramethyl dihydrochloride (TMBZ) was prepared by dissolving 50 mg TMBZ in 25 ml of methanol. Later this solution was diluted with 75 ml of 0.25 mol/liter sodium acetate buffer with pH 5. 200 ul of TMBZ solution was added to the 100 ul of mosquito homogenate in each well followed by 25 ul of 3.0% hydrogen peroxide. The plates were read after 10 minutes using an Immunoassay Reader under 620 nm wavelengths. Optical density (OD) readings were compiled for analysis. Association of heme peroxidase with monooxygenase levels in mosquitoes was earlier demonstrated in *Anopheles albimanus* pyrethroid resistant strain [29]. A total of 87 and 117 whole mosquito samples for the reference and selected strains respectively were used in the MFO assay.

Electrophoretic Detection Non-Specific Esterases (NSEs)

Randomly selected adult mosquito samples from the reference and last selected generations were examined using vertical polyacrylamide gel slab electrophoresis. About 30 samples were analyzed for each of the normal and selected strains for *An. dirus* while 50 samples of each strain were analyzed from *An. minimus*. The methodology published by Apiwathnasorn, *et al.* and Sucharit, *et al.* was followed [30,31]. The loci were designated based on their relative mobilities. The designation is done to allow differentiation of proteins or isoenzymes from different samples across certain localities. The process was made to facilitate characterization of resistance mechanisms. The isoenzyme with the least anodal migration was designated locus 1 and the rest followed. Comparison between the activities of the enzymes present was also done by noting the staining intensities and frequency of the occurrence of the bands. A total of 71 and 96 whole mosquito samples for the reference and selected strains respectively, were used in the NSE assay.

Data Analysis

Susceptibility Tests: The Probit Analysis program developed by Raymond M, et al. [32] was used to analyze mortality data. This computer program was based on Probit Analysis developed by Finney JD, et al. [33]. The resistance ratio (RR) was calculated by dividing the LC_{50} of the selected strain by the LC_{50} obtained from baseline susceptibility testing.

Adults Size: Body weight and wing length measurements from the reference and selected strains of both *An. minimus* and *An. dirus* were compared using Student T-test for Independent samples by SPSS 7.5. (1997 Copyright, SPSS Inc.).

MFO Assays: Values from optical density (OD) readings were compared between the reference strain and last selected generation using Student T-test for independent samples of both species of *An. minimus* and *An. dirus*.

Results

Effects of Selection on Susceptibility

Anopheles minimus: Preliminary experiments showed variations in the effects of exposure on *An. minimus* Kanchanaburi strain to pyrethroid impregnated nets (LC_{10}) starting from the parent to the third selected generation. Briefly, based on probit analysis of the baseline susceptibility tests, the expected mortality when exposed to LC_{10} was 8.11% to 15.10% after 24 hours. However, the three-minute exposure to PIBNs affected blood feeding of the females. Only 23.60 to 41.67% fed fully; 19.25 to 43.57 % fed partially while 8.85 to 33.54 % did not feed at all (data not shown). Such that an additional mortality of 2.07 to 13.66 % was recorded after 48 hours. The normal strain showed 81.44% of fully engorged females. This value is considerably high compared to all the values obtained from exposed females starting from the parent to the third selected generation. Mosquito exposure to PIBNs decreased drastically the selected population. Continuous selection was only done in *An. minimus* Phrae strain. Other collections from Ratchaburi and Kanchanaburi failed to thrive under laboratory selection conditions. Seven selections made within nine generations of continuous colonization of *An. minimus* Phrae strain showed only slight development of tolerance (1.56 folds) at the last selected generation (S_9) (Tables 1& 2, Figure 3). Initial selections (S_1 to S_2) decreased susceptibility very slightly (RR_{50} = 1.18) but became even more susceptible at S_3. (RR_{50} = 0.87). There were no selection and susceptibility test done at S_4 and S_5 due to insufficient numbers. Following the release of selection pressure (no exposure to PIBNs) and blood feeding, the population regained almost equal susceptibility as the parent based on the LC_{50} values (RR_{50} = 1.07) calculated from S_6. The susceptibility even increased at S_7 (RR_{50} = 0.96) but started to decrease again at S_8 (RR_{50} = 1.41) and S_9 giving a final tolerance level of less than two folds (RR_{50} = 1.56). X^2 values showed that responses of the parent from the susceptibility test did not follow exactly a linear pattern which may indicate initial vigor at the start of the colonization (Figure 3). Succeeding generations of exposure however resulted in more linear responses.

Strain	LC_{50} (mg-ai/m²)	LC_{90} (mg-ai/m²)	RR_{50}	Slope ±SD	X^{2*}
Parent	28.85	45.96	-	6.34±2.18	23.41
S_1	28.15	40.01	0.98	8.39±1.19	2.03
S_2	33.99	59.21	1.18	5.32±1.09	1.59
S_3	25.14	44.01	0.87	5.27±0.87	1.1
S_6	30.81	49.42	1.07	6.25±0.97	5.06
S_7	27.79	50.54	0.96	4.94±0.86	0.32
S_8	40.8	78.86	1.41	4.48±1.21	10.26
S_9	45.05	78.82	1.56	5.28±0.76	5.22

* X^2> 10 not well represented by a line

Table 2: Probit mortality data from parent and selected generations of *Anopheles minimus*. Data were analyzed by Probit Analysis program Raymond M, *et al.* [32].

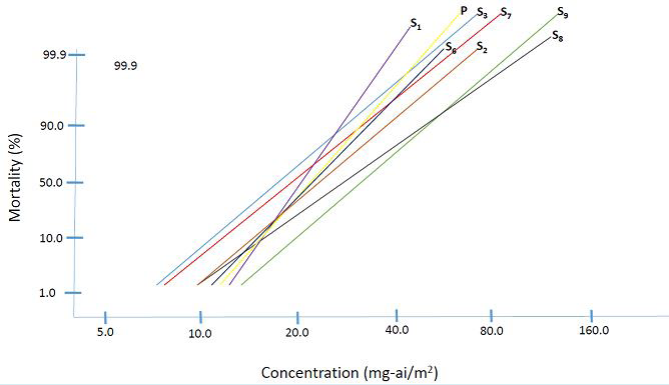

Figure 3: Probit lines derived from the parent (reference) and succeeding selected generations (S_1 to S_9) of *Anopheles minimus*.

***Anopheles dirus*:** Eleven generations of selection (except at S_3) in 12 generations of colonization of *An dirus* Ratchaburi strain resulted in low level resistance of 4.88 folds at the last selected generation (S_{12}). Parental strain gave an LC_{50} of 11.39 mg-ai/m^2 and LC_{90} of 68.53 mg-ai/m^2. Parental exposure until S_2 showed decreased susceptibility based on the resistance ratio calculated from LC_{50}. Absence of selection pressure at S_3 due to insufficient number of mosquitoes reverted susceptibility to a level almost equal to the parent strain (RR_{50} = 1.51) (Table 3 & Figure 4).

Strain	LC_{50} (mg-ai/m^2)	LC_{90} (mg-ai/m^2)	RR_{50}	Slope ±SD	X^{2*}
Parent	11.39	68.53	_	1.64±0.35	27.92
S_1	17.22	191.36	1.51	1.23±0.45	16.58
S_2	20.16	221.98	1.77	1.23±0.50	39.13
S_4	17.22	60.94	1.51	2.34±0.55	12.63
S_6	14.87	96.42	1.31	1.58±0.51	33.92
S_8	24.63	397.57	2.16	1.06±0.59	47.71
S_{12}	55.55	922.1	4.88	1.05±0.16	5.05

* X^2> 10 not well represented by a line

Table 3: Probit mortality data from parent and selected generations of *Anopheles dirus*. Data analyzed by Probit Analysis program Raymond M, et al. [32].

Continuous selection until S_6 showed decreased susceptibility at nearly equal level as S_2. However, at S_8, tolerance started to appear as indicated by a two-fold resistance ratio and continued increasing until S_{12}. There was no susceptibility test conducted at S_3, S_5, S_7, S_9 S_{10} and S_{11} of *An. dirus* due to low population turn out (Tables 1 & 3). Starting with the parent generation, the colony showed consistently variable response to permethrin as indicated by high X^2 values obtained. X^2 values higher than 10 indicated that mortality data do not exactly follow a linear pattern. Peak variation occurred at S_6 (X^2 = 33.92) and S_8 (X^2 = 47.71). Linear response was however obtained at S_{12} (X^2 = 5.05) which may indicate that the population is starting to develop a true resistance trait. The probit lines showed flat slopes (Figure 4).

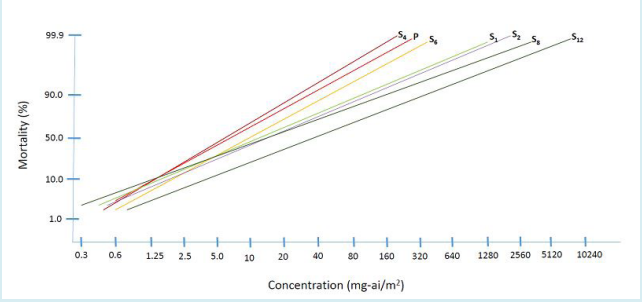

Figure 4: Probit lines derived from the parent (reference) and succeeding selected generations (S_1 to S_{12}) of *Anopheles dirus*.

Effects of Selection on Adult Size

Anopheles minimus. Results showed that seven times of selection for resistance in nine generations of *An. minimus* do not show any significant effect on both body weight and wing length parameters ($p>0.05$). Student T-test for unequal variances showed that there is no significant difference between the mean body weights of the reference (0.95 ±0.03 mg) and S_9 (1.04 ±0.05mg) females. Same statistical test showed no significant difference between wing lengths of reference (2.18± 0.01mm) and S_9 (2.43± 0.02 mm) females.

Anopheles dirus. Varying results were obtained from the two parameters used to compare adult size of *An dirus*. Student T-test for unequal variances showed that body weight was significantly increased at S_{12} (1.40±0.30 mg) when compared to the reference (1.30 ±0.30 mg) ($p<0.05$). However, Student T-test for equal variances showed no significant difference between the wing lengths of S_{12} (3.55 ±0.01mm) and reference (3.52 ±0.03mm) strains.

Effects of Selection on the Activity of Detoxifying Enzymes

Mixed Function Oxidases (MFOs) Enzyme Assay

Anopheles minimus. Selection for permethrin resistance resulted in increased activity of mixed function oxidase enzymes at S_9. Activity of MFOs after 10 minutes (T_{10}) recorded an average 0.3384±0.02 and 0.4283±0.02 OD values for reference and selected strains; respectively ($p<0.05$).

Anopheles dirus. Selection for permethrin resistance resulted in significant increase in the activity of mixed function oxidase enzymes at S_{12}. After 10 minutes, recorded OD values were 0.22±0.01 and 0.35±0.01 for reference and selected strains, respectively ($p<0.05$).

Electrophoretic Detection Non-Specific Esterases (NSEs)

Anopheles minimus. Electrophoretic results showed similar banding patterns for both the reference and selected strains (Figure. 5). The electrophoretic pattern showed presence of five loci producing non-specific esterase enzymes. Loci 1, 2, and 5 were all monomorphic with two bands (alleles) while locus 4 was observed to have only one band. Locus no. 3 showed some specific variations of one or two bands. Locus no.3 also showed visibly higher staining intensity compared to other loci present. No visible difference, however, can be observed in the overall banding pattern from the five loci between reference and selected (S_9) strains.

Anopheles dirus. Both the reference and selected strains showed five loci for NSEs (Figure 6). Loci 1, 2 and 4 were monomorphic with a single band. Locus no.5 showed the presence of two bands (alleles). There was no noticeable difference in staining intensities from loci 1, 2, 4 and 5 between the reference and selected strains indicating presence of enzymes almost at the same level. Slightly lower staining intensities of the bands at locus no. 3 of the selected strains may indicate lesser production of enzymes as a result of selection. This may also suggest that selection pressure is directed towards other mechanisms and may indicate non-involvement of non-specific esterases.

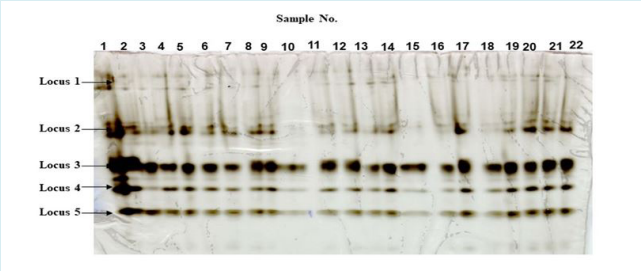

Figure 5: Non-specific esterases (alpha-napthyl acetate) banding patterns of the 4th instar larvae (sample at lane no. 1), reference (samples 2-11) and selected- S_9 (sample 12-22) adult females of *Anopheles minimus* on polyacrylamide gel.

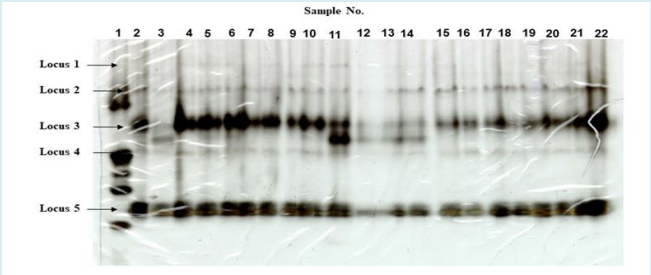

Figure 6: Non-specific esterases (alpha-napthyl acetate) banding patterns of the 4th instar larvae (sample at lane no. 1), reference (samples 2-11) and selected- S_{12} (sample 12-22) adult females of *Anopheles dirus* on polyacrylamide gel.

Discussion

Effects of Selection on Susceptibility

Preliminary selection exposures showed that three minutes of exposure to low concentration (20 mg-ai/m^2) of permethrin resulted in partial female engorgement or total inability to feed the survivors of *An. minimus* (Kanchanaburi strain) causing failure of the colony. Feeding

inhibition, deterrence and irritation have been shown to be a major mechanism as to how the use of PIBNs contributes to disease reduction [13,19,23,34]. Additionally, altered feeding may result in shorter life span, lower fecundity, irregular and lengthened oviposition cycle of mosquito vectors in communities using PIBNs [35,36]. This was presented to provide support and further explanation to the published findings regarding the use of PIBNs. The results showed innate toxicity of permethrin towards *An. minimus* as compared to *An. dirus*. (Figures 3 & 4) *An. minimus* showed steeper slopes ranging from 4.48 to 8.39 indicating greater increase in mortality for every ten-fold increase in the logarithmic concentration of permethrin. Flatter slope on the other hand was observed from *An. dirus* ranging from 1 to 2.34. Theoretically, the population composed of almost entirely susceptible genotypes will produce lines at its steepest. Selection of populations with heterozygous genotypes however will show probit lines decreasing in slope as it moves rightward [37]. This could probably explain variable response and flatter slopes calculated from the probit regression lines of *An. dirus*.

Results showed that several generations of selection by exposing females alone are needed to induce low level resistance in *An. dirus* or reduce susceptibility in the case of *An. minimus*. The experiment showed unique responses of different species to the selection which means individual studies must be made and cannot be directly extrapolated between different species. These findings agree with most laboratory selection experiments undertaken. Laboratory induction experiments of exposing only females of *An. maculatus* and *Aedes aegypti* showed only slight development of tolerance after several generations of selection [38]. Pyrethroids however were simultaneously used for both crop protection and public health purposes. The wide scale and continued use of insecticides for both purposes has posed intensive selection pressure on *Anopheles* vectors. This has resulted in the high levels of deltamethrin resistance in *An. sinensis* populations from China and Korea [39-42]. Resistance induction in the larval stage or adult stage exposing both sexes on the other hand showed considerable success. These successful resistance induction experiments done in the laboratory preceded discoveries of resistance in field populations of both anopheline and culicine mosquitoes [13,43,44]. In virtually all of the above-cited cases, dosage-mortality line moves to the right in successive generations and consequently LC_{50} levels increase, depicting similar trends as in *An. minimus* and *An. dirus*.

Effects of Selection on Adult Size

No significant effect was observed on adult sizes of the progenies of selected *An. minimus* (S_9) and reference strains. Varying results however were observed from *An. dirus* with body weight significantly increased at S_{12} compared to the reference strain while no significant difference was observed between the wing length measurements of reference and selected (S_{12}) strains. Theoretically, adult size is primarily determined by larval nutrition and environmental conditions. With a standardized procedure, changes may occur as a functional adaptation; probably as part of resistance development. Body size can also be associated with survival and dispersal capabilities of some mosquito species [45]. Further work must be done to determine how the change in size due to insecticide selection may affect fitness of the vectors.

Effects of Selection on the Activity of Detoxifying Enzymes

Mixed Function Oxidases: Results show that there is a significant increase in mixed function oxidase activity for both species of *An. minimus* and *An. dirus*. *An. dirus* showed low level resistance of 4.88 folds while *An. minimus* only showed reduced susceptibility (1.56 folds). Selection exposures to permethrin may have resulted in the increased production of the MFOs but further studies are needed to determine if this mechanism contributes to the development of resistance. MFOs have been reported as probable resistance mechanisms of *An. darlingi* for deltamethrin and DDT [46] as well as contributing resistance mechanisms for *Anopheles* as *An. gambiae* [47] and other *Aedes* vectors as well [48].

Nonspecific Esterases

Non-specific esterases (NSEs) have been reported to have evolved as one mechanism of pyrethroid resistance in *An. albimanus* from Mexico [49]. This study revealed that there is no significant difference in the patterns and visible staining intensities of the bands obtained from polyacrylamide electrophoresis of non-specific esterases for *An. minimus*. However for *An. dirus*, slightly lower staining intensity was observed from locus no.3 of the selected strain. Lower staining intensity indicates reduced amount of enzyme which may or may not be linked to permethrin selection. Further studies are needed for a more conclusive outcome. Like MFOs, NSEs have been implicated as resistance mechanisms of *Anopheles* [46,47,49] and *Aedes* vectors [45,48] to several classes of insecticides.

Conclusion

Seven selections made within nine generations of *An. minimus* Phrae strain resulted in reduced susceptibility (1.56 folds) at the last selected generation (S_9). Eleven selections made within 12 generations of *An. dirus* resulted in the development of low-level resistance of 4.88 folds at the last selected generation (S_{12}). Permethrin selection significantly

increased *An. dirus* adult size based on the body weights but not in terms of wing lengths. Significant increase in the levels of the mixed function oxidases (MFOs) enzymes were both detected from selected strains of *An. minimus* and *An. dirus*. Increased MFOs may be responsible for the reduced susceptibility of *An. minimus* and low-level resistance of *An. dirus*. Further studies should be conducted to characterize resistance development using molecular biology tools to better predict the manifestation. Certainty on the presence of resistance genes will facilitate determination of the degree and expression of resistant phenotype in filial generations through laboratory induction approximating probable appearance in the field populations.

The limitation of the study includes use of laboratory grown population implying limited population gene pool when compared to the actual wild populations of the species. It is also recommended that WHO impregnated papers be used in the susceptibility tests to ensure adherence and comparability to the standards.

Conflict of interest: The authors declare that they have no conflict of interests.

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