Tropical Biomedicine 24(1): 63–75 (2007)

Characterization on malathion and permethrin resistance by bioassays and the variation of esterase activity with the life stages of the mosquito *Culex quinquefasciatus*

Selvi, S.[1], Edah, M.A.[1], Nazni, W.A.[2], Lee, H.L.[2] and Azahari, A.H.[2]
[1] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur
[2] Entomology Division, Infectious Diseases Research Centre (IDRC), Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur
E-mail: selvi_subramaniam@yahoo.com
Received 2 February 2007; received in revised form 5 March 2007; accepted 7 March 2007

Abstract. Larvae and adults of *Culex quinquefasciatus* were used for the test undertaken for malathion resistant strain (F61 – F65) and permethrin resistant strain (F54 – F58). The results showed that the LC50 for both malathion (F61 – F65) and permethrin (F54 – F58) resistant *Cx. quinquefasciatus* increased steadily throughout the subsequent five generations, indicating a marked development of resistance. The adult female malathion resistant strain have developed a high resistance level to malathion diagnostic dosage with a resistance ratio of 9.3 to 17.9 folds of resistance compared with the susceptible *Cx. quinquefasciatus*. Permethrin resistance ratio remained as 1.0 folds of resistance at every generation. It was obvious that malathion resistance developed at a higher rate in adult females compared to permethrin. Enzyme-based metabolic mechanisms of insecticide resistance were investigated based on the biochemical assay principle. From the results obtained obviously shows that there is a significant difference ($p<0.05$) in esterase level in both malathion and permethrin selected strains. Female malathion selected strain has the higher level of esterase activity compared to the female permethrin selected strain at (0.8 to 1.04) α-Na µmol/min/mg protein versus (0.15 to 0.24) α-Na µmol/min/mg protein respectively. This indicated increased level of non-specific esterase is playing an important role in resistance mechanism in female malathion selected strain. Permethrin selected strain exhibited non-specific esterase activity at a very low level throughout the different life stages compared to malathion selected strain. This study suggests that life stages play a predominant role in conferring malathion and permethrin resistance in *Cx. quinquefasciatus*.

INTRODUCTION

Culex quinquefasciatus is one of the most common mosquitoes found in human habitations in the Tropics and Subtropics of the world. Throughout most of its range the female feeds intensely and actively only at night and it causes nuisance (Richard & David, 1959) and are vectors of urban filariasis and Japanese encephalitis. Resistance to insecticide has appeared in the major insect vector of every genus. In 1992 it has been documented the list of insecticide resistant vector species included 56 anopheline and 39 culicine mosquitoes (WHO, 1992). These documentations were mostly done via the conventional detection methods using World Health Organization (WHO) standard testing procedure mainly based on susceptibility tests which are dosage-mortality (bioassay) experiments done in the laboratory (Lee *et al*., 1996).

Since insecticides, particularly organophosphate (OP), pyrethroid and carbamate are still an integral part of vector management strategies, evaluation of vector management programs must regularly be done to determine the rate at which they are contributing or enhancing resistance

development (Brown & Brogdon, 1987). Insecticide resistance are more likely to be associated with biochemical basis of resistance and even small genetic changes can alter the target's shape, so the protein in the mosquito is no longer susceptible to the insecticide. Basically, enzyme-based resistance occurs when enhanced levels or modified activities of esterases, oxidases or glutathione S-transferase (GST) prevent the insecticide from reaching its site of action.

In *Culex* mosquitoes and aphids elevated esterases confer organophosphate resistance through gene amplification (Hemingway, 2002). Study conducted by Karunaratne & Hemingway (2001), revealed that malathion resistance in *Cx. quinquefasciatus* and *Culex tritaeniorhynchus* is linked to broad spectrum resistance to organophosphorus compounds is due to elevated levels of esterases that sequester malaoxon, but are unable to metabolize malathion. Meanwhile, hydrolysis of ester plays significant role in permethrin and deltamethrin-resistant strains of *Cx. quinquefasciatus* as evident from the esterase profiles of larvae and adult female mosquitoes revealed from the study conducted by Pillai *et al.* (1994) The Entomologist, Janet Hemingway in her verbal citation has described that the mechanisms for esterase-driven resistance have been uncovered, those that lead to the overproduction of the mono-oxygenases and gluthathione have remained more elusive. In Havana City Cuba, the resistance mechanisms for elevated non-specific esterase and altered AChE in *Cx. quinquefasciatus* is still present in relatively high frequencies despite the replacement of malathion by pyrethroid insecticides in mosquito control programme (Bisset *et al.*, 1991). It is relatively easy to monitor resistance with direct biochemical assays. In future, molecular based mechanisms study might help in screening the proteins that produce resistance and find the genes that are responsible for resistant phenotypes.

The objectives of this study are to determine the rate of resistance development to the insecticide malathion (OP) and permethrin (pyrethroid) in the presence of intense selection pressure and to identify the esterase resistance mechanism whether it is influenced by the development of the life stages of a mosquito by using biochemical analysis.

MATERIALS AND METHODS

Mosquitoes and insecticides

Adult *Cx. quinquefasciatus* were bred in the Insectarium of Division of Medical Entomology, IMR and maintained in 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 *Cx. quinquefasciatus*, laboratory bred Penang strain reared for about 30 years were 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) and permethrin 10.9% a.i. (Shell) were used in this study. The insecticides used in the adult susceptibility test were diagnostic dosages of WHO impregnated papers malathion 5.0% and permethrin 0.75% impregnated papers were purchased from Vector Control Research Unit, Penang, Malaysia.

WHO larval bioasay

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 and permethrin was 1000 mg/L. Each 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 and permethrin 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 each malathion and permethrin resistant *Cx. quinquefasciatus* mosquitoes were 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% and permethrin 0.75%. Exposed mosquitoes were covered with black cloth to make sure they would be resting on the impregnated paper. Exposure tubes with permethrin impregnated papers were laid horizontally throughout the test. Cumulative mortality was recorded after every 5 minutes for all the test insecticides with their respective exposure period which were 3 hours for malathion and 2 hours for permethrin and propoxur. 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.

Biochemical enzyme determination microassay

The level of non-specific esterases present in life stages of *Cx. quinquefasciatus* was determined using biochemical microplate assay. Esterase assay was conducted as described by Brogdon *et al.* (1988), Lee (1990) and Lee *et al.* (1992). Individual mosquitoes of different life stages (egg/ L1/ L2/ L3/ P1/ P2/ adult female/ adult male) was homogenized in 100µl 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 4° C. Aliquots of fifty micro liter was transferred into a microplate well, where each individual sample was followed by 50 µl substrate of α-naphthyl acetate (0.06g/ 10 ml acetone/ 500 µl buffer). Thereafter, 50µl 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 ± 2° C). The colour intensity result was expressed quantitatively as an absorbance (O.D.) at 450nm using enzyme microassay reader – Dynatec MR5000.

Data analysis

Lethal concentration (LC_{50}) for larvae and lethal time (LT_{50}) values for adults of each strain and insecticide was calculated using the Probit Analysis Program (Raymond, 1985). Based on the LC_{50} and LT_{50} values resistance ratio (RR) was determined by the ratio of resistant strain to the ratio of susceptible strain by adopting the method of Brown & Pal (1971). The enzyme activity was calculated at O.D. 450 nm/min/ mg protein. An 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 significant were determined at $p < 0.05$.

RESULTS

Larval bioassay

Bioassays of the five subsequent generations of malathion (F61 – F65) and permethrin (F54 – F58) are shown in Figure 1. Malathion selected strain exhibited LC_{50} values at the range between (0.8763 to 1.5788)mg/L. Meanwhile, for the permethrin selected strain LC_{50} value ranged from (0.9062 to 0.8394) mg/L throughout the five generations compared to the malathion selected strain. The results showed LC_{50} for both the strains increased steadily indicating marked development of resistance rate though it showed various susceptibility with higher and lower values of LC_{50} (mg/L) throughout the five consecutive generations.

After subjection to selection pressure with malathion and permethrin, it was found that permethrin resistance was developing significantly at a higher rate ($p < 0.05$) compared to malathion (Figure 1). Malathion and permethrin resistant strains have the highest level of resistance values, with LC_{50}: 1.578mg/L at 3rd generation and 1.8394 at 5th generation respectively. After intense selection of five generations for malathion and permethrin revealed resistance ratio 70.7 to 79.9 folds and 32.8 to 35.6 folds respectively.

Adult bioassay for malathion

The susceptibility test of adult mosquitoes to diagnostic concentration for malathion 5.0% impregnated paper showed a potential resistance development at LT_{50} ranging from 255.5 to 26010.6 minutes and the decrease of LT_{50} values in each successive generations was not consistent for malathion (Table 1). At the fourth generation (F64) it was observed that this strain has the highest level of malathion resistance. The resistance ratio after five generations of selection pressure gradually increased from 9.3 to 17.9 folds of resistance compared to the susceptible strain.

Adult bioassay for permethrin

As selection progressed, there was significant decrease at LT_{50} in permethrin selected population. Slope values of the regression line was also more steep and decreased with each successive generation. This permethrin strain more or less exhibited intense of susceptibility when compared to malathion strain with LT_{50} ranging between 20.0 to 27.3 minutes. Hence, the resistance ratio also remained unchanged for all the five selection population with 1.0 fold.

24 hours post-exposure treatment

At 24 hours recovery period, survivorship increased in malathion resistant *Cx. quinquefasciatus* strain upon exposure to 5.0% malathion with mortality rate varying from 38.3% to 68.3% (Table 1). Whereas, permethrin resistant *Cx. quinquefasciatus* strain with 0.75% diagnostic dosage showed complete 100% mortality throughout all the five selection of generations (Table 2). According to WHO if the mortality rate is below 80% it is indicative of a significant increase of resistance. Thus, the resistance intensity indicated by percentage of 24 hours post exposure mortality was greater for malathion strain compared to permethrin strain.

Biochemical enzyme microassay

The enzyme assay of non-specific esterases for malathion and permethrin is presented in Table 3 & Table 4. The mean esterase activity is expressed in µmol/min/mg protein. Interestingly, the mean value of the esterase expression increased gradually in ascending order from the lower life stages (egg, L1) to the higher life stages (adults). The results obviously showed that there is a significant increase ($p < 0.05$) in esterase levels of different life stages in both strains as compared to the laboratory strain (Table 3 & Table 4).

As shown in Table 5 & Table 6, mean non-specific esterase activity ranged from 0.65 to 0.88 and 0.28 to 0.12 α-Na µmol/min/mg protein in male *Cx. quinquefasciatus* of malathion and permethrin selected strains respectively. This indicated that esterase activity increases significantly in male malathion strain but decreases in male permethrin strain.

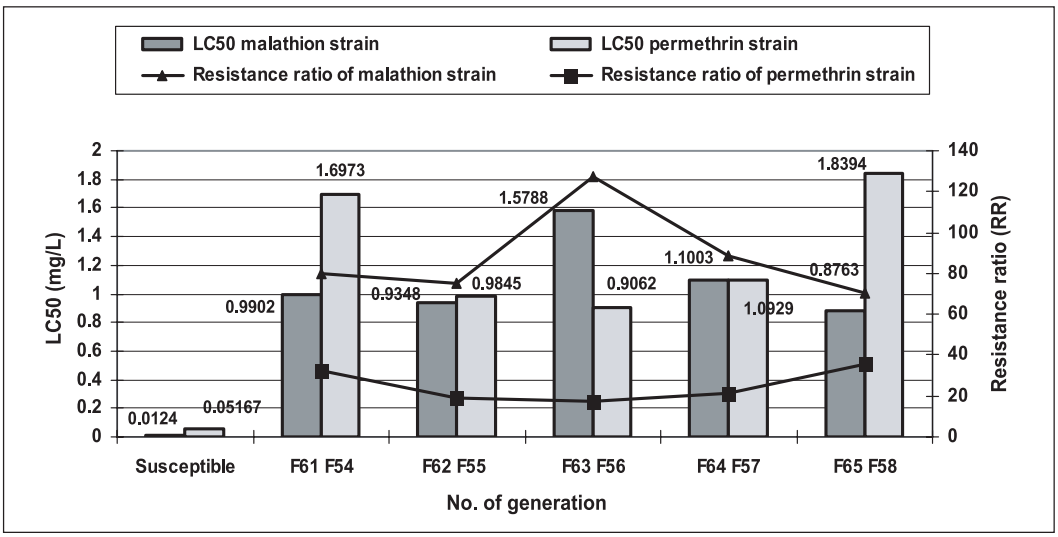

Figure 1. Comparison between LC_{50} values and resistance ratio of malathion and permethrin resistant *Culex quinquefasciatus* for larval bioassay at the five subsequent generations

Table 1. LT_{50} (min) values and 24 hours post-exposure mortality of malathion resistant *Culex quinquefasciatus* adult female mosquitoes of 10 subsequent generation exposed against WHO diagnostic dosage of malathion 5.0%

MALATHION 5.0% (3 Hours of Exposure Time)					24 hours post-exposure mortality (%)
Species/ Strain *Culex quinquefasciatus*	Generation	LT_{50} (mg/L) 95% (C.L)	Regression line	Resistance Ratio (RR-S)	
Susceptible	F 657	21.0 (20.2–21.7)	Y=5.55x – 57.80	–	100
Resistant	F 61	7557.4 (–)	Y=1.03x – 9.29	–	68.3
Susceptible	F 658	27.4 (25.9–28.9)	Y=6.54x – 69.80	–	100
Resistant	F 62	255.5 (229.5–446.4)	Y=12.14x – 145.67	9.3	45
Susceptible	F 659	31.4 (30.3–32.4)	Y=11.34x – 125.32	–	100
Resistant	F 63	1648.5 (711.6–17041.6)	Y=0.96x – 7.69	52.5	48.3
Susceptible	F 660	38.3 (37.3–39.4)	Y=12.5x – 139.73	–	98.3
Resistant	F 64	26010.6 (–)	Y=0.73x – 5.53	–	40
Susceptible	F 661	32.0 (30.5–33.2)	Y=13.11x – 145.81	–	100
Resistant	F 65	571.8 (283.4–22548960000)	Y=2.34x – 24.86	17.9	38.3
Mean ± SE for LT_{50}		7668.76 ± 4739.15	–	26.6	47.98 ± 5.38

(RR-S) = resistance ratio in comparison with laboratory strain

Table 2. LT_{50} (min) values and 24 hours post-exposure mortality of permethrin resistant *Culex quinquefasciatus* adult female mosquitoes of 10 subsequent generation exposed against WHO diagnostic dosage of permethrin 0.75%

PERMETHRIN 0.75% (2 Hours of Exposure Time)					24 hours post-exposure mortality (%)
Species/ Strain *Culex quinquefasciatus*	Generation	LT_{50} (mg/L) 95% (C.L)	Regression line	Resistance Ratio (RR-S)	
Susceptible	F 657	19.5 (18.9–20.3)	Y=8.19x – 87.47	–	100
Resistant	F 54	20.0 (18.3–21.9)	Y=5.12x – 52.88	1.0	100
Susceptible	F 658	22.6 (22.0–23.3)	Y=7.79x – 83.47	–	100
Resistant	F 55	27.3 (26.0–28.7)	Y=5.95x – 63.02	1.2	100
Susceptible	F 659	22.6 (21.9–23.3)	Y=7.64x – 81.47	–	100
Resistant	F 56	22.9 (21.9–24.0)	Y=7.05x – 75.04	1.0	100
Susceptible	F 660	23.3 (21.8–24.9)	Y=8.10x – 87.07	–	98.3
Resistant	F 57	23.7 (22.8–24.5)	Y=7.22x – 77.13	1.0	100
Susceptible	F 661	21.2 (20.5–21.8)	Y=6.68x – 70.69	–	100
Resistant	F 58	20.2 (17.2–23.7)	Y=6.48x – 68.26	1.0	100
Mean ± SE for LT_{50}	22.82 ± 1.34	–	1.0	100.00 ± 0.00	

(RR-S) = resistance ratio in comparison with laboratory strain

Obviously the same phenomenon occurred for female mosquito where the mean esterase activity increased from 0.80 to 1.04 α-Na µmol/min/mg protein at the rate of 1.3 folds and decreases from 0.24 to 0.15 α-Na µmol/min/mg protein at the rate of 1.6 folds for malathion and permethrin strains respectively.

Resistance ratio of the elevated levels of non-specific esterases in the female malathion strain were in the range between 5.71 to 7.43 folds whereas in the male malathion strain the resistance ratios were 0.65 to 0.88 folds. Meanwhile, both the female and male permethrin strains exhibited a decrease in resistance ratio from 1.71 to 1.07 folds and from 2.54 to 1.09 folds respectively throughout the five selection generations (Table 3 & Table 4).

The peak of the mean esterase activity in the larval stage was at third (L3) instar respectively 0.54 to 0.69 and 0.13 to 0.20 α-Na µmol/min/mg protein for malathion and permethrin strains. The activity of esterases increased dramatically in the pupal stage. The same pattern was observed for both the strains at first and fifth generations in comparison to the susceptible strain (Figure 2). As shown in Figure 2, the esterase activity in first generation of both malathion (F61) and pemethrin (F54) strains declined gradually in the adult stage of male and female, however it increased intensely at the fifth generation.

Generally, the correlation was observed in association to the resistance ratio of LC_{50} and total non-specific mean esterase

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

Life stages	Mean ± SD (α– Na µmol/ min/mg protein)	Resistance ratio (RR-S)
	Culex quinquefasciatus Susceptible Strain	
Egg	0.09 ± 0.01[a] (4)	–
1st instar	0.06 ± 0.01[a] (24)	–
2nd instar	0.07 ± 0.00[a] (24)	–
3rd instar	0.09 ± 0.02[a] (24)	–
Pupa 1	0.15 ± 0.02[a] (24)	–
Pupa 2	0.17 ± 0.06[a] (24)	–
Male (♂)	0.11 ± 0.02[a] (24)	–
Female (♀)	0.14 ± 0.03[a] (24)	–
	Culex quinquefasciatus (F 61)	
Egg	0.20 ± 0.01[a] (4)	2.22
1st instar	0.07 ± 0.00[a] (24)	1.17
2nd instar	0.57 ± 0.12[a] (24)	8.14
3rd instar	0.69 ± 0.10[a] (24)	7.67
Pupa 1	0.92 ± 0.07[a](24)	6.13
Pupa 2	0.93 ± 0.08[a] (24)	5.47
Male (♂)	0.65 ± 0.17[a] (24)	5.90
Female (♀)	0.80 ± 0.10[a] (24)	5.71
	Culex quinquefasciatus (F 62)	
1st instar	0.09 ± 0.02[a] (24)	1.50
2nd instar	0.19 ± 0.06[a] (24)	2.71
3rd instar	0.54 ± 0.10[a] (24)	6.00
Pupa 1	0.86 ± 0.13[a] (24)	5.73
Pupa 2	0.82 ± 0.11[a] (24)	4.82
Male (♂)	0.85 ± 0.13[a] (24)	7.73
Female (♀)	0.93 ± 0.04[a] (24)	6.64
	Culex quinquefasciatus (F 63)	
1st instar	0.10 ± 0.01[a] (24)	1.67
2nd instar	0.29 ± 0.08[a] (24)	4.14
3rd instar	0.36 ± 0.07[a] (24)	4.00
Pupa 1	1.03 ± 0.24[a] (24)	6.87
Pupa 2	0.69 ± 0.19[a] (24)	4.06
Male (♂)	0.82 ± 0.17[a] (24)	7.45
Female (♀)	1.24 ± 0.10[a] (24)	8.86
	Culex quinquefasciatus (F 64)	
1st instar	0.06 ± 0.00[a] (4)	1.00
2nd instar	0.08 ± 0.00[a] (24)	1.14
3rd instar	0.24 ± 0.11[a] (24)	2.67
Pupa 1	0.82 ± 0.77[a] (24)	5.47
Pupa 2	0.41 ± 0.27[a] (24)	2.41
Male (♂)	0.42 ± 0.19[a] (24)	3.82
Female (♀)	0.74 ± 0.14[a] (24)	5.29
	Culex quinquefasciatus (F 65)	
Egg	0.12 ± 0.01[a] (4)	1.33
1st instar	0.08 ± 0.00[a] (24)	1.33
2nd instar	0.09 ± 0.01[a] (24)	1.29
3rd instar	0.54 ± 0.10[a] (24)	6.00
Pupa 1	0.78 ± 0.13[a] (24)	5.20
Pupa 2	0.73 ± 0.16[a] (24)	4.29
Male (♂)	0.88 ± 0.17[a] (24)	8.00
Female (♀)	1.04 ± 0.11[a] (24)	7.43

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

Table 4. Mean optical density (OD) of non-specific esterases towards life stages of *Culex quinquefasciastus* susceptible control and permethrin selected five subsequent test populations against α– naphthyl acetate

Life stages	Mean ± SD (α– Na µmol/ min/mg protein)	Resistance ratio (RR-S)
	Culex quinquefasciatus Susceptible Strain	
Egg	0.09 ± 0.01[a] (4)	–
1st instar	0.06 ± 0.01[a] (24)	–
2nd instar	0.07 ± 0.00[a] (24)	–
3rd instar	0.09 ± 0.02[a] (24)	–
Pupa 1	0.15 ± 0.02[a] (24)	–
Pupa 2	0.17 ± 0.06[a] (24)	–
Male (♂)	0.11 ± 0.02[a] (24)	–
Female (♀)	0.14 ± 0.03[a] (24)	–
	Culex quinquefasciatus (F 54)	
Egg	0.10 ± 0.01 [a] (24)	1.11
1st instar	0.07 ± 0.00 [a] (24)	1.17
2nd instar	0.12 ± 0.02 [a] (24)	1.71
3rd instar	0.20 ± 0.11 [a] (24)	2.22
Pupa 1	0.32 ± 0.17 [a] (24)	2.13
Pupa 2	0.32 ± 0.16 [a] (24)	1.88
Male (♂)	0.28 ± 0.12 [a] (24)	2.54
Female (♀)	0.24 ± 0.21 [a] (24)	1.71
	Culex quinquefasciatus (F 55)	
1st instar	0.08 ± 0.00 [a] (24)	1.33
2nd instar	0.10 ± 0.15 [a] (24)	1.43
3rd instar	0.14 ± 0.10 [a] (24)	1.56
Pupa 1	0.23 ± 0.10 [a] (24)	1.53
Pupa 2	0.18 ± 0.08 [a] (24)	1.06
Male (♂)	0.19 ± 0.13 [a] (24)	1.73
Female (♀)	0.20 ± 0.18 [a] (24)	1.43
	Culex quinquefasciatus (F 56)	
1st instar	0.10 ± 0.02 [a] (24)	1.67
2nd instar	0.12 ± 0.03 [a] (24)	1.71
3rd instar	0.18 ± 0.08 [a] (24)	2.00
Pupa 1	0.27 ± 0.08 [a] (24)	1.80
Pupa 2	0.32 ± 0.16 [a] (24)	1.88
Male (♂)	0.20 ± 0.11 [a] (24)	1.82
Female (♀)	0.27 ± 0.17 [a] (24)	1.93
	Culex quinquefasciatus (F 57)	
1st instar	0.07 ± 0.00 [a] (24)	1.17
2nd instar	0.07 ± 0.01 [a] (24)	1.00
3rd instar	0.11 ± 0.03 [a] (24)	1.22
Pupa 1	0.19 ± 0.10 [a] (24)	1.27
Pupa 2	0.17 ± 0.10 [a] (24)	1.00
Male (♂)	0.16 ± 0.13 [a] (24)	1.45
Female (♀)	0.22 ± 0.16 [a] (24)	1.57
	Culex quinquefasciatus (F 58)	
Egg	0.09 ± 0.01 [a] (4)	1.00
1st instar	0.06 ± 0.00 [a] (24)	1.00
2nd instar	0.07 ± 0.01 [a] (24)	1.00
3rd instar	0.13 ± 0.06 [a] (24)	1.44
Pupa 1	0.15 ± 0.05 [a] (24)	1.00
Pupa 2	0.23 ± 0.20 [a] (24)	1.35
Male (♂)	0.12 ± 0.09 [a] (24)	1.09
Female (♀)	0.15 ± 0.05 [a] (24)	1.07

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

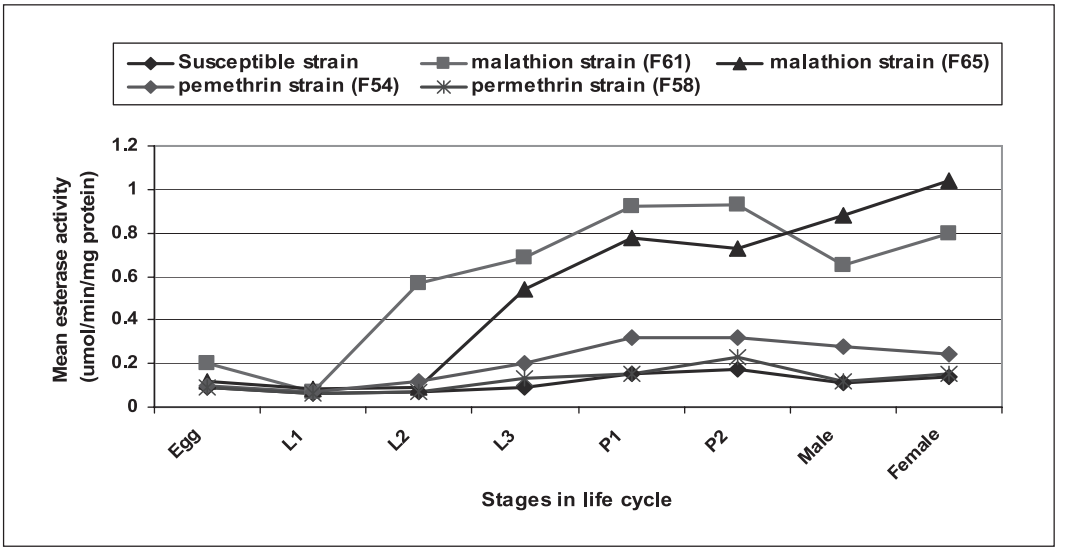

Figure 2. Esterase activity in life stages of malathion (F61, F65) and permethrin (F54, F58) selected *Culex quinquefasciatus* expressed in µmol /min/mg protein.

activity showed non-significant ($p > 0.05$) negative correlation ($r = - 0.524$, $p = 0.364$) and ($r = 0.125$, $p = 0.841$) for malathion and permethrin selected strains respectively (Figure 3 & Figure 4). There is no correlation between the LC_{50} values of malathion and permethrin and non-specific esterases in larval stage of *Cx. quinquefasciatus*. The present study also exhibited no correlation between the resistance ratios of LT_{50} and mean esterase activity in female permethrin strain. However, only in malathion strain with reference to the generation F63 the female had the highest level of non-specific esterase and this is significantly correlated well with the high LT50 value of resistance ratio of malathion insecticide at ($r = 0.960$) significant level $p < 0.05$ (Figure 4).

DISCUSSION

Both strains of malathion and permethrin selected of larvae exhibited a significant reduction in resistance towards LC_{50} values after few generation. It was not clear why such variations in LC_{50} values were found, however it can be verified by conducting biochemical test. It was obvious that selection pressure is important for maintaining resistance in the larval population (Selvi *et al.*,2005). It is likely the instability of resistance may be contributed by heterozygous population.

Adult bioassay results showed permethrin to be the most potent insecticide to produce high level of mortality rate in adults. Continuous selection pressure on malathion can cause resistance development at a higher rate in adults compared to permethrin (Selvi *et al.*, 2005). Analysis of the results of this study obviously indicate that it could be a resistant gene expression which was more active in larvae compared to adults in comparison with the resistance ratio of malathion and permethrin in 24 hours post-treatment. Similarly, study conducted by Selvi *et al.* (2006 a) against malathion and permethrin selected strains of *Aedes aegypti* showed that larval resistance ratio of LC_{50} ranged from 1.5 to 3.8 folds for malathion strain and 1.3 to 6.2 folds for permethrin strain compared to the adult resistance ratio of LT_{50} which ranged between 0.8 to 2.1 folds and 0.5 to 1.7 folds for malathion and permethrin strains respectively. Therefore,

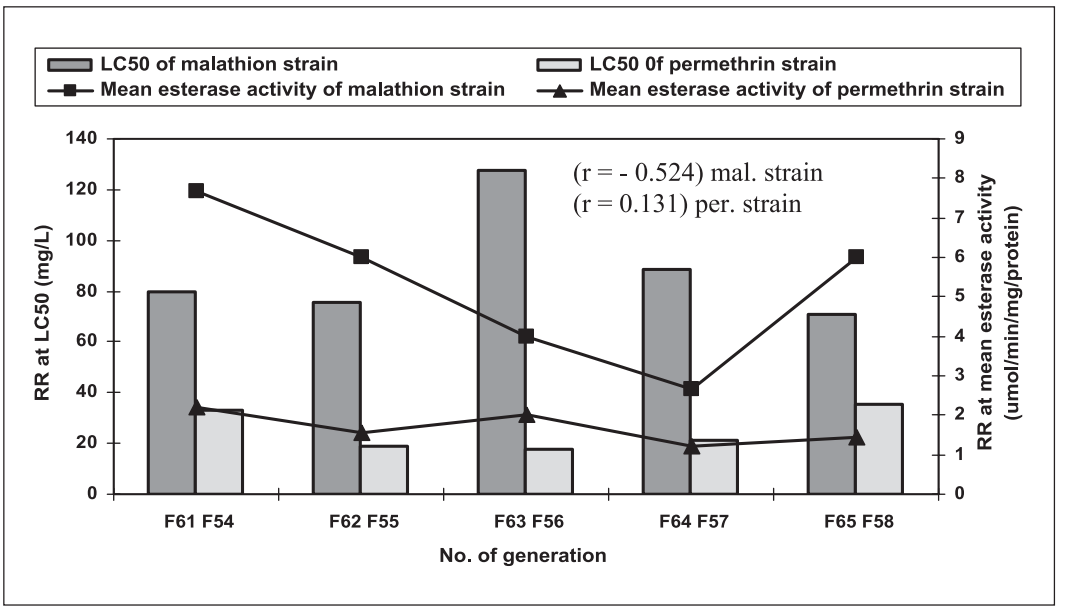

Figure 3. Correlation between LC_{50} value and mean esterase activity in *Culex quinquefasciatus* 3rd instars larvae of malathion (F61-F65) and permethrin (F54-F58) selected strains

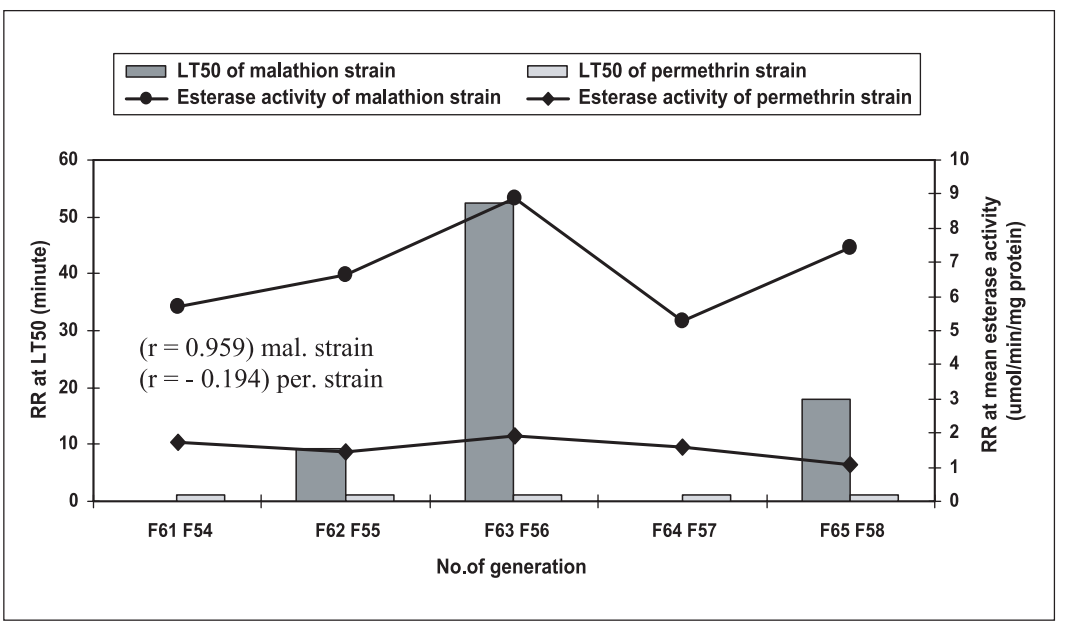

Figure 4. Correlation between LT_{50} value and mean esterase activity in *Culex quinquefasciatus* adult female of malathion (F61-F65) and permethrin (F54-F58) selected strains

it was evident that resistance does not depend upon one or the other stages of mosquitoes.

From the results obtained it is obvious that there is significant difference ($p < 0.05$) in esterase level in both

malathion and permethrin selected strains by using ANOVA. Both female malathion and permethrin selected strains had higher levels of esterase activity compared to male mosquito. This indicates increased level of esterases is playing an important role in resistance mechanism in female malathion selected strain. Similarly, Coto (2000) reported in *Cx. quinquefasciatus* synergist assays that esterases played an important role in malathion resistance but MFO's were not involved in causing malathion resistance in this species.

The activity of esterases increases in pupal stages for both malathion and permethrin selected strains. However, the pupae go through a short transitory period and as such possession of high esterase activity would not seem to offer much advantage (Selvi *et al.*, 2006 b). This study also shows that the metabolic enzyme was higher in the adult stages especially in female mosquito compared to the corresponding larval and pupal stages (Figure 2).

The difference of enzyme levels in females and males could be due to females having to undergo the process of egg production (Nazni *et al.*, 1999). Similarly, study by Devendra *et al.* (1993) also showed that esterase and gluthathione S-transferase activity declines after the emergence of adult in *Aedes aegypti*. The role of esterase and GST is also dependent upon the structure and concentration of insecticide and possibly the age of the insect under test (Perry & Buckman, 1970). The inconsistency of permethrin resistance in esterase activity may be attributed to esterase-mediated detoxification in *Cx. quinquefasciatus* and also to oxidative detoxification by mono-oxygenase as demonstrated by Kumar *et al.* (1991).

The increase in female esterase activity of malathion strain may be due to an overproduction of one or another isoenzyme form of esterase (Mouches *et al.*, 1987; Michel *et al.*, 1998). In Malaysia, Lee (1990) had confirmed by using biochemical tests that a major factor resulting in resistance in *Cx. quinquefasciatus* was due to elevated levels of esterases which correlated directly with malathion (OP) resistance. Besides this, Yu (2004) also had found that resistance development to malathion was highly associated with increased esterase activity which indicated that metabolic detoxification was likely the major resistance mechanism in insects other than mosquitoes such as plant bug, *Lygus lineolaris*.

The permethrin selected strain exhibited low level of esterase activity, thus it suggested that non-specific esterase was not associated with permethrin. Hence, there could be other detoxifying enzymes which may cause permethrin resistance in *Cx. quinquefasciatus* which can be detected using different biochemical enzyme test. Similarly, Hemingway *et al.* (1989) had found that pyrethroid resistance in Puerto Rican strain of *Ae. aegypti* was not associated with a quantitative change in esterase activity. Though non-specific esterases have been reported to be involved in pyrethroid metabolism in several insects and could play a role in the metabolism of permethrin in *Ae. aegypti* (Adriana *et al.*, 2005). This present study strongly indicates that the metabolic enzyme was higher in third (L3) instar larvae and adult female and male mosquitoes compared to the other life stages.

In conclusion, the findings of this present study indicated that permethrin selection of resistance was developing at a faster rate compared to malathion based on the LC_{50} values and malathion was a promising chemical larvicidal agent for the control of *Cx. quinquefasciatus* larvae. In contrast, permethrin is the most potent adulticide to produce high level of mortality in adult *Cx. quinquefasciatus*. In the overall trend, this study exhibits, malathion and permethirn resistance in these strains were not correlated distinctly between mean esterase activity against LC_{50} and LT_{50} values to malathion and permethrin selected strains except for LT_{50} malathion strain. The present data demonstrate non-specific esterases play a predominant role in different life stages in conferring malathion resistance in *Cx.*

quinquefasciatus. This was evident from the O.D. readings and the esterase activity directly had influenced the larval stages (L3) and adult stages which showed relatively higher value of O.D. compared to the permethrin selected strain. Native SDS-PAGE gel electrophoresis is being carried out as a further study to verify a better perception of life stages in relation to resistance development mechanism in *Cx. quinquefasciatus*.

Acknowledgements. Technical assistance rendered by the personnel of Division of Medical Entomology, Infectious Diseases Research Centre, IMR are gratefully acknowledged. This study was supported and funded by Vote-F research grant F0151/2003C and F0126/2004D by the University of Malaya.

REFERENCES

Adriana, E.F., Walter, A.V., Ildefonso, F.S., Mohammad, H.B., Haydee, L.B., Gustavo, P. G., Sau´l Lozano Fuentesa, William, G.B., William, C.B. & Barry, B. (2005). Elevated a-esterase levels associated with permethrin tolerance in *Aedes aegypti* (L.) from Baja California, Mexico. *Pesticide Biochemistry and Physiology* **82**: 66-78.

Bisset, J.A., Rodriquez, M.M., Hemingway, J., Diaz, C., Small, G.J. & Ortiz, E. (1991). Malathion and pyrethroid resistance in *Culex quinquefasciatus* from Cuba : efficacy of pirimiphos-methyl in the presence of at least three resistance mechanisms. *Medical and Veterinary Entomology* **5**: 223-228.

Brogdon, W.G., Beach, R.F., Stewart, J.M. & L. Castanaza (1988). Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanus*. *Bulletin of World Health Organization* **66**: 339-346.

Brown, A.W.A. & Pal, R. (1971). Insecticide resistance in arthropods. *World Health Organization Monograph Series* No. 38, 491 pp.

Brown, T.M. & Brogdon, W.G. (1987). Improve detection of insecticide resistance through conventional and molecular techniques. *Annual Review of Entomology* **32**: 145-162.

Coto, M.M., Lazcano, J.A., De Fernandez, D.M. & Soca, A. (2000). Malathion resistance in *Aedes aegypti* and *Culex quinquefasciatus* after its use in *Aedes aegypti* control programs. *Journal of American Mosquito Control Association* **16**: 324-330.

Devendra, T.M., Hemingway, J. & Leake, C.J. (1993). Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticide resistance. *Medical and Veterinary Entomology* **7**: 11-16.

Hemingway, J., Boddington, R.G. & Harris, J. (1989). Mechanisms of insecticide resistance in *Aedes aegypti* (L.) (Diptera : Culicidae) from Puerto Rico. *Bulletion of Entomological Research* **79**: 123-130.

Hemingway, J., Field, L. & Vontas, J. (2002). An overview of insecticide resistance. *Science* **298**: 96-97.

Karunaratne, S.H. & Hemingway, J. (2001). Malathion resistance and prevalence of the malathion carboxylesterase mechanism in populations of mosquito vectors of disease in Sri Lanka. *Bulletin of World Health Organization* **79**: 1060-1064.

Kumar, S., Thomas, A. & Pillai, M.K.K. (1991). Involvement of mono-oxygenases as a major mechanism of deltamethrin-resistance in larvae of three species of mosquitoes. *Indian Journal of Experimental Biology* **29**: 379-384.

Lee, H.L. (1990). A rapid and simple biochemical method for the detection of insecticide resistance due to elevated esterase activity in mosquito larvae of *Cx. quinquefasciatus*. *Tropical Biomedicine* **7**: 21-28.

Lee, H.L., Abimbola, O. & Inder Singh, K. (1992). Determination of insecticide susceptibility in *Cx. quinquefasciatus* Say adults by rapid enzyme micro-assay. *Southeast Asian Journal of*

Tropical Medicine and Public Health **23**(3): 458-463.

Lee, H.L., Salazar, F.V., Nazni, W.A. & Tadano, T. (1996). Biochemical monitoring and electroporetic characterization of organophospahate resistance in field strains of Malaysians *Cx. quinquefasciatus* Say adults using rapid enzyme microassays. *Tropical Biomedicine* **13**: 137-144.

Michel, R., Christine, C., Thomas, G., Thomas, L. & Nicole, P. (1998). An overview of the evolution of overproduced esterase in the mosquito *Culex pipens*. *Philosophical transactions of the Royal Society of London. Series B* **353**: 1707-1711.

Mouches, C., Magnin, M., Berge, J.B., de Silvestri, M., Betssat, V., Pasteur, N. & Georghiou, G.P. (1987). Overproduction of detoxifying esterases in organophosphate resistant *Culex* mosquitoes and their presence in other insects. *Proceedings of the National Academy of Sciences* **84**: 2113-2116.

Nazni, W.A., Qureishah, B., Lee, H.L. & Baharuddin, O. (1999). Changes in nonspecific esterases and glutathione-stransferases levels in life stages of the house fly *Musca domestica* (L.). *Tropical Biomedicine* **16**: 29-34.

Perry, A.S. & Buckman, A.J. (1970). Studies on microsomal cytochrome P-450 in resistant and susceptible house flies. *Life Sciences* **9**: 335-350.

Pillai, M.K.K., Arunima, S. & Sarita, K. (1994). Microplate assay of elevated esterase activity in individual pyrethroid-resistant mosquitoes. *Journal of Bioscience* **19** (2): 193-199.

Raymond, M. (1985). Log-probit analysis basic program of microcomputer. *Cahiers ORSTOM Entomologie Medicale et Parasitologie* **23**: 117-121.

Richard, H.F. & David, R.C. (1959). *Mosquitos of Medical Importance*. Washington D.C.: U.S. Department of Agriculture.

Selvi, S., Edah, M.A., Nazni, W.A., Lee, H.L. & Azahari, A.H. (2005). Resistance development and insecticide susceptibility in *Culex quinquefasciatus* against selection pressure of malathion and permethrin and its relationship to cross-resistance towards propoxur. *Tropical Biomedicine* **22**: 103-113.

Selvi, S., Edah, M.A., Nazni, W.A., Lee, H.L. & Azahari, A.H. (2006a). The development of resistance and susceptibility of *Aedes aegypti* larvae and adult mosquitoes against selection pressure to malathion, permethrin and temephos insecticides and its cross-resistance relationship against propoxur. *Malaysian Journal of Science* **25**: 1-13.

Selvi, S., Edah, M.A., Nazni, W.A., Lee, H.L. & Azahari, A.H. (2006 b). Bioassay and biochemical resistance detection and its mechanisms associated to the developmental stages influencing resistance level in insecticide selected of vector mosquito *Culex quinquefasciatus*. 1st USM – Penang International Postgraduate Convention : *3rd Life Sciences Postgraduate Conference – moving in synchrony with the changes in the World*, 24 – 27 May 2006, Universiti Sains Malaysia (USM), Penang, Malaysia, pp 1-7.

World Health Organization (1981). Instructions for determining the susceptibility of resistance of adult mosquitoes to organochlorines, organophosphates and carbamate insecticide diagnostic test. *World Health Organization Mimeograph WHO.VBC*/81.806.

World Health Organization (1992). Vector resistance to pesticides. 15th Report of the WHO Expert Committee on Vector Biology and Control. *World Health Organization Technical Report Series* **818**: 1-62.

Yu, C.Z., Gordon, L.S. & Ming, S.C. (2004). Enhanced esterase gene expression and activity in a malathion-resistant strain of the tarnished plant bug, *Lygus lineolaris*. *Insect Biochemistry and Molecular Biology* **34**: 1175-1186.