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Insecticide susceptibility and resistance development in malathion selected *Aedes albopictus* (Skuse)

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Abstract. *Aedes albopictus* was bioassayed to determine resistance development to malathion (OP). Two methods were applied, including WHO larval bioassay to determine the susceptibility to lethal concentration (LC), and adult bioassay to determine lethal time (LT). Larvae from colonies that had undergone selection pressure with malathion to yield 50% mortality were further subjected to selection for subsequent 10 generations. Selection of *Ae. albopictus* with malathion could relatively induce a consistent resistance ratio of 1.0 throughout 10 generations. It was noted that *Ae. albopictus* larvae showed less susceptibility to malathion compared to adults. The susceptibility test of adult mosquitoes to diagnostic dosage of 5.0% malathion-impregnated paper showed a variety of susceptibility to malathion when compared to the susceptible strain. Bioassay results indicated that the LT_{50} values of malathion-selected *Ae. albopictus* ranged between 11.5 – 58.8 minutes for ten consecutive generations. Biochemical enzyme studies indicated that there was a significant difference ($p < 0.05$) in esterase level in malathion-selected mosquitoes compared to non-selected control. Electrophoretic patterns of non-specific esterases at different life stages in malathion-selected *Ae. albopictus* suggested that non-specific esterases do not play a role in resistance of malathion-selected *Ae. albopictus*.

INTRODUCTION

The Asian tiger mosquito, *Aedes albopictus* is native to the tropical and subtropical areas of Southeast Asia. In the past couple of decades this species has invaded many countries throughout the World. It is well known throughout the World because of its vectorial role in Chikungunya and dengue outbreaks. There are about 434 species of mosquitoes in Malaysia belonging to 20 genera. In situations where the role of mosquitoes as vectors of threatening disease is minimal, their status as a nuisance is still prevalent (Yap *et al.*, 1997). The control of this vector relies largely on the use of chemicals which include organochlorine, organophosphate, carbamate compounds and recently the synthetic pyrethroids (Davidson & Herath, 1981; WHO, 1984). However, long term use of insecticides can lead to development of resistance. The World Health Organization (WHO) defines *resistance* as "the development of an ability in a strain of an organism to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species" (WHO, 1957). The development of mosquito resistance to chemical insecticides makes the control of mosquitoes and hence the diseases more difficult (Vythilingam *et al.*, 1992). The emergence of insecticide

resistance in these vectors has necessitated the development of resistance detection techniques (Lee *et al.*, 1992). Towards this end, standard resistance test kits were produced by the World Health Organization (WHO, 1981). These tests, though are easy to use especially with the inclusion of diagnostic dosages, are often time-consuming, requiring a large number of mosquitoes and limited number of insecticides or impregnated papers for testings (Lee & Tadano, 1994). The present WHO standardized bioassay which is based on insect survivorship following exposure to an insecticide, has been widely used for the past 2 decades and this test gives an indication of development and trends of resistance. However, several short-comings of the technique have prompted the development of biochemical assay methods. Biochemical techniques are essentially based on detection and qualitative analysis of enzymes known to be responsible for resistance. The importance of electrophoretic studies is to be able to demonstrate the esterase enzyme band patterns of mosquitoes. Chen & Sudderudin (1987) suggested that the level of insecticide tolerance was found correlated to the number of esterase bands.

This study describes the rate of resistance development to the insecticide malathion (OP) in *Ae. albopictus* in the presence of selection pressure. Furthermore, the study was conducted to verify level of non-specific esterase activity in resistant strain and to identify the presence of non-specific esterase bands at different developmental stages and in different sexes of *Ae. albopictus*. Such knowledge is essential in defining future control strategies against this medically important mosquito.

MATERIALS & METHODS

Mosquitoes and insecticides

Adult *Ae. albopictus* were bred in the Insectarium of Division of Medical Entomology, IMR and maintained in the rearing cages (23cm x 23cm x 23cm) at temperature 27 ± 2°C and RH 80% with a photoperiod of 14 hour of artificial daylight and 10 hour of darkness. The subsequent five generations of larval stage were subjected to selection pressure. To compare the resistance level of the resistant strains of *Ae. albopictus*, laboratory bred Jinjang strain reared for over 30 years was used as a standard susceptible strain. This strain has not been exposed to any insecticide or biological control agent. Malathion 93.3% a.i. (Cynamide) was used in this study. The insecticides used in the adult susceptibility test were diagnostic dosages of WHO impregnated papers malathion 5.0% was purchased from Vector Control Research Unit, Penang, Malaysia.

WHO larval bioassay

This test was conducted according to WHO (1981) larval susceptibility bioassay procedure. Twenty-five early fourth instar larvae were used for the larval bioassay test. The bioassay test was carried out in disposable paper cups of 300ml capacity. Stock solution of the insecticide was prepared as for malathion 2,500 mg/L. The insecticide consisted of five different concentrations in three replicates with ascending volume and three controls without insecticide. After introducing the larvae into paper cup, 100 ml water was added to make the final volume as 250ml.Larval mortality was recorded after 24 hours of exposure. Moribund larvae if any were counted as dead

Selection pressure test for mosquito larvae

The larval stages were subjected to selection pressure against malathion at every five generations (thousands of late fourth instars larvae were treated in 1 liter capacity beaker together with the larvae that survived from larval bioassay test) to the concentration which yield 50% mortality (LC_{50} in 24 hours) and the surviving larvae were reared to the next generation from the adults that emerged.

WHO adult bioassay

The female adults from malathion resistant *Ae. albopictus* mosquitoes was used in the test. Fifteen 10% sucrose fed females less than seven days old from each of the strains in four replicates and two controls were used. A diagnostic test using standard WHO Test Kits tube (2 cm x 4 cm) was conducted by means of tarsal exposure to papers impregnated with malathion 5.0%. Exposed mosquitoes were covered with black cloth to make sure they would be resting on the impregnated paper. Cumulative mortality was recorded after every 5 minutes for test insecticides with their respective exposure period was 1 hour for malathion. Mosquitoes that survived the exposure period were then transferred to holding tubes to observe the effect of post-treatment and mortality was recorded after 24 hours of recovery period. Cotton pads soaked in 10% sugar solution were provided during the 24 hours holding period. Controls were exposed to non-treated paper. All mosquitoes were exposed for 1 hr.

Biochemical enzyme determination microassay

The level of non-specific esterases present in life stages of *Ae. albopictus* was determined using biochemical microplate assay. Esterase assay was conducted as described by Brogdon (1987) and Lee (1990 & 1992). Individual mosquitoes of different life stages (egg/ L1/ L2/ L3/ P/ adult female/ adult male) was homogenized in 100 µl of 0.02M / 250 ml potassium phosphate buffer (pH 7.4) and further diluted with 400 µl buffer. The homogenate was centrifuged at 14,000 rpm for 10 min at 4ºC. Aliquots of fifty micro liter was transferred into a microplate well, where each individual sample followed by 50 µl substrate of α-naphthyl acetate (0.06g/ 10 ml acetone/ 500 µl buffer). Thereafter, 50 µl coupling agent 0.075g Fast Blue + 0.875g SDS in 50 ml of distilled water was added for colour indication. The test plate was incubated for 10 minutes at room temperature (27 ± 2ºC). The colour intensity result was expressed quantitatively as an absorbance (O.D.) at 450 nm using enzyme microassay reader – Dynatec MR5000 (Selvi *et al.*, 2007).

Preparation of enzyme extracts and electrophoresis

For the study of changes in esterase patterns during development, eggs, larvae, pupae and adults (separated by sex) were each collected at different life stages. Native PAGE was conducted using 30% polyacrylamide gels with 0.5 cm wells, contained within Bio-Rad Protean minigel system (Richmond, CA). Each samples (n = 15) was ground in 100 µl PBS (pH 7.4). The homogenates was centrifuged for 10 min at 4ºC. A volume of 50 µl of the supernatant was used as enzyme source for electrophoresis with 30% polyacrylamide gel and mixed with 5% of 15 µl xylene cyanole marker (as a dye). 14 µl of sample aliquots were loaded into each well of stacking gel. Electrophoresis was performed in electrode buffer at a constant 150 V (± 60mA) and lasted for 1.5 h in a 4ºC cold chamber. After electrophoresis, the gels were removed from electrophoresis unit and were placed in 50ml PBS for esterase activity staining for incubation period of 10 min. After that, 20 mg α-naphthyl and 20 mg β-naphthyl were dissolved in 1 ml acetone and 0.07 g Fast Blue RR salt dissolved in 3ml distilled water were added to stain the esterase enzyme and to visualize esterase bands. Approximately 15 min later, gels were removed and fixed in 10% acetic acid.

Data analysis

The bioassay data were pooled and analyzed with personal computer programmed with standard probit analysis (Raymond, 1985). Results are presented as lethal concentration i.e., LC_{50} for larval bioassay and lethal time LT_{50} for adult bioassay. Based on the LC_{50} and LT_{50} values resistance ratio (RR) was determined by the ratio of resistant strain and field strain to the ratio of susceptible strain by adopting the method of Brown & Pal (1971). Values of resistance ratio (RR) greater than 1 is an indication of resistance and values less

than or equal to 1 are considered as susceptible. In all cases, the LC50 and LT 50 values were expressed in mg/L and minutes respectively. The resistance ratio (RR-S and RR-F were determined as follows,

$$\text{Resistance Ratio (RR-S)} = \frac{\text{LC 50 /LT 50 of resistant (selection pressure) strain}}{\text{LC 50/ LT 50 of laboratory strain (susceptible)}}$$

$$\text{Resistance Ratio (RR-F)} = \frac{\text{LC 50 /LT 50 field strain}}{\text{LC 50 /LT 50 of resistant (selection pressure) strain}}$$

The following criteria was used for interpretation of adult susceptibility test as per recommended by WHO (1998),

98–100% mortality indicates susceptibility
80–97% mortality suggests the possibility of resistance that needs to be further confirmed
<80% mortality suggests resistance

The enzyme activity was calculated at O.D. 450 nm/min/mg protein. A one way analysis of variance (ANOVA) was used to compare the enzyme expression levels between life stages of insecticide selected strain to susceptible strain. All levels of statistical significance were determined at $p < 0.05$. The percentage of frequency absorbance histograms plotted for non-specific esterase activity of α–naphthyl acetate at different life stages (subsequently 1st, 5th and 10th generations) of selected populations against absorbance 450 nm expressed as µmol/min/mg protein. Individuals with esterase levels above that threshold are less susceptible. The upper range limit at 450 nm is determined as 0.20. This becomes the resistance threshold. The resistance threshold for upper range limit was determined based on the intensity of final esterase colour and its direct link to the absorbance value (Selvi, 2009). For analysis of band patterns the gels were photographed using Image kit digital (Alpha 2200), monitor com (Dell E771p), printer (Sony UDDB 1395) for further analysis and documentation (Selvi 2009).

RESULTS

Larval bioassay
Bioassay results of LC_{50} (mg/L) values in ten subsequent generations of *Ae. albopictus* exposed continuously to malathion for 24 hours is presented in Table 1. After subjection to selection pressure with malathion for 10 generations, it was found that malathion could induce a consistent resistance ratio of approximately 1.0 throughout mosquito generations.

The field strain appeared to be less susceptible to malathion with the LC_{50} value of 0.1332 mg/L compared to the selected strains. The higher RR was observed, for instance the F1 generation had a RR-S value of 1.3 versus RR-F of 2.8 as indicated in Table 1. Malathion resistant strain has the highest level of resistance, measured as LC_{50} value of 1.233mg/L at 6th generation. After intense selection for ten generations, resistance ratio was shown with the values ranging from 0.5 to 1.2.

Adult bioassay for malathion
The susceptibility test of adult mosquitoes to diagnostic concentration (5.0%) of malathion impregnated paper showed a potential resistance development at LT_{50} ranging from 11.5 to 58.8 minutes (Table 2). High levels of RR-S and RR-F was observed from F3 – 3.0 fold and F5 – 4.7 fold respectively. The resistance ratio after ten generations of selection pressure decreased from 1.7 to 1.2 folds of resistance compared with the susceptible strain (Table 2). It was worth noting that the resistance measured as LT_{50} value fluctuated and was inconsistent throughout the selected generations. Nevertheless, as shown in Table 2 after 10 generations of selection, the malathion-selected strain had shown no remarkable change in the percentage of 24 hours adult post-exposure mortality, consistently at the rate of 86.7 – 100%. Mean LT_{50} value exhibited 29.36 ± 4.74 minutes and mean RR-S was lower i.e.

Table 1. LC_{50} (mg/L) values in insecticide test of early fourth instar larvae of laboratory and resistant strains of *Aedes albopictus* of 10 subsequent generation exposed continuously for 24 hours to malathion

MALATHION					
Species/Strain *Aedes albopictus*	Generation	LC_{50} (mg/L) 95% (C.L)	Regression line	Resistance Ratio (RR-S)	Resistance Ratio (RR-F)
Susceptible	F19	0.1042 (0.0966 – 0.1148)	Y = 4.78x – 38.11	–	–
Field	F1	0.1332 (0.1280 – 0.1375)	Y = 9.77x – 84.13	1.3	–
Resistant	F1	0.0472 (0.0431 – 0.0720)	Y = 16.00x – 133.71	0.5	2.8
Resistant	F2	**	–	–	–
Resistant	F3	0.0843 (0.0685 – 0.1121)	Y = 9.16x – 76.75	0.8	1.6
Resistant	F4	0.1173 (0.0968 – 0.6714)	Y = 7.05x – 58.95	1.1	1.1
Resistant	F5	0.1074 (0.1024 – 0.1200)	Y = 13.83x – 119.91	1.0	1.2
Resistant	F6	1.233 (0.1147 – 0.1159)	Y = 13.21x – 115.3	1.2	1.1
Resistant	F7	0.1135 (0.1098 – 0.1162)	Y = 13.05x – 113.12	1.1	1.2
Resistant	F8	0.1164 (0.1115 – 0.1192)	Y = 12.60x – 109.25	1.1	1.1
Resistant	F9	0.1163 (0.1129 – 0.1186)	Y = 16.00x – 140.09	1.1	1.1
Resistant	F10	0.1250 (0.1215 – 0.1286)	Y = 31.69x – 283.15	1.2	1.0
Mean ± S.E. for LC 50 (R)		0.11 ± 0.01	–	1.0	1.2

** - cannot compute by probit
RR - S: Resistance ratio to susceptible strain
RR - F: Resistance ratio to field strain

1.6 fold compared to mean RR-F 2.3 fold. As shown in Table 2, malathion selected *Ae. albopictus* indicated moderate level of resistance development to malathion diagnostic dosage, throughout the selected generations, with the mean 24 hours post-exposure mortality of 95.14 ± 1.81%.

24 hours post-exposure treatment

Malathion insecticide concentration used in this study killed the susceptible strain at a maximal rate. All the malathion-selected strain at different generations died at a faster rate, within 45 minutes of exposure time (Figure 1). It was noted that there was no significant difference ($p > 0.05$, $F = 0.28$) in the mean 24 hours post-exposure treatment throughout the selected generations (Table 2). The same concentration of malathion could kill field strain at minimal rate, within 1 hour of exposure time, of 57% of the mosquitoes and no significant difference ($p > 0.05$, $F = 7.38$) was observed (Table 2).

Table 2. LT_{50}(min) values and 24 hours post-exposure mortality of malathion resistant *Aedes albopictus* adult female mosquitoes of 10 subsequent generation exposed against WHO diagnostic dosage of malathion 5.0%

MALATHION 5.0% (1 HOUR OF EXPOSURE TIME)						
Species/Strain *Aedes albopictus*	Generation	LT_{50} (minute) 95%(C.L)	Regression line	Resistance Ratio (RR-S)	Resistance Ratio (RR-F)	24 hours post-exposure mortality (%)
Susceptible	F10	21.9 (21.3 – 22.5)	Y = 9.76x – 105.72	–	–	100[a]
Field	F1	53.8 (50.5 – 58.7)	Y = 5.11x – 54.96	2.5	–	80[b]
Resistant	F1	37.3 (34.3 – 40.2)	Y = 5.65x – 60.36	1.7	1.4	86.7[b]
Susceptible	F11	15.2 (11.3 – 20.6)	Y = 6.00x – 62.11	–	–	98.3[a]
Resistant	F2	47.1 (44.0 – 50.4)	Y = 7.84x – 86.5	3.1	1.1	90[b]
Susceptible	F12	19.3 (16.2 – 22.9)	Y = 9.57x – 102.97	–	–	98.3[a]
Resistant	F3	58.8 (51.4 – 70.8)	Y = 2.31x – 22.20	3.0	0.9	88[b]
Susceptible	F13	15.9 (15.2 – 16.6)	Y = 7.33x – 77.12	–	–	100[a]
Resistant	F4	35.0 (32.5 – 37.7)	Y = 3.3x – 33.1	2.2	1.5	90[b]
Susceptible	F14	19.0 (18.5 – 19.5)	Y = 10.72x – 115.90	–	–	97.5[b]
Resistant	F5	11.5 (10.4 – 12.4)	Y = 5.06x – 50.95	0.6	4.7	100[a]
Susceptible	F15	18.9 (18.2 – 19.7)	Y = 5.10x – 52.49	–	–	98.3[a]
Resistant	F6	22.0 (19.7 – 24.5)	Y = 5.82x – 60.99	1.2	2.4	96.7[b]
Susceptible	F16	24.7 (24.1 – 25.4)	Y = 12.99x – 142.98	–	–	98.3[a]
Resistant	F7	16.7 (15.6 – 17.8)	Y = 6.40x – 66.78	0.7	3.2	100[a]
Susceptible	F17	18.3 (17.9 – 19.9)	Y = 9.03x – 96.68	–	–	100[a]
Resistant	F8	18.9 (17.9 – 19.9)	Y = 8.33x – 88.89	1.0	2.8	100[a]
Susceptible	F18	15.8 (15.4 – 16.2)	Y = 8.47x – 89.82	–	–	100[a]
Resistant	F9	26.5 (22.8 – 30.8)	Y = 4.45x – 45.82	1.7	2.0	100[a]
Susceptible	F19	16.7 (16.2 – 17.2)	Y = 7.16x – 75.37	–	–	100[a]
Resistant	F10	19.8 (18.7 – 20.8)	Y = 7.39x – 78.49	1.2	2.7	100[a]
Mean ± S.E. for LT 50 (R)		29.36 ± 4.74	–	1.6	2.3	95.14 ±1.81[b]

RR - S : Resistance ratio to susceptible strain
RR - F : Resistance ratio to field strain

%[a] : susceptible
%[b] : moderately resistant
%[c] : resistant

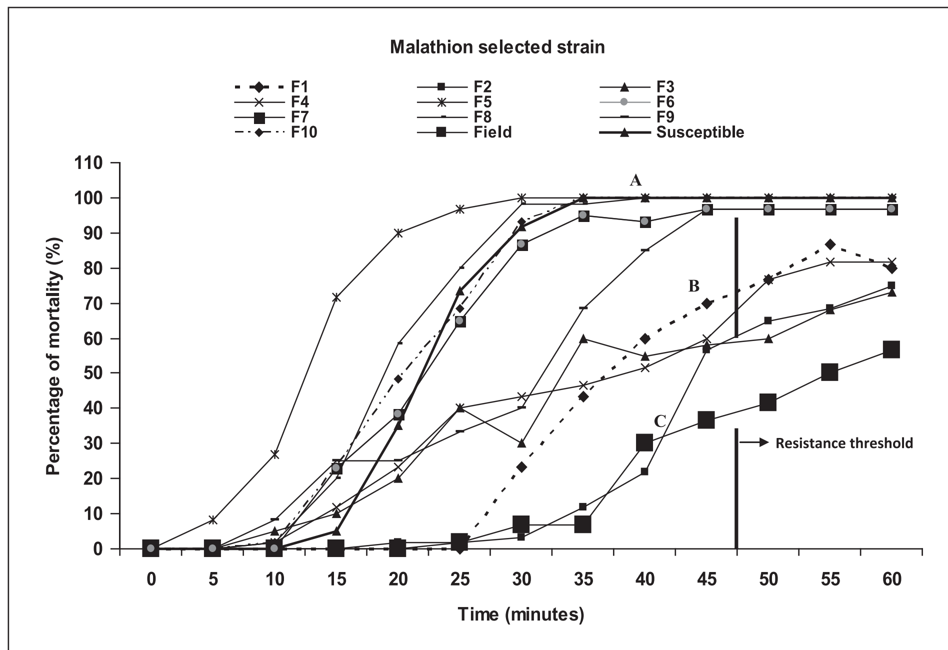

Figure 1. Percent mortality and resistance threshold for *Ae. albopictus* malathion selected strain exposed to diagnostic dosage of 5.0% malathion for 1 hour

A – malathion insecticide concentration killed all the **susceptible strain** at a maximal rate
B – all the **malathion selected strain** at different generations died at a faster rate within 45 minutes of exposure time
C – malathion insecticide concentration killed **field strain** at minimal rate within 1 hour of exposure time to kill 57% of the mosquitoes

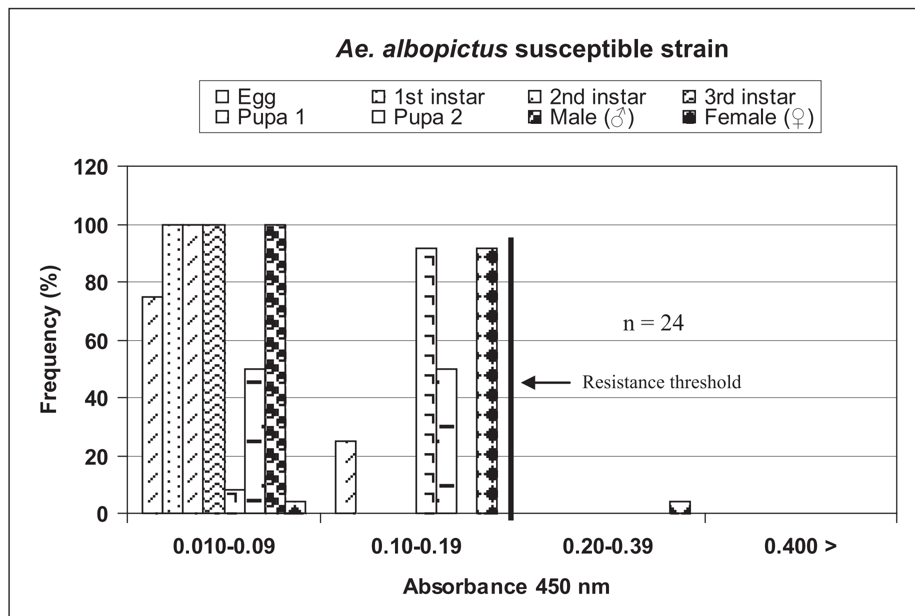

Figure 2. Non-specific esterase O.D. value and resistance threshold in life stages for *Ae. albopictus* susceptible strain at absorbance 450 nm

Table 3. Optical density (OD) of non-specific esterases towards developmental stages of *Ae. albopictus* susceptible control and malathion selected five subsequent test populations against α-naphthyl acetate

Life stages	Mean ± SD (α – Na µ mol / min/mg protein	Resistance ratio (RR-S)	Resistance ratio (RR-F)	Life stages	Mean ± SD (α – Na µ mol / min/mg protein	Resistance ratio (RR-S)	Resistance ratio (RR-F)
	Ae. albopictus Susceptible Strain				Ae. albopictus (F 3)		
Egg	0.34 ± 0.14 [a] (4)	-	-	1st instar	0.08 ± 0.00 [a] (24)	1.14	1.14
1st instar	0.07 ± 0.00 [a] (24)	-	-	2nd instar	0.08 ± 0.00 [a] (24)	1.00	1.33
2nd instar	0.08 ± 0.00 [a] (24)	-	-	3rd instar	0.13 ± 0.03 [a] (24)	1.08	1.08
3rd instar	0.12 ± 0.01 [a] (24)	-	-	Pupa 1	0.12 ± 0.02 [a] (24)	0.80	1.00
Pupa 1	0.15 ± 0.02 [a] (24)	-	-	Pupa 2	0.11 ± 0.01 [a] (24)	0.58	0.65
Pupa 2	0.19 ± 0.04 [a] (24)	-	-	Male (♂)	0.12 ± 0.02 [a] (24)	0.75	1.09
Male (♂)	0.16 ± 0.02 [a] (24)	-	-	Female (♀)	0.14 ± 0.02 [a] (24)	1.00	0.88
Female (♀)	0.14 ± 0.03 [a] (24)	-	-				
	Ae. albopictus Field Strain				Ae. albopictus (F 4)		
Egg	0.29 ± 0.10 [a] (4)	0.85	-	1st instar	0.08 ± 0.00 [a] (24)	1.00	1.33
1st instar	0.07 ± 0.01 [a] (24)	1.00	-	2nd instar	0.11 ± 0.03 [a] (24)	0.92	0.92
2nd instar	0.06 ± 0.00 [a] (24)	0.75	-	3rd instar	0.15 ± 0.02 [a] (24)	1.00	1.25
3rd instar	0.12 ± 0.03 [a] (24)	1.00	-	Pupa 1	0.08 ± 0.00 [a] (24)	0.42	0.47
Pupa 1	0.12 ± 0.03 [a] (24)	0.80	-	Pupa 2	0.08 ± 0.00 [a] (24)	0.50	0.73
Pupa 2	0.17 ± 0.04 [a] (24)	0.89	-	Male (♂)	0.08 ± 0.00 [a] (24)	0.57	0.50
Male (♂)	0.11 ± 0.01 [a] (24)	0.69	-	Female (♀)	0.06 ± 0.00 [a] (24)	0.86	0.86
Female (♀)	0.16 ± 0.02 [a] (24)	1.14	-				
	Ae. albopictus (F 1)				Ae. albopictus (F 5)		
Egg	0.18 ± 0.04 [a] (4)	0.53	0.62	Egg	0.23 ± 0.01 [a] (4)	0.68	0.79
1st instar	0.07 ± 0.00 [a] (24)	1.00	1.00	1st instar	0.07 ± 0.00 [a] (24)	1.00	1.00
2nd instar	0.08 ± 0.00 [a] (24)	1.00	1.33	2nd instar	0.08 ± 0.00 [a] (24)	1.00	1.33
3rd instar	0.13 ± 0.03 [a] (24)	1.08	1.08	3rd instar	0.11 ± 0.03 [a] (24)	0.92	0.92
Pupa 1	0.12 ± 0.02 [a] (24)	0.80	1.00	Pupa 1	0.12 ± 0.02 [a] (24)	0.80	1.00
Pupa 2	0.16 ± 0.04 [a] (24)	0.84	0.94	Pupa 2	0.11 ± 0.02 [a] (24)	0.58	0.65
Male (♂)	0.09 ± 0.01 [a] (24)	0.56	0.82	Male (♂)	0.08 ± 0.01 [a] (24)	0.50	0.73
Female (♀)	0.11 ± 0.01 [a] (24)	0.79	0.69	Female (♀)	0.10 ± 0.01 [a] (24)	0.71	0.63
	Ae. albopictus (F 2)				Ae. albopictus (F 6)		
1st instar	0.07 ± 0.00 [a] (24)	1.00	1.00	1st instar	0.08 ± 0.01 [a] (24)	1.14	1.14
2nd instar	0.08 ± 0.00 [a] (24)	1.00	1.33	2nd instar	0.09 ± 0.01 [a] (24)	1.13	1.50
3rd instar	0.13 ± 0.03 [a] (24)	1.08	1.08	3rd instar	0.11 ± 0.04 [a] (24)	0.92	0.92
Pupa 1	0.15 ± 0.02 [a] (24)	1.0	1.25	Pupa 1	0.13 ± 0.02 [a] (24)	0.87	1.08
Pupa 2	0.12 ± 0.02 [a] (24)	0.63	0.71	Pupa 2	0.12 ± 0.01 [a] (24)	0.63	0.71
Male (♂)	0.08 ± 0.00 [a] (24)	0.50	0.73	Male (♂)	0.09 ± 0.01 [a] (24)	0.56	0.82
Female (♀)	0.11 ± 0.01 [a] (24)	0.79	0.69	Female (♀)	0.11 ± 0.01 [a] (24)	0.79	0.69

[a] denotes significant difference at 0.05 level of probability
(n) = sample size in parenthesis
(RR-S) = resistance ratio in comparison to laboratory strain
(RR-F) = resistance ratio in comparison to field strain

Biochemical enzyme microassay

The results of non-specific esterases enzyme assay on the malathion-selected mosquito is presented in Figure 2. Biochemical assays indicated that there was a significant difference ($p < 0.05$) in esterase level upon malathion selection, as demonstrated in Table 3. Results of Table 3 and Figures 2-6 showed that the selected strains exhibited heterogeneously susceptible status at all life stages, where there was also presence of some non-specific esterase activity in less than 50% of the population at life stages of larvae, pupae and females. Upon comparison of the esterase profile to the susceptible populations, non-specific esterase activities at all life stages were below the resistance threshold < 0.20 with the mean esterase activity of 0.07 – 0.34 α-Na µmol/min/mg protein. However in egg stage, 50% of the population indicated presence of

Table 3: Continued…

Life stages	Mean ± SD (α – Na µ mol / min/mg protein	Resistance ratio (RR-S)	Resistance ratio (RR-F)
	Ae. albopictus (F 7)		
1st instar	0.08 ± 0.00 [a] (24)	1.14	1.14
2nd instar	0.10 ± 0.01 [a] (24)	1.25	1.67
3rd instar	0.13 ± 0.03 [a] (24)	1.08	1.08
Pupa 1	0.12 ± 0.01 [a] (24)	0.80	1.00
Pupa 2	0.11 ± 0.01 [a] (24)	0.58	0.65
Male (♂)	0.11 ± 0.01 [a] (24)	0.69	1.00
Female (♀)	0.13 ± 0.01 [a] (24)	0.93	0.81
	Ae. albopictus (F 8)		
1st instar	0.09 ± 0.01 [a] (24)	1.29	1.29
2nd instar	0.09 ± 0.00 [a] (24)	1.13	1.50
3rd instar	0.12 ± 0.02 [a] (24)	1.00	1.00
Pupa 1	0.11 ± 0.02 [a] (24)	0.73	0.92
Pupa 2	0.12 ± 0.02 [a] (24)	0.63	0.71
Male (♂)	0.08 ± 0.01 [a] (24)	0.50	0.73
Female (♀)	0.07 ± 0.01 [a] (24)	0.50	0.44
	Ae. albopictus (F 9)		
1st instar	0.09 ± 0.00 [a] (24)	1.29	1.29
2nd instar	0.11 ± 0.02 [a] (24)	1.38	1.83
3rd instar	0.11 ± 0.04 [a] (24)	0.92	0.92
Pupa 1	0.11 ± 0.03 [a] (24)	0.73	0.92
Pupa 2	0.15 ± 0.03 [a] (24)	0.79	0.88
Male (♂)	0.08 ± 0.00 [a] (24)	0.50	0.73
Female (♀)	0.10 ± 0.01 [a] (24)	0.71	0.63
	Ae. albopictus (F10)		
Egg	0.16 ± 0.01 [a] (4)	0.47	0.55
1st instar	0.07 ± 0.00 [a] (24)	1.00	1.00
2nd instar	0.11 ± 0.03 [a] (24)	1.38	1.83
3rd instar	0.19 ± 0.04 [a] (24)	1.58	1.58
Pupa 1	0.12 ± 0.04 [a] (24)	0.80	1.00
Pupa 2	0.10 ± 0.01 [a] (24)	0.52	0.59
Male (♂)	0.10 ± 0.01 [a] (24)	0.63	0.91
Female (♀)	0.12 ± 0.01 [a] (24)	0.86	0.75

[a] denotes significant difference at 0.05 level of probability
(n) = sample size in parenthesis
(RR-S) = resistance ratio in comparison to laboratory strain
(RR-F) = resistance ratio in comparison to field strain

esterase activity at 0.34 ± 0.14 α-Na µmol/min/mg protein (Table 3 and Figure 2). In the field collected *Ae. albopictus*, there was presence of non-specific esterase activities at egg, pupae and adult female stages, with the mean esterase activity of (0.29 ± 0.10), (0.17 ± 0.04) and (0.16 ± 0.02) µ-Na µmol /min/mg protein, respectively (Table 3 and Figure 3). The mean esterase activity did not differ greatly by life stages and there was a significant difference ($p < 0.05$) from one generation to subsequent generations although inconsistency values were observed (Table 3). In most cases, there was no significant differences between the esterase activity of males and females.

The RR(S) of the elevated levels of non-specific esterases activity in malathion-selected mosquito in comparison to susceptible strain was in the range of 0.59 – 1.88 folds. There was no distinct variation in RR(F) compared to RR(S) in mean esterase activity in selected strain. Moreover, there was no significant correlation ($r = 0.012$, $p > 0.05$) between LC_{50} values to total non-specific mean esterase activities in the larvae of malathion selected *Ae. albopictus*, nor adult females.

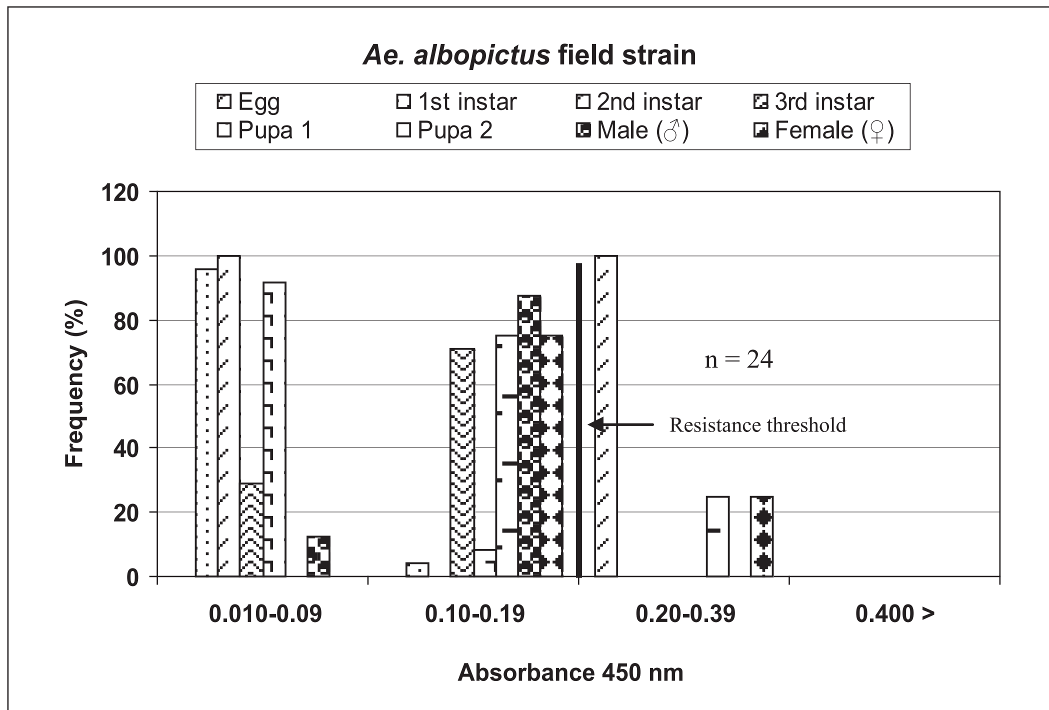

Figure 3. Non-specific esterase O.D. value and resistance threshold in life stages for *Ae. albopictus* field strain at absorbance 450 nm

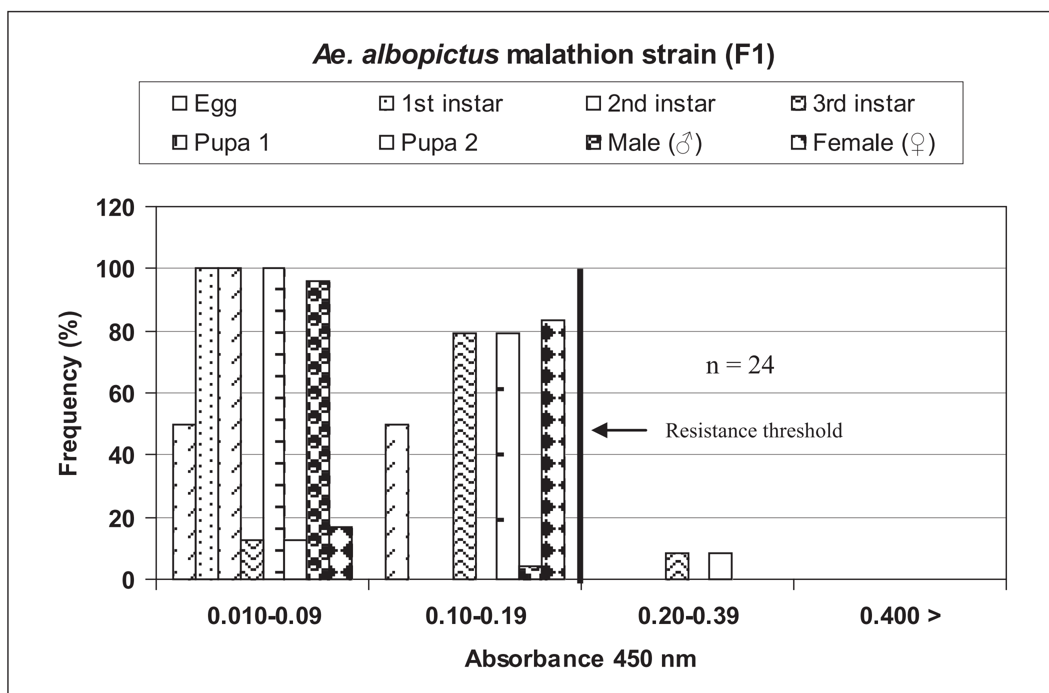

Figure 4. Non-specific esterase O.D. value and resistance threshold in life stages for *Ae. albopictus* malathion selected strain (F1) at absorbance 450 nm

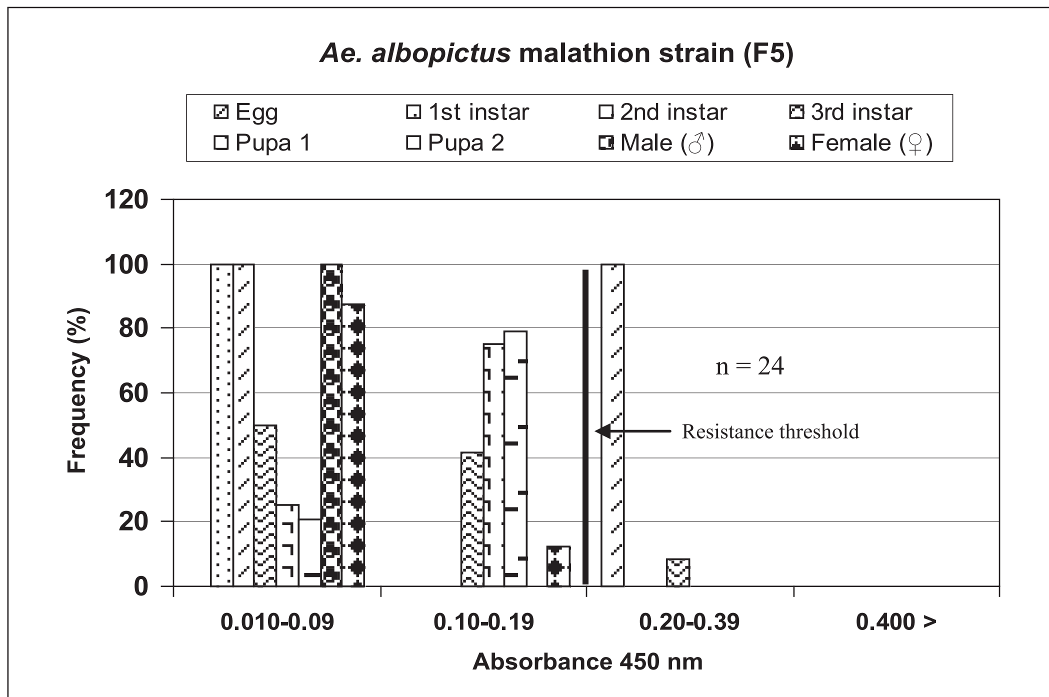

Figure 5. Non-specific esterase O.D. value and resistance threshold in life stages for *Ae. albopictus* malathion selected strain (F5) at absorbance 450 nm

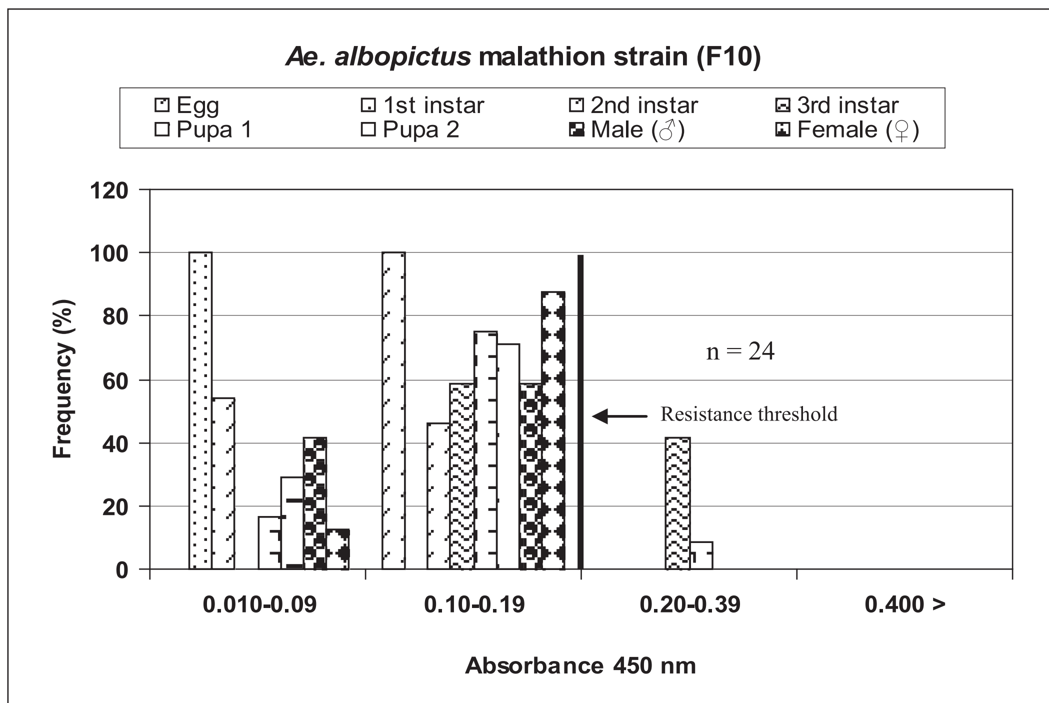

Figure 6. Non-specific esterase O.D. value and resistance threshold in life stages for *Ae. albopictus* malathion selected strain (F10) at absorbance 450 nm

Electrophoretic patterns of non-specific esterases in *Ae. albopictus*

Non-specific esterase enzyme activity was assessed at different life stages (egg, 1st instar larva {L1}, 2nd instar larva{L2}, 3rd instar larva{L3}, Pupa stage 1{P1}, Pupa stage 2{P2}, Male & Female). The non-specific esterase banding pattern of *Ae. albopictus* was characterized using α- and β- naphthyl acetate. Each number of esterase bands varied depending on the strains and sex and these bands were designated as E1, E2, E3, E4 and E5. Figure 7 shows electrophoretic patterns of esterases with clear bands at different life stages in malathion resistant strains of *Ae. albopictus*. Distinctive differences in the electrophoretic patterns were not observed when major developmental stages of mosquitoes were compared to eggs, larvae, pupae and adults. The banding pattern was not consistent within each major stage as compared to different generations (Figures 7–8). These banding patterns were summarized into a table to indicate presence of bands with different level of esterase and in different life stages, based on esterase activity rating (Tables 4 – 6). *Aedes albopictus* malathion-resistant strain exhibited E1, E2 at L3 stage, and at P1 and P2 exhibited E2 and E4. Males exhibited only E3 band and females exhibited E2 and

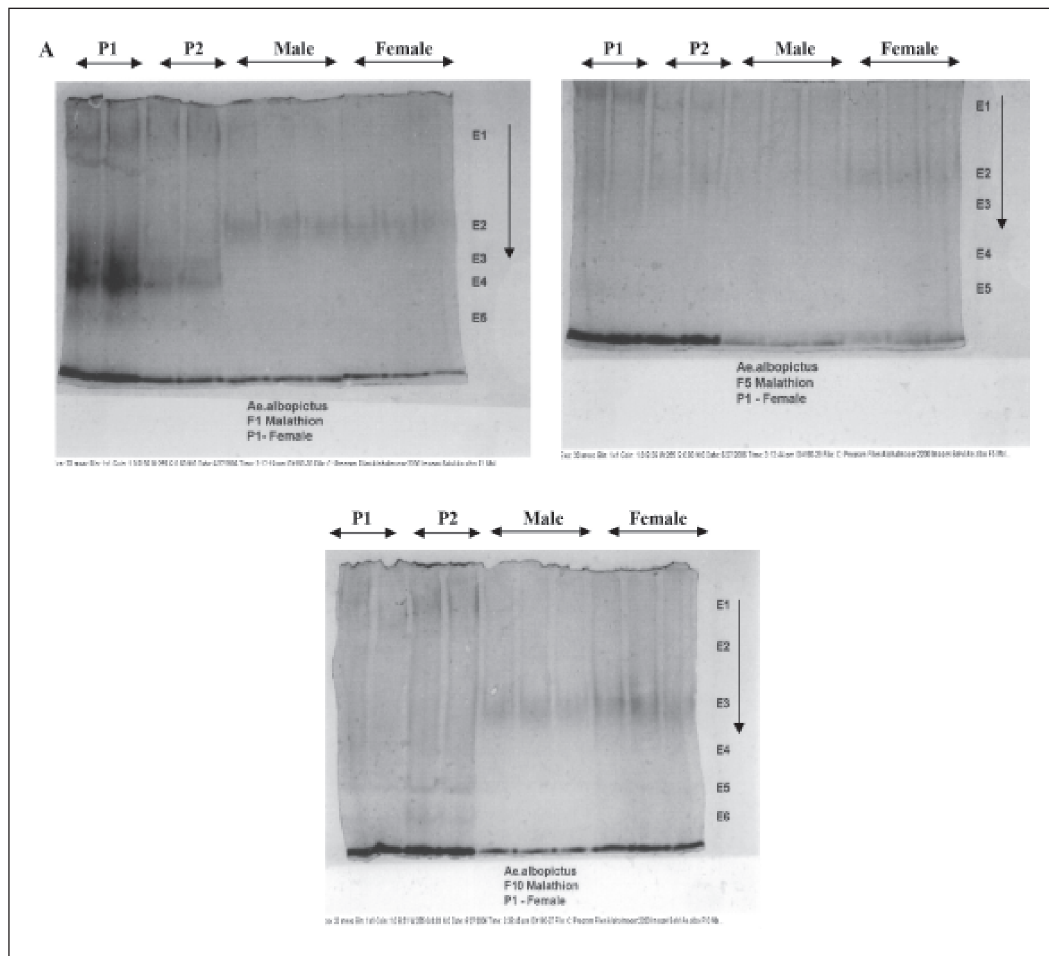

Figure 7. Non-specific esterase isoenzymes of *Aedes albopictus* of malathion resistant strains by 1st (F1), 5th (F5) and 10th (F10) generations separated according to the life stages by native polyacrylamide gel electrophoresis

A – Pupa 1 (lanes 1-2), Pupa 2 (lanes 3-4), Male (lanes 5-7), Female (lanes 8-10)

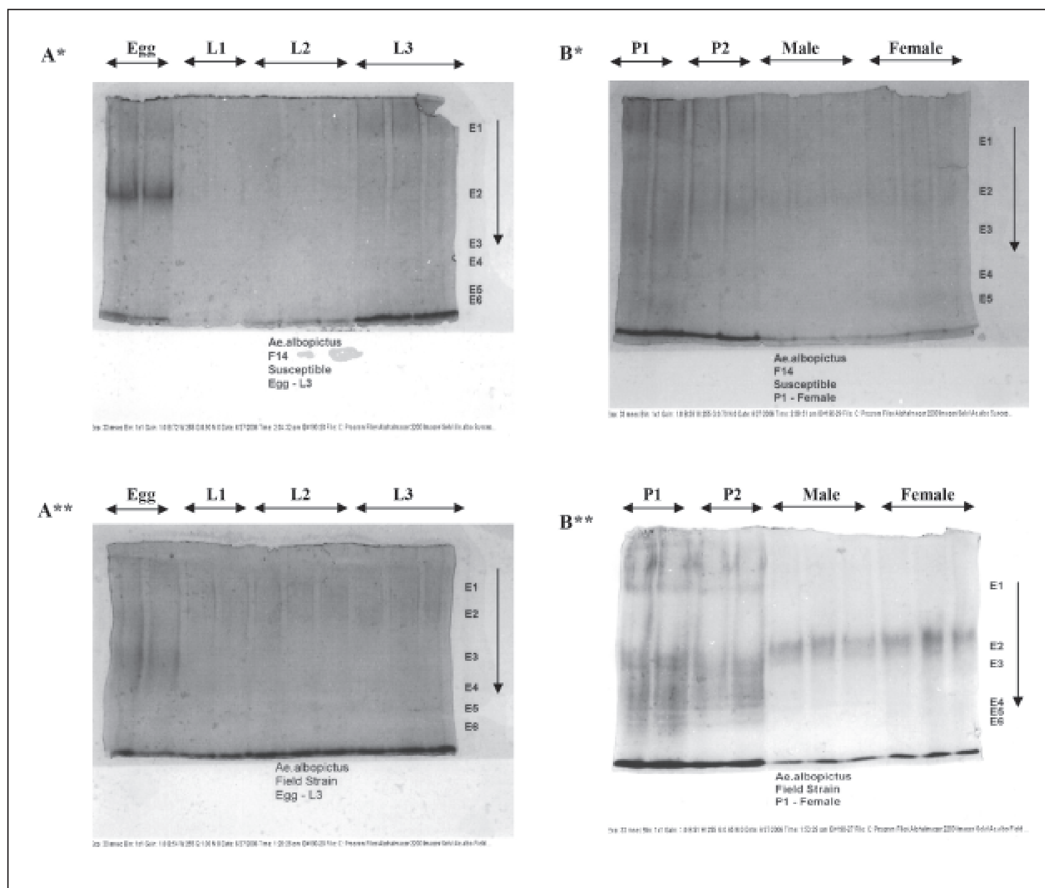

Figure 8. Non-specific esterase isoenzymes of *Aedes albopictus* of susceptible strain F14 and field strain separated according to the life stages by native polyacrylamide gel electrophoresis

A* & A** – Egg (lanes 1-2), 1st instar (lanes 3-4), 2nd instar (lanes 5-7), 3rd instar (lanes 8-10)
B* & B** – Pupa 1 (lanes 1-2), Pupa 2 (lanes 3-4), Male (lanes 5-7), Female (lanes 8-10)

Table 4. Summary of non-specific esterase activity bands in *Ae. albopictus* malathion resistant strain

Esterase Bands	Egg	L1	L2	L3	P1	P2	♂	♀
E1				+	+	+		
E2				+			+	+
E3								
E4					+	+		
E5					+	+		

Table 5. Summary of non-specific esterase activity bands in *Ae. albopictus* suceptible strain

Esterase Bands	Egg	L1	L2	L3	P1	P2	♂	♀
E1					+	+		
E2	++				+	+	+	+
E3								
E4								
E5							+	+

Esterase activity rating, + = low
++ = medium
+++ = very high

Table 6. Summary of non-specific esterase activity bands in *Ae. albopictus* field strain

Esterase Bands	Egg	L1	L2	L3	P1	P2	♂	♀
E1				+	+	+		
E2				+			+	+
E3	+							
E4					+	+		
E5					+	+		

Esterase activity rating, + = low
++ = medium
+++ = very high

E3 bands. No bands were observed in eggs, L1 and L2 stages. There was no noticeable major common band observed in *Ae. albopictus* malathion-selected strain. All the bands were lightly stained and this indicating that non-specific esterase may not play a role in malathion resistance in *Ae. albopictus*.

Susceptible strain exhibited different patterns of electrophoretic bands, and there was absence of band at L3 stage (Figure 8). The E1 and E2 bands were detected at P1 and P2 stages, while the adult males and females similarly exhibited E2 and E4 esterase bands, and the moderately stained E2 band appeared in egg stage. Field collected *Ae. albopictus* did not exhibit intense esterase band in any developmental stages (Figure 8) and the band pattern was different from all other strain tested in this study. Collectively E1 and E2 bands were detected at L3 stage, E2 and E4 in P1 and P2 stages, E3 in male adults, and both E2 and E3 bands in female adults.

DISCUSSION

There was no marked difference in the resistance pattern to malathion resistant strain, as demonstrated by the LC_{50} values, and this strain was resistant at a low rate. All the larvae exhibited a significant decline in the resistance after subjection to selection pressure for a few generations. It was not clear why variation on the LC_{50} values was found and probably this could be contributed by heterozygous genes in the population which caused quick dilution of resistant genotypes resulting in the decline of resistance level (Selvi *et al.*, 2006).

Based on LC_{50} values, *Ae. albopictus* selected strain and field strain generally appeared susceptible to malathion. As far as is known, the malathion selected strain is originally collected from the field and had been exposed to laboratory rearing for 20 generations before selection pressure was applied for testing. On the other hand, the field strain has a high probability of previous exposure to insecticides in the field and may therefore be expected to exhibit tolerance for malathion as observed in larval bioassay and adult bioassay. It may be noted that the RR-S and RR-F however did not differ by more than 4.6 folds.

Certain trends are evident from the *Ae. albopictus* adult susceptibility where it was moderately resistant to malathion. Variation exists between geographic strains in response to insecticide application (Wesson, 1990). In none of the strains did the LT_{50} approach to the diagnostic dosage that the World Health Organization (1992) recommended. A quick perusal of the data from the present study indicates that malathion is less toxic at diagnostic dasage against *Ae. albopictus*. In contrast, Rohani *et al.* (2001) reported that *Ae. albopictus* from Kuala Lumpur was highly resistant to permethrin. However, the strain from Kelantan and Johor showed moderate resistance to permethrin. Rohani *et al.* (1998) also found multiple resistance to both permethrin and DDT in an urban strain of *Ae. albopictus* in Kuala Lumpur city.

Results of the non-specific esterase enzyme studies had exhibited heterogeneous susceptible status at all life stages in this mosquito. However, it did not show high variation in the mean esterase activity. Therefore, inconsistencies in absorbance values at different life stages may be attributed to other enzyme mechanisms or other resistance enzymes (Lee, 1990).

Laboratory strains are known to be generally pure for resistance, all the individuals being homogenous for resistance allele, but resistant field population almost invariably contain some heterogeneous and the susceptible alleles are always infiltrating from surrounding untreated areas (Georghiou, 1980). Initial scoring of the results into resistant and susceptible categories by eye before spectrophotometric readings are taken will avoid any problem of mis-classification of heterozygotes as susceptible. However, it was not possible to differentiate by eyes between heterozygous individuals with high activity and homozygous-resistant ones with low activity (Hemingway, 1986).

In addition, *Ae. albopictus* field collected strain exhibited presence of non-specific esterase activity at certain life stages with no great variation compared to laboratory susceptible strain. The enzyme activity in the field strain could reflect local history of insecticide employment. A study conducted by Nazni (2000) reported enzyme microassay of esterases and oxidases in laboratory and field strains of *Ae. albopictus*, and showed that field strains had higher levels of esterases activities, that is 2.4 X compared to laboratory strain. In this study, non-specific esterases may not be involved in resistance at different life stages of *Ae. albopictus*, there could be other detoxification enzyme involved in conferring malathion resistance in *Ae. albopictus* such as insensitive AChE.

The esterase bands varied with no clear trends of greater esterase activity and it was apparent from visual observation in this study that there was no distinguishable occurrence of bands at different life stages (Selvi, 2009), reflecting absence of inheritance characteristic band patterns upon insecticide selection. The results thus showed that non-specific esterases do not play a role in malathion resistance, neither influence resistance development at different life stages.

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