Tropical Biomedicine 28(2): 425–437 (2011)

Insecticide resistance development in *Aedes aegypti* upon selection pressure with malathion

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Received 11 June 2010; received in revised form 15 August 2010; accepted 19 August 2010
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Abstract. Bioassay test against malathion had been carried out with larval and adult stages of *Aedes aegypti*. The mosquitoes were under selection pressure against malathion for forty-five consecutive generations. The rate of resistance development was measured by LC_{50} and LT_{50} values. The larvae and adult females, after subjection to malathion selection for 45 generations, developed high resistance level to malathion, with resistance ratio of 52.7 and 3.24 folds, respectively over control mosquitoes. Cross-resistance towards the same and different groups of insecticides was determined using the F44 and F45 malathion-selected adult females. Insecticides tested were DDT (4.0%), permethrin (0.75%), propoxur (0.1%), fenitrothion (1%), λ-cyhalothrin (0.05%) and cyfluthrin (0.15%). Results indicated that the mosquitoes were highly resistant to DDT and fenitrothion, moderately resistant to propoxur, tolerant to permethrin and λ-cyhalothrin, and very low resistant to cyfluthrin.

INTRODUCTION

The *Aedes aegypti* mosquito also known as *Stegomyia aegypti* (Reinert *et al.*, 2004), is the main vector for dengue fever (Smith, 1956; Hammon, 1966; Rudnick, 1967; Boromisa *et al.*, 1987; Gubbler *et al.*, 1987; Rohani *et al.*, 1997, 2001) and Chikungunya (Peters & Dalrymple, 1990; Bodenmann & Genton, 2006; Pialoux *et al.*, 2007; Sourisseau *et al.*, 2007; de Lamballerie *et al.*, 2008). *Aedes aegypti* is an invasive species that is currently widespread throughout tropical to temperate regions of the globe. The ability of this mosquito species to breed in artificial containers has facilitated its passive spread in the last decades through main transportation routes (Vezzani & Carbajo, 2008). The *Ae. aegypti* mosquitoes coexist in man-made containers in urban, suburban and rural settlements in tropical and subtropical regions (Rohani *et al.*, 2001; Gomes *et al.*, 2005; Jirakanjanakit *et al.*, 2007).

Effectiveness of chemical control is threatened by development of resistance to insecticides as described for *Ae. aegypti* (Georghiou *et al.*, 1987; Rawlins 1998, Wirth & Georghiou, 1999; Lima *et al.*, 2003; Macoris *et al.*, 2007). The organophosphorous compounds including malathion have been widely used in vector control programme and development of resistance against these compounds has been reported in different mosquito vectors (Gopalan *et al.*, 1996; Bisset *et al.*, 1997; Poopathi *et al.*, 2000). Resistance is defined as the acquired ability of an insect population to tolerate doses of insecticides which can kill the majority of individuals in a normal population of the same species (WHO, 1957; Paeporn *et al.*, 2004; Dennehy & Dunley, 2009). Resistance is relatively easy to monitor with direct insecticide bioassays done in the laboratory (Lee, 1996). Development of resistance to a particular insecticide may be accompanied by development of high levels of resistance or hypersensitivity to other insecticides, via

similar (i.e., cross-resistance) or different (i.e., multiple cross-resistance) resistance mechanisms (Leonard *et al.*, 1988; Pittendrigh *et al.*, 2000). Previous selection with insecticides can confer resistance to new insecticides through cross-resistance (Golenda & Forgash, 1985; Scott, 1989; Bisset *et al.*, 1997) and could cause serious impact on the control of insect pests by reducing effectiveness of many new insecticides. Cross-resistance occurs when a population of insects that has developed resistance to one insecticide exhibits resistance to one or more insecticide(s) it has never encountered. Cross-resistance is different from multiple resistance, which occurs when insects develop resistance to compounds of several chemical classes by expressing non-specific or multiple resistance mechanisms (Metcalf, 1989; Dennehy & Dunley, 2009). Generally, insecticides within a chemical group are sharing a common mode of action. Frequent applications of compounds belonging to one group can dramatically increase the risk of cross-resistance, and may thus select for target-site resistance.

The objective of this study was to determine the rate of resistance development to malathion in the presence of malathion selection pressure and to verify whether it can result in cross-resistance to other insecticides in this strain. Improving our understanding of resistance and cross-resistance mechanisms will help to develop a successful programme for minimizing or preventing resistance development.

MATERIALS AND METHODS

Mosquitoes

Aedes aegypti larvae from Selangor, which have been maintained in the insectarium of Unit of Medical Entomology, Institute for Medical Research for many years and have not been exposed to any insecticide and/or biological control agent was used and designated as F0. The mosquitoes were bred and reared in the insectarium. The F0 and the subsequent larval stage generations were subjected to selection pressure. Malathion 93.3% a.i. was used in the study.

Bioassay test for mosquito larvae

The larval stages were subjected to selection pressure against malathion at the concentrations that caused 50% mortality (LC_{50}), using the WHO (1981a) standard bioassay. Selection was applied to mosquitoes for 45 consecutive generations. Briefly, bioassays were carried out in triplicates, with five different malathion concentrations by serial dilutions. Controls without insecticide were done in three repetitions. Bioassays were carried out on twenty-five early fourth instar larvae, in paper cups, each with 250 ml insecticide solution. Larval mortality was recorded after 24 hours of exposure. Surviving larvae were reared and bred to subsequent generations.

Bioassay test for adult mosquitoes

Adult female malathion resistant *Ae. aegypti* mosquitoes were used in the test. The susceptibility of the mosquitoes to malathion were conducted using WHO (1981b) Test Kits with some modifications. Fifteen non-blood-fed females, 1-7 days old, were exposed to 5% malathion impregnated papers for 32 consecutive generations. The tests were carried out in triplicates, and duplicated controls without insecticide was performed. Mortality of mosquitoes was recorded every five minutes for 1 hour exposure time in the WHO standard exposure tubes. Mosquitoes that survived the exposure period were kept in holding tubes to observe the effect of post-treatment and mortality was recorded after 24 hours of holding period. Cotton pads soaked in 10% sugar solution were provided during the 24 hours holding period.

Adult bioassay test for cross-resistance

For determining cross-resistance, female adults of F44 and F45 malathion resistant *Ae. aegypti* were used in bioassays using standard WHO Test Kits against permethrin (0.75%/1 h), DDT (4.0%/1 h), propoxur (0.1%/1 h), fenitrothion (1%/1 h), λ-cyhalothrin (0.05%/1h) and cyfluthrin (0.15%/1 h) (WHO, 1998). Insecticide-impregnated papers were purchased from Vector Control Research Unit, Penang, Malaysia.

Data analysis for susceptibility test

Bioassay results were subjected to Probit Analysis (Finney, 1971), using a computerized programme of Raymond (1985) to obtain LC_{50} and LT_{50} values. Based on the LC_{50} and LT_{50} values, resistance ratio of resistant strain to susceptible strain was calculated by adopting the method of Brown & Pal (1971). The resistance ratio (RR) was determined as follow:

$$\text{Resistance ratio (RR)} = \frac{LC_{50}/\ LT_{50} \text{ of tested generation}}{LC_{50}/LT_{50} \text{ of } F_0 \text{ generation}}$$

RESULTS AND DISCUSSION

The malathion resistance level of *Ae. aegypti* increased steadily, upon malathion selection treatment on mosquitoes for 45 subsequent generations. As shown in Table 1, a marked resistance was observed throughout selected mosquito generations. The final resistance ratio to malathion was 52.7 folds, compared to F0 generation. It was not possible to calculate rate of selection in each generation due to inconsistent larval LC_{50} values. Previous reports have defined that a population may be termed resistant when its larval LC_{50} has increased by 10 times (Knipling, 1950; Nazni *et al*., 2005), thus results reported herein indicated that a high level of resistance to malathion in *Ae. aegypti* had developed under insecticide selective pressure in the laboratory. Studies by Bisset *et al*. (1991) and Gopalan *et al*. (1996) demonstrated 1,208-fold resistance after 22 generations, and 2,036-fold resistance after 25 generations of selection with malathion. In our studies, the adult female malathion-resistant mosquitoes developed high resistance level to malathion and LT_{50} values increased from 19.50 minutes to 53.36 minutes and resistance ratio after 32 generations of selection pressure was 2.74 folds over F0 generation (Table 2). Resistance was shown by low mortalities of offspring from parents which survived selective pressures in preceding generations. In the presence of the pesticide, it is these substantially less susceptible individuals that survive and reproduce. The results could be due to genetic make up, enabling the mosquito to survive. This genetic makeup is then passed on to their offspring, resulting in shifts in the population's susceptibility via pesticide-induced selection. The speed and degree of development of resistance depends on the frequency of resistance gene(s) in the population, the type of gene which is responsible for resistance, the insecticide dosage applied, and the frequency of application (Nazni *et al*.,1998; Hidayati *et al*., 2005). In the present study, the resistance ratio in larvae was higher than adults, upon selection for 32 generations, similar to those reported by Tadano & Brown (1966). It could be possible that the larval stages could detoxify malathion at faster rate than adults (Lee *et al*., 1998; Rohani *et al*., 2001). However, resistance is not restricted to one or other stages (Nazni *et al*., 2005).

The resistance ratio after 45 generations of malathion selection pressure was 3.24 folds compared to the F0 generation. To test whether cross-resistance against other insecticides could occur after a time selection against malathion, the F44 and F45 generations were tested against permethrin (0.75%/1 h), DDT (4.0%/1 h), propoxur (0.1%/1 h), fenitrothion (1%/1 h), λ-cyhalothrin (0.05%/1h) and cyfluthrin (0.15%/1 h). The criteria of interpretation was proposed by Davidson & Zahar (1973), and modified by WHO (1998) as that susceptible insects present 98-100% mortality and resistant insects present mortality below 80%. The results shown in Table 3 indicated that the malathion resistant mosquitoes were highly resistant to the organochlorine DDT and the organophosphate fenitrothion with low or no mortality within 60 minutes in both generations (Figure 1 and 2), to which the mosquito population had never been exposed.

It is possible that elevated levels of resistance to fenitrothion in these mosquitoes may result from continuous exposure to the selection pressure with malathion which is the same insecticide class. The results that the mosquitoes are more resistant to fenitrothion compared to malathion are similar to the study of Bracco *et al.* (1999). They found that *Culex quinquefasciatus* from

Table 1. LC_{50} values of malathion against laboratory selected *Ae. aegypti* larvae

Aedes aegypti larvae malathion strain			
Generation	LC_{50}(mg/l)	95% (Level of confidence)	RR
F0	0.06006	0.04877 – 0.06993	–
F1	0.15267	0.13958 – 0.16880	2.54
F2	0.13833	0.12584 – 0.15352	2.30
F3	0.15842	0.14608 – 0.17153	2.64
F4	0.15295	0.14173 – 0.16488	2.55
F5	0.17717	0.16217 – 0.19321	2.95
F6	Nd	–	–
F7	Nd	–	–
F8	Nd	–	–
F9	0.11618	0.06480 – 0.15316	1.93
F10	0.09866	0.08029 – 0.11349	1.64
F11	Nd	–	–
F12	0.07383	0.04596 – 0.09943	1.23
F13	0.12326	0.10105 – 0.14508	2.05
F14	0.14187	0.11309 – 0.16994	2.36
F15	0.13956	0.12023 – 0.15976	2.32
F16	Nd	–	–
F17	0.21331	0.18467 – 0.23782	3.55
F18	0.25070	0.22839 – 0.27191	4.17
F19	Nd	–	–
F20	Nd	–	–
F21	0.23891	0.21298 – 0.26244	3.98
F22	Nd	–	–
F23	0.24290	0.21760 – 0.26609	4.04
F24	0.27659	0.25156 – 0.30065	4.60
F25	0.26171	0.23471 – 0.28684	4.36
F26	0.23074	0.20664 – 0.25260	3.84
F27	0.26865	0.24575 – 0.29079	4.47
F28	0.25434	0.23199 – 0.27401	4.23
F29	0.27198	0.24861 – 0.29283	4.53
F30	0.23386	0.20145 – 0.26037	3.89
F31	0.30101	0.27817 – 0.32219	5.01
F32	0.29824	0.27615 – 0.31873	4.97
F33	0.30009	0.27740 – 0.32113	5.00
F34	0.31195	0.28908 – 0.33349	5.19
F35	0.33089	0.30988 – 0.35129	5.51
F36	0.32361	0.30382 – 0.34270	5.39
F37	0.34748	0.32770 – 0.36707	5.79
F38	0.34804	0.32745 – 0.36848	5.79
F39	0.36098	0.34096 – 0.38112	6.01
F40	0.36537	0.34538 – 0.38556	6.08
F41	0.36868	0.34782 – 0.38992	6.14
F42	0.36290	0.34262 – 0.38335	6.04
F43	0.37537	0.35521 – 0.39598	6.25
F44	0.36981	0.35019 – 0.38968	6.16
F45	0.37668	0.35756 – 0.39610	6.27

C.L = Conference Limit
RR = Resistance ratio
Nd = Not done

Table 2. LT_{50} (min) values and 24 hours post-exposure mortality of malathion against laboratory selected *Ae. aegypti* adult

Generation	LT_{50} (min)	95% (C.L)	24 hours post-exposure mortality (%)	RR
F_0	19.50705	18.13854 – 20.85778	100	–
F_1	28.98558	26.94174 – 31.19667	100	1.49
F_2	30.95599	29.49766 – 32.41785	100	1.59
F_3	31.02635	29.29150 – 32.82357	100	1.59
F_4	31.03112	29.41849 – 32.62939	100	1.59
F_5	31.43363	30.43181 – 32.40746	100	1.61
F_6	38.43427	36.98564 – 39.93929	100	1.97
F_7	Nd	–	–	–
F_8	Nd	–	–	–
F_9	41.71824	40.30156 – 43.20806	100	2.14
F_{10}	40.80668	39.57576 – 42.06506	97.0	2.09
F_{11}	44.92260	43.78996 – 46.07746	100	2.30
F_{12}	37.80398	36.42355 – 39.42363	100	1.94
F_{13}	39.20830	38.18509 – 40.21722	100	2.01
F_{14}	Nd	–	–	–
F_{15}	39.25974	37.71394 – 40.89171	100	2.01
F_{16}	43.00900	41.32263 – 44.83026	100	2.20
F_{17}	42.09232	40.47051 – 43.78776	97.8	2.16
F_{18}	39.84652	38.57235 – 41.14726	100	2.04
F_{19}	38.91205	(37.87510 – 39.95157	100	1.99
F_{20}	44.44964	43.22381 – 45.78867	100	2.28
F_{21}	45.84996	44.31125 – 47.49898	100	2.35
F_{22}	Nd	–	–	–
F_{23}	42.97056	41.75134 – 44.10806	100	2.20
F_{24}	Nd	–	–	–
F_{25}	44.38379	42.99822 – 45.85508	100	2.28
F_{26}	50.29687	49.16133 – 51.49074	100	2.58
F_{27}	50.38785	49.01560 – 51.93747	100	2.58
F_{28}	50.83128	49.34119 – 52.55136	97.7	2.61
F_{29}	Nd	–	–	–
F_{30}	50.39172	49.01431 – 51.93690	95.5	2.58
F_{31}	53.12757	51.30361 – 55.38157	96.5	2.72
F_{32}	53.36516	51.91957 – 55.09170	95.6	2.74

C.L = Conference Limit
RR = Resistance ratio
Nd = Not done

Table 3. LT_{50}, regression line and resistance ratio of *Ae. aegypti* malathion strain exposed to different insecticides

Insecticide	Generation	LT_{50} (C.L)	24 hours post-exposure mortality (%)	RR
Malathion (5.0%)	F0	19.50705 (18.13854 – 20.85778)	100	1.0
	F44	64.49703 (60.65099 – 73.01113)	88.9	3.31
	F45	63.22931 (60.02967 – 69.57816)	86.7	3.24
Permethrin (0.75%)	F44	23.19501 (21.71022 – 24.45387)	100	1.19
	F45	18.55102 (17.32652 – 19.75349)	100	0.95
DDT (4.0%)	F44	R*	53.3	–
	F45	R*	24.4	–
Fenitrothion (1.0%)	F44	R*	53.6	–
	F45	R*	100	–
λ-Cyhalothrin (0.05%)	F44	NA	100	–
	F45	28.86009 (28.03094 – 29.68441)	100	1.48
Cyfluthrin (0.15%)	F44	12.53049 (11.81857 – 13.19337)	100	0.64
	F45	9.65130 (8.32195 – 10.62206)	100	0.5
Propoxur (0.1%)	F44	36.50859 (35.21983 – 37.79270)	95.6	1.87
	F45	33.59721 (32.36727 – 34.81835)	95.6	1.72

C.L = Conference Limit
RR = Resistance ratio
R* = Highly resistance i.e. cannot compute by Probit analysis
NA = Not Available i.e. data not enough to compute by Probit analysis

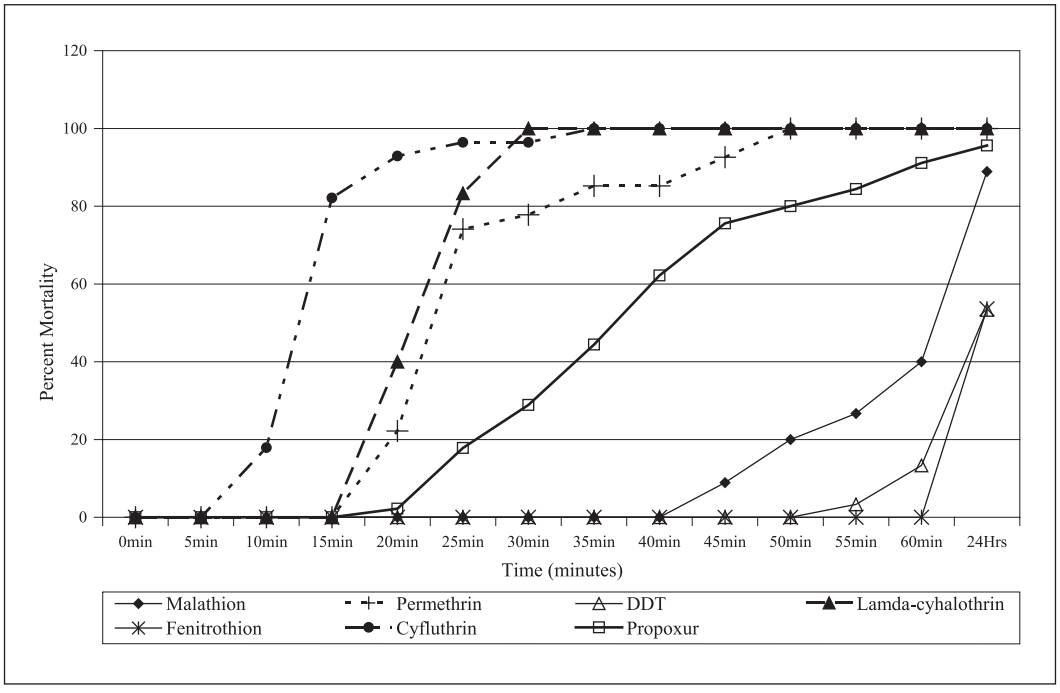

Figure 1. Percent mortality of *Ae. aegypti* malathion strain (F44) exposed to different insecticides

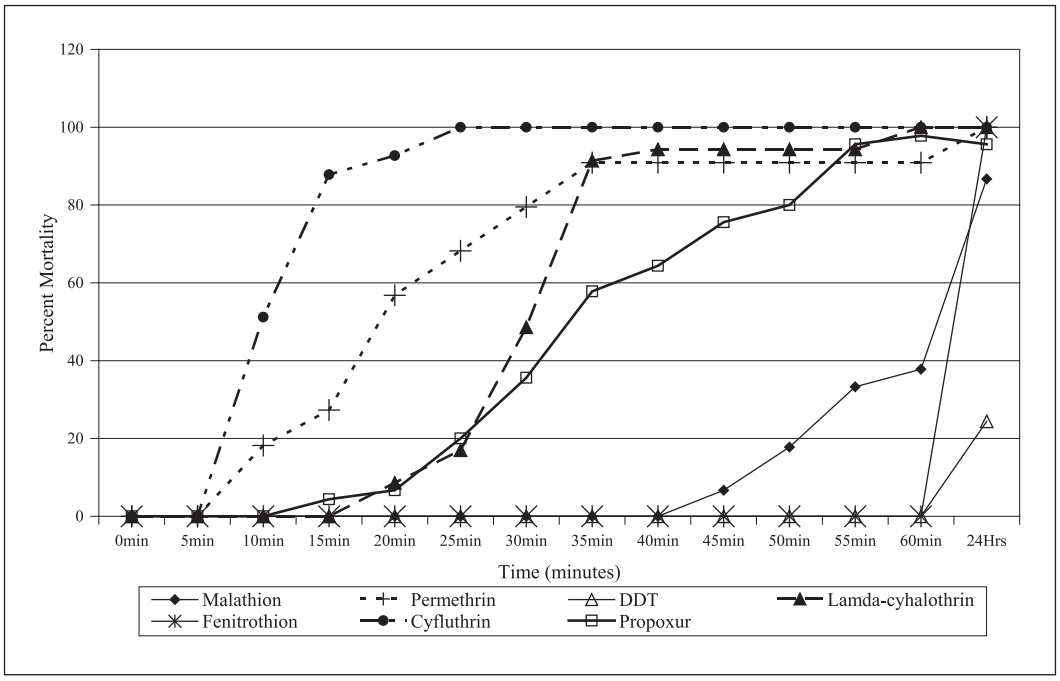

Figure 2. Percent mortality of *Ae. aegypti* malathion strain (F45) exposed to different insecticides

R* = Highly resistance i.e. cannot compute by Probit analysis
NA = Not Available i.e. data not enough to compute by Probit analysis

São Paulo City developed high resistance to both malathion and fenitrothion to which the insecticides had never been used for mosquito control in the area. This study is comparable to that reported by Charoverty & Kalyanasundaram (1992) in India in that *Anopheles stephensi*, after some generations of selection pressure with permethrin in laboratory, showed increased resistance ratio to 13 folds compared with a susceptible strain. In addition, *An. stephensi* showed cross-resistance towards cypermethrin and deltamethrin with resistance ratio of 7 and 10 folds, respectively, and further resistance toward 4% DDT had also been detected (Charoverty & Kalyanasundaram, 1992). High level of physiological resistance to DDT in *Ae. aegypti* could be related to previous use of DDT in agriculture and public health (Brown, 1961; Nazni *et al.*, 2000), before being maintained as susceptible strain in the insectory. The natural tolerance of *Aedes* to DDT had been known in Malaya since 1950's prior to the wide-spread use of DDT in malaria eradication programmes (Lee *et al.*, 1998). Coker (1958) had studied the genetic basis of a Malayan strain of *Ae. aegypti* which was about 6 times resistant to DDT compared with a susceptible strain. It could be that if the mosquitoes are kept insecticide–free for a long period the resistance can be reversed (Nazni *et al.*, 2005). How cross-resistance of organophosphate and DDT insecticides occurred in this study is not known, although it could be possibly due to efficiently increased detoxification by enzymes such as glutathione-*S*-transferases (Bull, 1981; Oppenoorth, 1985; Ahmad, 2007).

The LT_{50} result in Figure 3 showed moderate resistance in F44 and F45 generations to propoxur, with LT_{50} values of 36.51 minutes and 33.60 minutes, and with resistance ratio of 1.87 and 1.72 folds for respectively, in comparison to the F0 generation. Acetylcholinesterase (AChE) is a common target for organophosphates and carbamates. Cross-resistance to organophosphates and carbamates can arise from the target site insensitivity of AChE. This resistance mechanism has occurred in

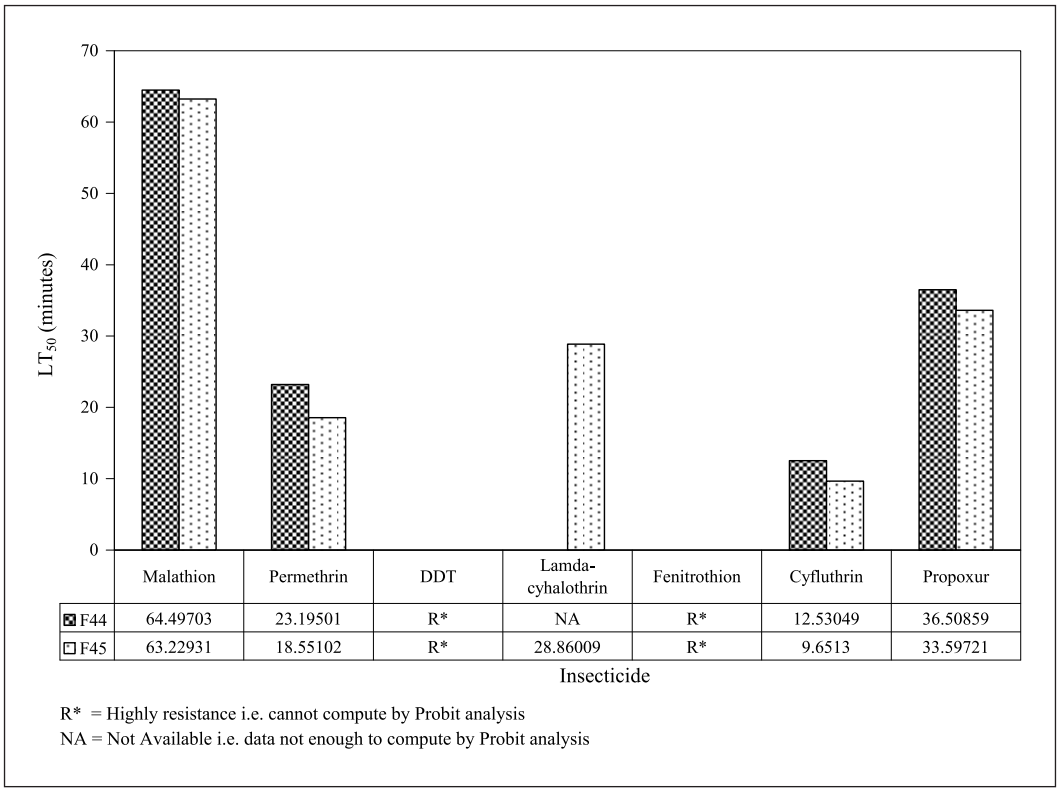

Figure 3. LT_{50} of *Ae. aegypti* malathion-resistant F44 and F45 generations exposed to different insecticides

several mosquito species (Ayad & Georghiou 1975; Hemingway, 1982; 1985; Liu *et al.*, 2004). Usually, when a resistant strain is selected with an insecticide, resistance extends to other compounds of the same class of insecticides or to compounds with similar modes of action (Liu *et al.*, 2004). Much resistances are conferred by a single major genetic factor that differs between resistant and susceptible pests. When a single factor confers resistance to more than one insecticide, this is cross-resistance by which a single mechanism is responsible for resistance to more than one insecticide. Cross-resistance occurs when selection with one insecticide causes resistance to other insecticides that have never been used. In most cases, cross-resistance involves structurally similar insecticides. Cross resistance also means that other classes of pesticides, e.g. organophosphates and carbamates, would be less effective where resistance occurs to either compounds.

Tolerance to the permethrin and λ-cyhalothrin was observed in F44 generation, with LT_{50} value of 23.19 minutes with resistance ratio of 1.19 folds against permethrin. Resistance ratios in F45 generation were 0.95 folds and 1.48 folds with the LT_{50} values of 18.55 minutes and 28.86 minutes for permethrin and λ-cyhalothrin, respectively. Both generations showed similar susceptibility or lower tolerance to cyfluthrin, with LT_{50} values of 0.64 and 0.5 folds higher than the F0 generation of *Ae. aegypti* malathion resistant strain.

Based on bioassays on house flies, *Musca domestica* L., and German cockroaches, *Blattella germanica* (L.), Scott & Wen (1997) proposed that an insect could be referred to as cross-resistant when the resistance ratio was > 4. It has also been suggested that insects should not be considered resistant until a resistance ratio of 10 is exhibited (Valles *et al.*, 1997; Liu *et al.*, 2004). Accordingly, we would consider that the

malathion resistant strain of *Ae. aegypti* in this study was tolerant rather than cross-resistant towards those insecticides, except for DDT and fenitrothion.

Genetics and intensive application of insecticides are responsible for rapid development of resistance in many insects and mites. Selection by an insecticide allows some insects with resistance genes to survive and pass the resistance trait onto their off springs (Hoskins, 1959; Brown, 1960; Laurence, 1960). The proportion of resistant insects in a population continues to increase as the susceptible insects are eliminated by insecticide (Brown, 1986; de Carvalho *et al*., 2004). Eventually, resistant insects out number susceptible insects and the insecticide is no longer effective. The rate at which insecticide resistance develops in insects depends on several factors, including how rapidly the insects reproduce, the insects' level of resistance, migration and host range of the insects, the insecticide's persistence and specificity, and the rate, timing and number of applications of insecticide (Mallet, 1989; de Carvalho *et al*., 2004). Resistance increases faster in places such as greenhouses, where insects or mites reproduce rapidly, the place where there is little or no immigration of susceptible insects (Dennehy & Dunley, 2009), and in situation that growers may spray frequently with the same insecticide or insecticides from the same chemical class (de Carvalho *et al*., 2004; Coleman & Hemingway, 2007).

In summary, the exact physiological mechanism for organophosphate (malathion) resistance in mosquitoes remains to be explored to develop alternate measures to manage resistance in mosquitoes. Cross-resistance studies are important and may result in cross-resistance pattern, which in turn are essential in order to fine-tune management recommendations, for example alternation of insecticides to avoid continuous selection for the same resistance gene or mechanism. Control programme should thus be aware of cross-resistance to the same or related synthetic compounds against mosquito populations and agricultural pests. Studies of resistance mechanisms as well as cross-resistance, therefore, will enable us not only to develop a successful programme for overcoming resistance but also to design novel strategies to prevent or minimize the spread and evolution of resistance. Study of cross-resistance will also provide useful information for identifying known resistance mechanisms (Scott, 1990).

Acknowledgement. The authors wish to thank the Director, Institute for Medical Research, Kuala Lumpur for permission to publish, and staff of Medical Entomology Unit, Institute for Medical Research, Kuala Lumpur for their help. This is a partial study for PhD thesis, University Malaya, Kuala Lumpur. The study was supported by the IRPA 06-05-01-0115.

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