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Multiple resistance of *Culex vishnui* Theobald against four major classes of insecticides in an agricultural area in Sekinchan, Selangor, Malaysia

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Abstract. The resistance status of riceland *Culex vishnui* against four major groups of insecticides (i.e., organochlorines, carbamates, organophosphates and pyrethroids) was investigated. Biochemical assays (ESTα, ESTβ, MFO and GST) were also conducted to detect the resistance levels. Throughout a 12-month study period, multiple insecticide resistance was observed in both larvae and adult *Cx vishnui*. *Culex vishnui* larvae exhibited low resistance against malathion, temephos and permethrin with resistance ratio (RR) values < 5. In adult bioassay, *Cx. vishnui* were highly resistant against all tested adulticides with 24h post-treatment mortality < 70%. Correlations between permethrin and malathion resistance, as well as between deltamethrin and cyfluthrin resistance were found in *Cx. vishnui*. The results indicated that mixed function oxidases activity of *Cx. vishnui* was the highest compared to ESTα, ESTβ and GST. Spearman rank-order analysis showed that ESTα, ESTβ and GST were involved in multiple resistances in *Cx. vishnui*. The findings of this study established a baseline of insecticide susceptibility and revealed the effects of agricultural insecticide pressure on the vectors of Japanese encephalitis in Malaysia.

INTRODUCTION

Approximately 17% of the estimated global burden of infectious diseases is caused by vector borne diseases and it is one of the major causes of morbidity and mortality in tropical and subtropical countries (WHO, 2012). Among various prevention/control approaches, chemical control involving the use of insecticides appears to be the norm for both public health and household insect pest control (Yap *et al.*, 2000). This is also the case in Malaysia, where chemical pesticides are commonly used to control mosquitoes.

Insecticides have been frequently used for vector control programs throughout the world because insecticides can kill a great number of vectors within a short time and suppress the vector population. However, continuous use of insecticides for extended periods may cause the development of adaptation (Mittal *et al.*, 2004) and insecticide resistance in the mosquitoes (Sathantriphop *et al.*, 2006a). The development of resistance, particularly to multiple insecticides, is an important issue. Mosquitoes have adapted themselves to insecticides that are commonly used for long periods for their survival (Saelim *et al.*, 2005). In mosquitoes, insecticide resistance is caused by two mechanisms: increased detoxification and target site insensitivity (Tantely *et al.*, 2010). As the exposure to the insecticides increase, the mosquitoes build up immunity against the insecticides used; therefore to kill mosquitoes with a certain immunity to insecticides, a higher dose is required (Hidayati, 2010). For successful mosquito

control programs, overreliance on the same insecticides should be avoided and this is one of the principles in insecticides resistance management. Unfortunately, effective insecticides resistance management has been restrained due to limited option of insecticides available for vector control. WHO has only approved four classes of insecticide, two of which are sharing the same mode of action (WHO, 2013). The two modes of actions that are shared by two classes of insecticides are sodium channels gating in myelinated nerves for organochlorine and pyrethroid insecticides (Vijverberg *et al.*, 1982; Davies *et al.*, 2007) and inhibition of acetylcholinesterase for organophosphorus and carbamate insecticides (Fukuto, 1990; Jin *et al.*, 2004).

Mosquitoes are able to express multi-insecticides resistance through mutation at target sites and increase of single or multiple detoxifying mechanisms (Perera *et al.*, 2008; Fonseca-Gonzalez *et al.*, 2009; Edi *et al.*, 2012; Nwane *et al.*, 2013). Such as the mutation of *kdr* (knockdown resistance) alleles and *Ace-1*^{R} (acetylcholinesterase gene) in mosquitoes has resulted in multi-insecticides ressitance (Yewhalaw *et al.*, 2011). Also the occurrence of monooxygenases/mixed function oxidases (MFO), esterases (EST), glutathione S-tranferases (GST) and acethycholine (AChE) enzymes in mosquitoes were found to be responsible for multi-insecticides resistance (Hemingway & Ranson, 2000).

In Malaysia, many studies have been conducted to investigate the susceptibility status of mosquitoes obtained from different ecological habitats (Nazni *et al.*, 1998; Loke *et al.*, 2012; Low *et al.*, 2013); however, no study has been conducted on mosquitoes from rice cultivation areas that are usually treated with insecticides. Furthermore, studies on the susceptibility status of *Culex vishnui* in the region is scarce, and has not been updated for a long period. A previous study which was conducted in Andhra Pradesh, India in 2002, only focuses on temephos and deltamethrin (Sharma *et al.*, 2003). In Malaysia, a previous study was conducted against *Cx. vishnui* obtained from a pig farm in Tanjung Sepat, Selangor. However, only the susceptibility status of adult female *Cx. vishnui* was conducted (Chen *et al.*, 2013).

The objective of the present study was to determine the susceptibility of *Cx. vishnui* collected from a rice cultivation area in Sekinchan, Selangor, Malaysia to four major classes of insecticides.

MATERIALS AND METHODS

Study site

The study was carried out in a village adjacent to a paddy field in Sekinchan, Selangor, Malaysia. The paddy field is about 50 meters apart from the mosquito collection site (3º31'23.70"N, 101º8'31.29"E).

Field strains of mosquitoes

Adult female mosquitoes were collected from January to December 2011 by using human landing catch (HLC) technique. Informed consent was obtained from volunteers before the start of the study. HLC was conducted for 4 hours each month for a period of 12-months. The collectors sat near mosquito breeding or resting sites. All mosquitoes landing on humans were collected by using a small tube (50mm X 19 mm) which was subsequently plugged with cotton wool. The collected mosquitoes were then identified and segregated according to species. The mosquitoes were identified using the keys of Knight & Stone (1977); Das *et al.* (1990) and Reuben *et al.* (1994). Field collected *Cx. vishnui* were then brought to the insectary and bred to obtain first filial generation 1 (F1) which was subsequently used for the adult bioassays.

Reference susceptible strain of mosquitoes

A reference strain for *Cx. vishnui* was obtained from the insectary of the Institute of Biological Sciences (ISB), Faculty of Science, University of Malaya and maintained for 10 generations. The

reference strain was not exposed to any control agents since it was colonized in the insectary.

Insecticides

Three active ingredients representing two major insecticides classes were used in the larval susceptibility tests, namely malathion and temephos (organophosphate) and peremrthin (pyrethroid). Temephos 156.25mg/L, malathion 8% and permethrin 0.5% in solution were purchased from the Vector Control Research Unit, University Sains Malaysia, Penang, a WHOPES Collaborating Centre. For the adult susceptibility test the WHO diagnostic dose of insecticides impregnated on Whatman no 1 filter papers purchased from the WHOPES Collaborating Centre in University Sains Malaysia, Penang were used. The adults were tested against 10 insecticides, namely organophosphates (malathion 5%, fenitrothion 1%), pyrethroids (permethrin 0.25%, etofenprox 0.05%, deltamethrin 0.025%, lambdacyhalothrin 0.025%, cyfluthrin 0.15%), organochlorines (DDT 4%, dieldrin 4%) and carbamate (propoxur 0.1%) (Table 1).

WHO Larval Bioassay

Larval bioassay was performed according to the WHO standard method (WHO, 1981a). Stock solutions of each insecticide were made up in ethanol and further diluted to the desired concentrations. Larval bioassay was carried out using a total of 5 to 10 concentrations ranging from 0.160 to 0.400mg/L for malathion, 0.010 to 0.300 mg/L for temephos and 0.020 to 0.200mg/L for permethrin to obtain 50% and 90% lethal concentration (LC_{50} and LC_{90}). The insecticide test concentrations were prepared by pipetting the appropriate standard insecticide solution into 300mL drinking paper cups and filled up to the 250mL mark with distilled water. A total of 25 late 3rd or early 4th instar *Cx vishnui* larvae were collected in paper cups with sufficient larval food. Any larvae showing abnormalities were discarded and replaced with normal larvae. These larvae were then introduced into the cups with different concentrations of insecticides for 3 replicates. The cups were held at room temperature of 28 ± 1°C and 70 ± 10% relative humidity. The control (untreated) was set up by adding 1mL of ethanol into the distilled water. The larval mortality in both treated and control were recorded after 24 hours.

WHO adult bioassay

Sucrose-fed 2-5 days old adult female *Cx vishnui* were used for the study. The procedure for the Bioassay was carried out using the WHO protocol (WHO 1981b). The adult mosquitoes were exposed to the diagnostic dosage of standard WHO

Table 1. Exposure period (minutes) of all insecticides tested against *Cx vishnui*

Insecticides		Exposure period (min)
Organochlorine	DDT (4%)	240
	Dieldrin (4%)	60
Carbamate	Propoxur (0.1%)	120
Organophasphate	Malathion (5%)	60
	Fenitrothion (1%)	120
Pyrethoid	Permethrin (0.25%)	180
	Deltamethrin (0.025%)	60
	Lambdacyhalothrin (0.025%)	60
	Cyfluthrin (0.15%)	60
	Etofenprox (0.05%)	60

insecticide paper in batches of 15 individuals, with the appropriate exposure time used according to the insecticides tested and recommended by WHO adult bioassay procedures (Table 1). All tests were conducted at 25 ± 2°C. Cumulative knockdown was recorded every minute until the end of respective exposure periods of the different insecticides. After the exposure period, the mosquitoes were transferred into a paper cup covered with netting and provided with 10% sucrose solution. Both test mosquitoes and their respective controls were held for 24h before the final mortality was recorded. All insecticides were tested in three replicates.

Biochemical assays

Adult females *Cx. vishnui* were selected for microassays with three enzymes (non-specific esterases, mixed function oxidases and gluththione-S-transferases). Due to the limited number of mosquitoes available, only these three enzymes were studied.

Esterase assays

The esterase was assayed following Brogdon *et al.* (1988) and Lee (1990). Individual adult female mosquitoes were first homogenized in 100 µL phosphate buffer and further diluted with 400 µL buffer. The homogenate was centrifuged at 1500 rpm for 10 minutes at 4°C. By using a micropipette, 50 µL homogenate was transferred to a well in a microtiter plate. A total of 4 replicate aliquots of the homogenate from a single mosquito were used for this assay. A total of 24 adult mosquitoes were used and incubated for 1 minute, followed by the addition of 50 µL indicator solution (3% hydrogen peroxide). The reaction was allowed to continue for 10 minutes and was stopped by adding 50 µL 10% acetic acid. The absorbance of the reaction mixture was measured using an immunoassay reader (Dynatech, Model MR5000) at wavelength 450 nm to quantify the enzyme activity.

Mixed function oxidases

Mixed function oxidases activities were measured according to Brogdon *et al.*, (1997). Individual adult female mosquitoes were homogenized in 100 µL sodium acetate buffer and further diluted to 1 mL with the addition of 900 µL of buffer. By using a micropipette, 100 µL homogenate was transferred to a microtiter plate. A total of 4 replicate aliquots of the homogenate from a single mosquito (24 totals) were used for this assay. 3,3'5,5'-tetramethylbenzidine (TMBZ) solution (200 µL) was then added into each well in the microtiter plate, followed by 25 µL of 3% hydrogen peroxide. The reactions were incubated for 5 minutes at room temperature. The colour intensities were then read using an immunoassay reader (Dynatech, Model MR 5000) at wavelength 630 nm to quantify the enzyme activity. The activity was expressed as the optical density.

Glutathione S-transferases

Glutathione-S-transferase enzyme activities were determined using the procedures of Lee & Chong (1995). An individual adult female mosquito was homogenized in a microcentrifuge tube filled with 100 µL potassium phosphate buffer. Each homogenate was diluted to the final volume of 500 µL with 400 µL buffer. The homogenate was then centrifuged at 1400 rpm, 4°C for 10 minutes. The clear homogenate (50 µL) was then added into the wells of a microtiter plate. Using this technique, 4 replicates (50 µL) from the homogenate from a single mosquito were used for testing. Substrate solution (GSH) (50 µL) and CDNB (50 µL) were then added into each well. The reaction was allowed to continue for 30 minutes. The colour intensities were read using an immunoassay reader (Dynatech, Model MR5000) at wavelength 410 nm and optical density values were obtained. GST activity was calculated based on the protein concentration and expressed as CDNA-Kŋmol/min/mg protein.

Data analysis

Larval bioassay data within the range of 5-95% were subjected to probit analysis (Finney, 1971) using a computerized

program, PROBIT (National Center for Scientific Research, France) developed by Raymond (1993). Resistance ratios (RR) were calculated by dividing values for the field strain with the reference strain based on the LC_{50} obtained from the larval bioassays (Brown & Pal, 1971). Levels of resistance were calculated according to Mazarri & Georghiou (1995) where calculated RR values of < 5 are expressed as low resistance, 5 – 10 are expressed as medium resistance and > 10 are express as high resistance. The associations between the RR values in larval bioassays were assessed by Spearman rank-order correlation, for the determination of cross resistance (Low *et al.*, 2013).

A specific time for each chemical's knockdown analysis was conducted according to the Knockdown time of the reference strain. The percentage mortality at 24 h post-treatment was used to determine the susceptibility status based on the WHO susceptibility criterion of mortality rate of >98%, whereas resistant represents a mortality rate of <80%; a mortality rate between 80 – 98% is a sign of tolerant/intermediate resistance (WHO, 2009). If the control mortality was > 5%, the percentage mortalities would be corrected by Abbott's (1925) formula. Comparative measure of knockdown and mortality between the study sites was performed using ANOVA. Turkey's test was used to separate means in significant ANOVAs, $P < 0.05$. Cross-resistance of mosquitoes was determined by Spearman rank-order correlation between the percentage mortality in the bioassays (Low *et al.*, 2013).

With regards to enzyme assays, the values for the colour intensities of each enzyme assay for all strains of female mosquitoes were pooled and analysed using SPSS v.19. Resistance ratios (RR) were calculated by dividing values for the field strain with the reference strain based on the mean enzyme activity obtained from the enzyme assays. Resistance values of > 1 indicated resistance, while values ≤ 1 were considered susceptible. Spearman-rank order was used to determine the correlation between survival rate of mosquitoes against various insecticides and enzyme levels in field collected *Cx. vishnui* strain.

RESULTS

Table 2 shows the susceptibility status and resistance ratio (RR) of *Cx. vishnui* larvae against malathion, temephos and permethrin. The LC_{50} of malathion, temephos and permethrin against *Cx. vishnui* ranged from 0.214 – 0.296 mg/L, 0.053 – 0.119 mg/L and 0.042 – 0.076 mg/L, respectively. According to the mean LC_{50} values, *Cx. vishnui* larvae were more susceptible against permethrin (0.053 ± 0.03 mg/L), followed by temephos (0.090 ± 0.07 mg/L) and malathion (0.250 ± 0.008 mg/L). *Culex vishnui* larvae exhibited low resistance against all tested insecticides with RR_{50} values of < 5. *Culex vishnui* larvae were most resistant to temephos with the highest mean RR_{50} value at 1.70, followed by permethrin (1.66) and malathion (1.30). On the other hand, Spearman rank-order correlation indicated that no correlation was found with the other insecticides in the larval bioassay.

Due to the limited availability of mosquitoes it was not possible to conduct the exact concentration to determine the LC_{-99} (Lethal Concentration that kills 99% mosquitoes in a population) of the tested insecticides; the diagnostic dosage of the tested insecticides against *Culex* mosquitoes was used to test the susceptibility of *Cx. vishnui* in the present study. In the adult bioassays, due to the low numbers of knockdown/no-knockdown of adult females *Cx. vishnui* within the exposure period against all tested insecticides, the KT_{50} and KT_{90} for most of the strains were not determined. The bioassays revealed that dieldrin, malathion, fenitrothion, cyfluthrin, deltamaethrin, etofenprox and lambdacyhalothrin resistance phenotypes were expressed most frequently among all insecticides evaluated, as no knockdown of *Cx. vishnui* were recorded during the

Table 2. Malathion, temephos and permethrin susceptibility for *Cx. vishnui* larval strain

Cx. vishnui collected	Malathion		Temephos		Permethrin	
	LC_{50} (mg/L) (95% C.L.)	Resistance Ratio (RR)	LC_{50} (mg/L) (95% C.L.)	Resistance Ratio (RR)	LC_{50} (mg/L) (95% C.L.)	Resistance Ratio (RR)
Ref. strain	0.194 (0.175-0.215)	–	0.053 (0.030-0.081)	–	0.032 (0.021-0.043)	–
January	0.241 (0.215-0.277)	1.24[a]	0.077 (0.008-0.253)	1.45[a]	0.044 (0.032-0.060)	1.38[a]
February	0.228 (0.206-0.257)	1.18[a]	0.077 (0.052-0.109)	1.45 [a]	0.053 (0.040-0.072)	1.66[a]
March	0.227 (0.202-0.258)	1.17[a]	0.068 (0.042-0.101)	1.28 [a]	0.050 (0.036-0.072)	1.56[a]
April	0.236 (0.213-0.266)	1.22[a]	0.138 (0.039-1.509)	2.60 [a]	0.060 (0.047-0.081)	1.88[a]
May	0.273 (0.244-0.307)	1.41[a]	0.112 (0.075-0.166)	2.11 [a]	0.072 (0.055-0.096)	2.25[a]
June	0.219 (0.193-0.250)	1.13[a]	0.085 (0.057-0.122)	1.60 [a]	0.045 (0.034-0.059)	1.41[a]
July	0.241 (0.209-0.282)	1.24[a]	0.102 (0.007-0.945)	1.93 [a]	0.047 (0.037-0.059)	1.47[a]
August	0.214 (0.188-0.243)	1.10	0.076 (0.050-0.109)	1.43 [a]	0.046 (0.033-0.063)	1.44 [a]
September	0.296 (0.263-0.346)	1.53[a]	0.119 (0.011-1.780)	2.25 [a]	0.076 (0.057-0.112)	2.38[a]
October	0.278 (0.252-0.309)	1.43[a]	0.077 (0.051-0.109)	1.45 [a]	0.047 (0.036-0.062)	1.47[a]
November	0.268 (0.237-0.304)	1.38[a]	0.070 (0.044-0.102)	1.32 [a]	0.042 (0.032-0.055)	1.31[a]
December	0.274 (0.244-0.309)	1.41[a]	0.075 (0.003-0.295)	1.42 [a]	0.051 (0.039-0.068)	1.59[a]
Mean	0.250 ± 0.008[b]	1.30	0.090 ± 0.07[b]	1.70	0.053 ± 0.03[b]	1.66

[a] CL does not overlap with the reference strain and significantly different from the reference strain.
[b] LC_{50} values of all strains were not significantly different between each month, F = 0.049; df = 11,24; P = 1.00, Turkey test.
C.L. = Confidence Limit
Ref. = Reference

exposure period. On the other hand, across the study periods, no knockdown of *Cx. vishnui* was recorded from DDT (4 months), propoxur (4 months) and permethrin (7 months) within the exposure periods (Table 3).

The results from the susceptibility tests revealed that adults of *Cx. vishnui* from the study site were highly resistant to all tested insecticides with a mean percentage mortality of < 50% (Table 4) compared to the reference strain.

The Spearman rank order correlation showed significant positive correlations between cyfluthrin and deltamethrin as well as permethrin and malathion in terms of mortality rate in females *Cx. vishnui* in the study site (Figure 1 & Figure 2).

Table 3. Percentage knockdown of *Cx. vishnui* adults exposed to various adulticides within a certain exposure period

Cx. vishnui collected	Knockdown (%)									
	Organochlorine		Carbamate	Organophasphate		Pyrethoid				
	DDT 4%	Dieldrin 4%	Propoxur 0.1%	Malathion 5%	Fenitrothion 1%	Permethrin 0.25%	Cyfluthrin 0.15%	Deltamethrin 0.025%	Etofenprox 0.05%	Lambdacyhalothrin 0.025%
Ref. Strain	20.01 ± 6.67	15.56 ± 2.22	35.57 ± 5.88	13.34 ± 6.67	28.90 ± 2.22	51.14 ± 8.02	51.14 ± 15.56	66.70 ± 20.38	46.69 ± 16.79	53.36 ± 20.38
January	22.00 ± 2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
February	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
March	2.22 ± 2.22	0.00	4.45 ± 4.45	0.00	0.00	2.22 ± 2.22	0.00	0.00	0.00	0.00
April	24.46 ± 13.52	0.00	4.45 ± 2.22	0.00	4.45 ± 4.45	15.56 ± 4.45	0.00	2.22 ± 2.22	0.00	0.00
May	5.45 ± 5.45	0.00	6.67 ± 3.85	2.22 ± 2.22	0.00	2.22 ± 2.22	0.00	0.00	0.00	0.00
June	0.00	0.00	0.00	0.00	0.00	2.22 ± 2.22	0.00	0.00	0.00	0.00
July	0.00	0.00	6.67 ± 6.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00
August	2.22 ± 2.22	0.00	6.67 ± 3.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00
September	0.00	0.00	2.22 ± 2.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00
October	11.12 ± 8.02	0.00	2.22 ± 2.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00
November	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
December	11.12 ± 11.12	0.00	0.00	2.22 ± 2.22	2.22 ± 2.22	4.45 ± 4.45	0.00	0.00	0.00	0.00
Mean	6.55 ± 2.54[a]	0.00[a]	2.78 ± 0.83[a]	0.37 ± 0.25[a]	0.56 ± 0.40[a]	2.23 ± 1.28[a]	0.00[a]	0.19 ± 0.19[a]	0.00[a]	0.00[a]

[a] Percentage knockdown were no significantly different between each month, F = 1.284; df = 11,108; P = 0.244, Turkey test.
Ref. = Reference

Table 4. Percentage mortality of *Cx. vishnui* exposed to various adulticides 24 h post-treatment

Cx. vishnui collected	Mortality (%)									
	Organochlorine		Carbamate	Organophasphate		Pyrethoid				
	DDT 4%	Dieldrin 4%	Propoxur 0.1%	Malathion 5%	Fenitrothion 1%	Permethrin 0.25%	Cyfluthrin 0.15%	Deltamethrin 0.025%	Etofenprox 0.05%	Lambdacyhalothrin 0.025%
Ref. Strain	80.04 ± 3.85	66.70 ± 3.85	73.37 ± 6.67	82.26 ± 2.22	91.14 ± 4.43	93.36 ± 3.84	88.92 ± 5.87	82.26 ± 11.12	88.92 ± 5.87	86.69 ± 7.69
Jan	60.11 ± 3.76	13.34 ± 3.85	35.55 ± 9.69	37.78 ± 2.22	4.45 ± 4.45	44.44 ± 5.88	17.79 ± 2.22	37.80 ± 2.22	40.02 ± 6.67	48.91 ± 5.88
Feb	23.35 ± 3.34	10.01 ± 10.01	6.67 ± 6.67	43.36 ± 3.34	16.68 ± 16.68	56.70 ± 3.34	50.03 ± 3.34	43.36 ± 3.34	16.68 ± 3.33	36.69 ± 3.34
Mar	35.58 ± 13.52	31.13 ± 5.88	24.46 ± 5.88	26.68 ± 7.70	42.24 ± 5.88	28.90 ± 4.45	28.90 ± 4.45	48.91 ± 5.88	55.58 ± 4.45	60.03 ± 7.70
Apr	57.81 ± 5.88	40.02 ± 3.85	24.46 ± 5.88	22.23 ± 5.88	44.47 ± 2.22	42.24 ± 5.88	42.24 ± 5.88	44.47 ± 9.69	55.58 ± 4.45	60.03 ± 7.70
May	35.57 ± 5.88	22.23 ± 2.22	35.57 ± 4.45	28.90 ± 11.76	40.02 ± 7.70	40.02 ± 3.85	13.34 ± 7.70	31.13 ± 5.88	17.79 ± 5.88	35.57 ± 2.22
Jun	33.35 ± 6.67	30.02 ± 3.34	26.68 ± 6.67	33.35 ± 13.34	36.69 ± 3.34	40.02 ± 6.67	40.02 ± 6.67	53.36 ± 6.67	36.69 ± 3.34	33.35 ± 6.67
Jul	28.90 ± 5.88	31.13 ± 8.02	33.35 ± 3.85	37.80 ± 5.88	51.14 ± 2.22	37.80 ± 5.88	44.47 ± 4.45	44.47 ± 2.22	37.80 ± 5.88	48.91 ± 5.88
Aug	24.46 ± 2.22	35.57 ± 4.45	37.80 ± 2.22	24.46 ± 2.22	37.80 ± 4.45	35.57 ± 4.45	48.91 ± 9.69	46.69 ± 3.85	44.47 ± 5.88	44.47 ± 8.01
Sept	23.25 ± 3.34	46.69 ± 6.67	23.25 ± 3.34	33.35 ± 6.67	30.02 ± 3.34	30.02 ± 10.01	46.69 ± 6.67	53.36 ± 6.67	33.35 ± 6.67	46.69 ± 6.67
Oct	46.69 ± 7.70	24.46 ± 2.22	17.79 ± 8.02	42.24 ± 8.89	48.91 ± 17.36	48.91 ± 9.69	64.48 ± 5.88	46.69 ± 13.88	33.35 ± 0.00	48.91 ± 5.88
Nov	30.02 ± 3.34	36.69 ± 3.34	40.02 ± 0.00	33.35 ± 6.67	23.35 ± 3.34	43.36 ± 3.34	43.36 ± 3.34	36.69 ± 3.34	26.68 ± 6.67	56.70 ± 3.34
Dec	46.69 ± 13.88	42.24 ± 14.58	60.03 ± 6.67	42.24 ± 2.22	42.24 ± 2.22	42.24 ± 5.88	33.35 ± 7.70	40.02 ± 0.00	44.47 ± 4.45	37.80 ± 12.38
Mean	37.14 ± 3.71[a]	30.29 ± 3.22[a]	30.47 ± 3.83[a]	33.81 ± 2.06[a]	34.83 ± 4.00[a]	37.52 ± 3.71[a]	39.47 ± 4.11[a]	43.93 ± 1.92[a]	36.87 ± 3.62[a]	44.01 ± 4.41[a]

Percentage mortality recorded 24 h after the initial exposure time.
[a] Percentage mortality were no significantly different between each month, F = 1.425; df = 11,108; $P = 0.172$, Turkey test.
Ref = Reference

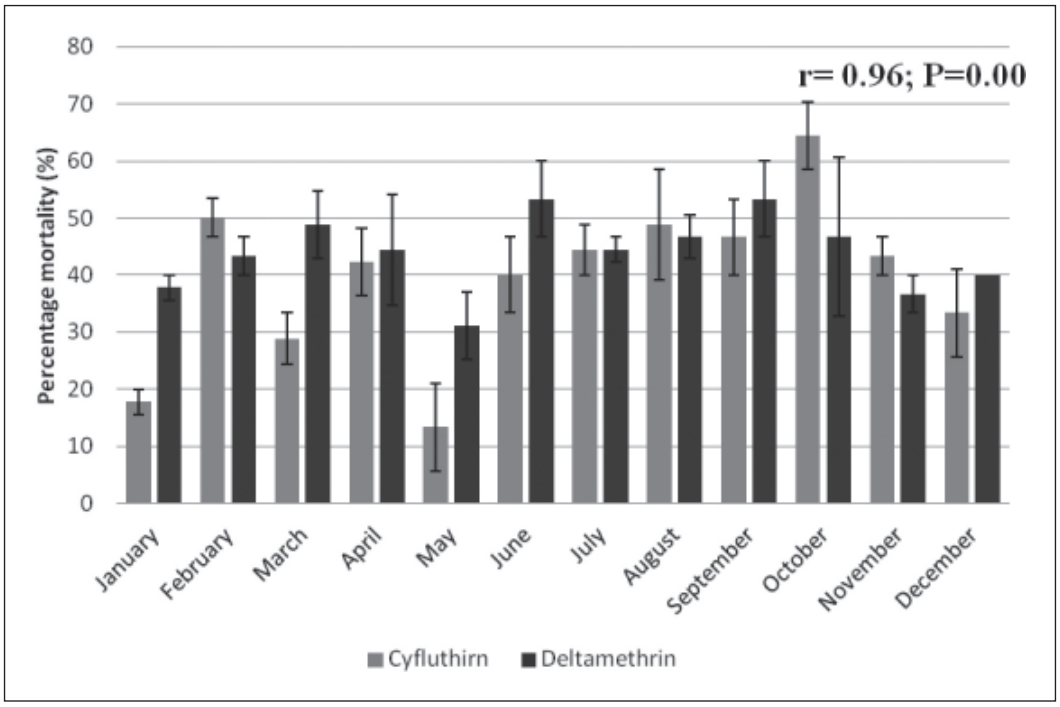

Figure 1. Spearman rank-order correlation between percentage mortality of *Culex vishnui* exposed to cyfluthrin and deltamethrin

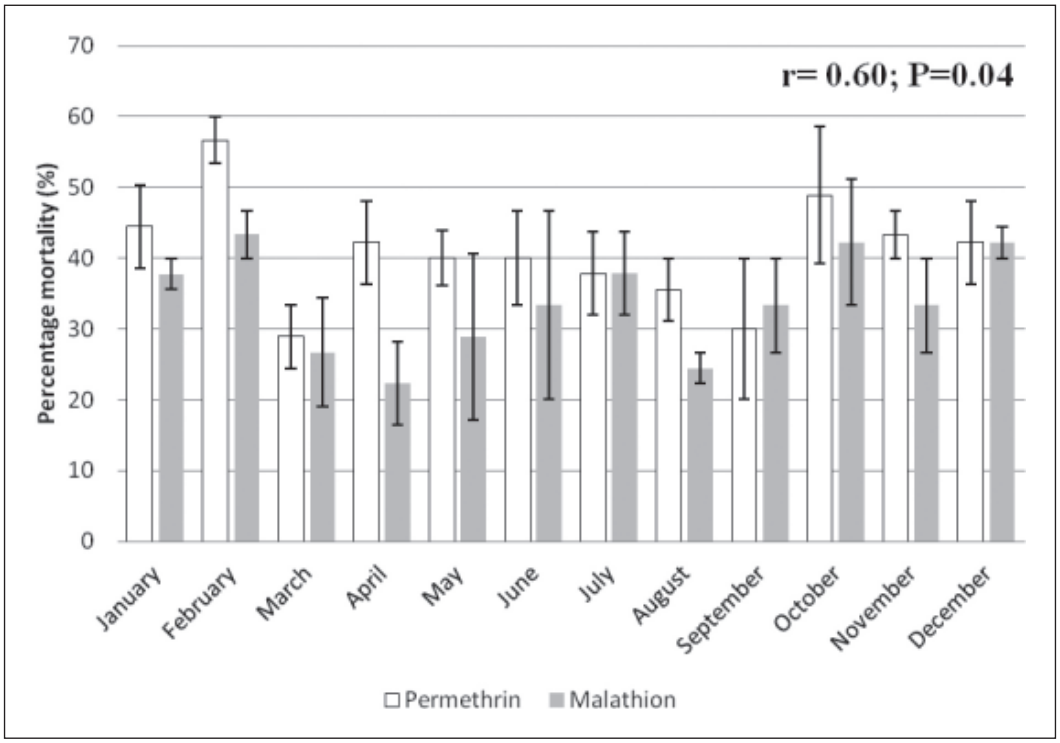

Figure 2. Spearman rank-order correlation between percentage mortality of *Culex vishnui* exposed to permethrin and malathion

Table 5. Mean MFO, GST, ESTα and ESTβ in population of *Cx. vishnui*

Cx. vishnui collected	MFO (Absorbance 630nm)	RR (MFO)	GST (CDNB-ŋmol/min/mg protein)	RR (GST)	ESTα (α-naphtol ŋmol/min/mg protein)	RR (EST-α)	ESTβ (α-naphtol ŋmol/min/mg protein)	RR (EST-β)
Ref. strain	0.57 ± 0.02	–	0.15 ± 0.01	–	0.28 ± 0.01	–	0.24 ± 0.00	–
January	0.49 ± 0.03	0.86	0.11 ± 0.00	0.73	0.30 ± 0.01	1.07	0.25 ± 0.01	1.04
February	0.50 ± 0.24	0.88	0.17 ± 0.00	1.13	0.37 ± 0.02	1.32	0.26 ± 0.01	1.08
March	0.65 ± 0.03	1.14	0.12 ± 0.01	0.80	0.26 ± 0.01	0.93	0.22 ± 0.01	0.91
April	0.58 ± 0.03	1.02	0.11 ± 0.01	0.73	0.21 ± 0.00	0.75	0.20 ± 0.00	0.83
May	0.64 ± 0.04	1.12	0.13 ± 0.01	0.87	0.25 ± 0.01	0.89	0.22 ± 0.01	0.92
June	0.64 ± 0.03	1.12	0.13 ± 0.01	0.87	0.28 ± 0.02	1.00	0.23 ± 0.01	0.96
July	0.71 ± 0.04	1.25	0.13 ± 0.00	0.87	0.23 ± 0.01	0.82	0.20 ± 0.01	0.83
August	0.46 ± 0.02	0.81	0.12 ± 0.00	0.80	0.24 ± 0.01	0.86	0.21 ± 0.01	0.88
September	0.52 ± 0.03	0.91	0.12 ± 0.00	0.80	0.26 ± 0.02	0.93	0.21 ± 0.01	0.88
October	0.79 ± 0.03	1.39	0.14 ± 0.00	0.93	0.22 ± 0.01	0.79	0.21 ± 0.01	0.88
November	0.53 ± 0.02	0.93	0.12 ± 0.01	0.80	0.23 ± 0.01	0.82	0.20 ± 0.00	0.83
December	0.63 ± 0.03	1.11	0.14 ± 0.01	0.93	0.34 ± 0.02	1.21	0.21 ± 0.00	0.88
Mean	0.59 ± 0.03	1.04	0.13 ± 0.00	0.87	0.27 ± 0.01	0.96	0.22 ± 0.01	0.92

The mean mixed oxidase function (MFO), glutathione S-transferases (GST) and non-specific esterases α and β (ESTα and ESTβ) activities in *Cx. vishnui* are shown in Table 5. The mean values of MFO, GST, ESTα and ESTβ activities in *Cx. vishnui* ranged from 0.46–0.79 at absorbance 630nm, 0.49–0.79 CDNB-ŋmol/min/mg protein, 0.11- 0.17 CDNB-ŋmol/min/mg protein, 0.21- 0.37 α-naphtol ŋmol/min/mg protein and 0.20-0.26 α-naphtol ŋmol/min/mg protein, respectively. It is important to highlight that the GST activity of *Cx. vishnui* was lower with resistance ratio values < 1 when compared with the reference strain. On the other hand, 7 out of the 12 populations of field collected *Cx. vishnui* showed resistance ratio of > 1 when compared with the reference strain and in terms of the MFO absorbance of 630nm expressed. Meanwhile, the low resistance ratio of ESTα and ESTβ in field collected *Cx. vishnui* ranged from 0.75-1.32 and 0.83-1.08, respectively.

The Spearman rank-order correlation indicated a significant link between the survival rate of *Cx. vishnui* exposed to lambdacyhalothrin and the GST activity (r = 0.71; P = 0.02), ESTα activity (r = 0.64; P = 0.05), and ESTβ activity (r = 0.69; P = 0.03) in the biochemical assays (Figure 3). On the other hand, there is a significant correlation between the survival rate of *Cx. vishnui* exposed to fenitrothion and the ESTα activity (r = 0.61; P = 0.05) and ESTβ activity (r = 0.64; P = 0.04) respectively in the biochemical assays (Figure 4). There is a significant correlation between the survival rate of *Cx. vishnui* exposed to dieldrin and the ESTβ activity (r = 0.70; P = 0.01) (Figure 5). Meanwhile, no correlation was found between the other insecticides and enzymes in the biochemical assays (df = 11,47; F = 0.66; *P* = 1.00).

DISCUSSION

Insecticide application is generally regarded as an efficient way for pest/vector control. However, the development of resistance against insecticides has jeopardised this control method and failure has been shown in vector control programs in regions where resistance has been reported in vector insects (Fonseca-Gonzalez *et al.*, 2009).

In this study, we found that both the larvae and adults of *Cx. vishnui* obtained from the study site were highly resistant to all insecticides tested. This occurrence of

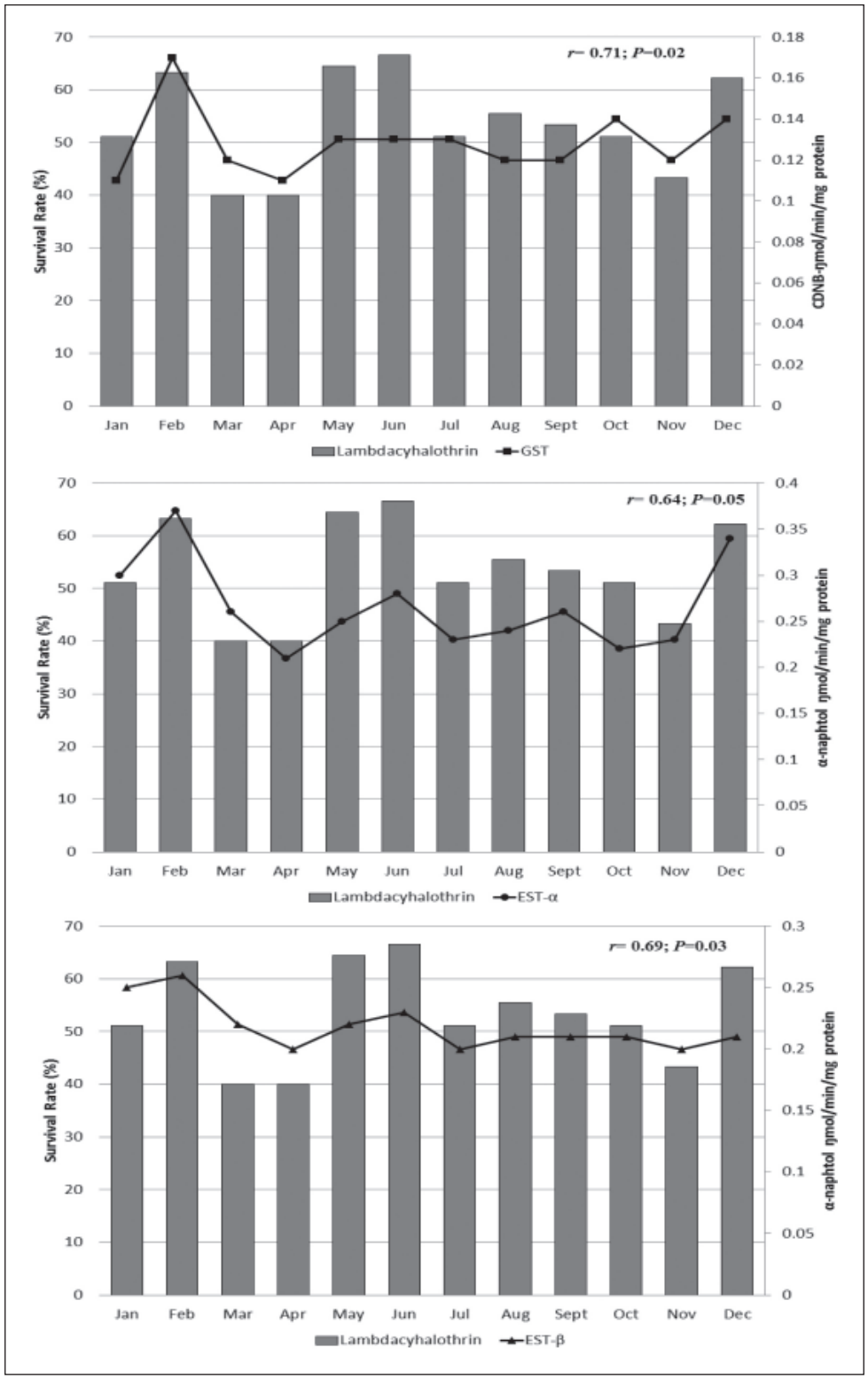

Figure 3. Spearman rank-order correlation between the survival rates of adult *Cx. vishnui* exposed to lambdacyhalothrin and GST, ESTα and ESTβ activities

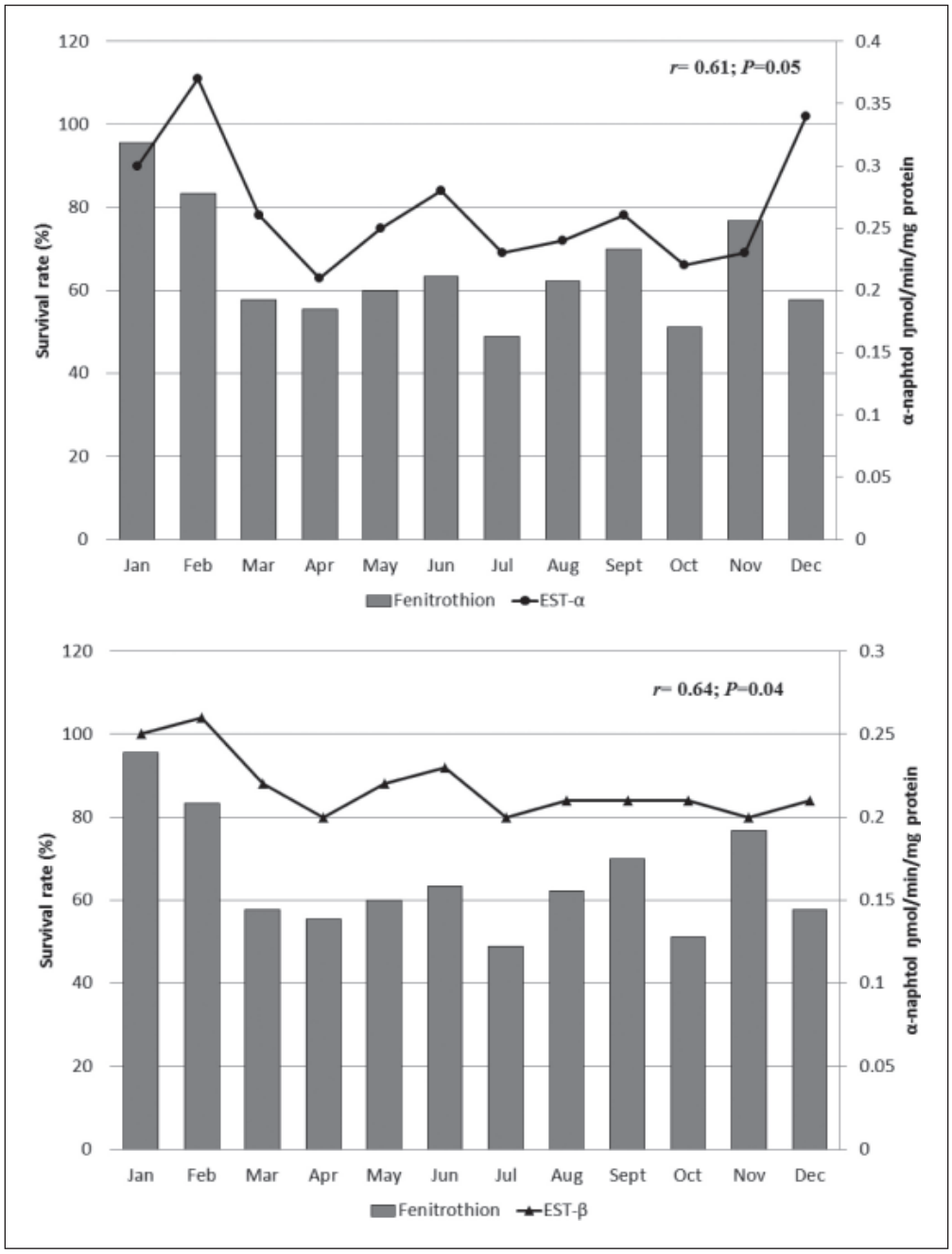

Figure 4. Spearman rank-order correlation between the survival rates of adult *Cx. vishnui* exposed to fenitrothion and ESTα and ESTβ activities

resistance against various insecticides might be due to the selection of insecticides as a result of agricultural activity in the study area. Continuous pressure by these insecticides will result in the development of one or more resistance genes in the mosquitoes because the compounds share the same target site or mode of action (Liu *et al.*, 2004; Selvi *et al.*, 2005). Apart from insecticides, certain fungicides and herbicides used for agricultural crops also synergistically

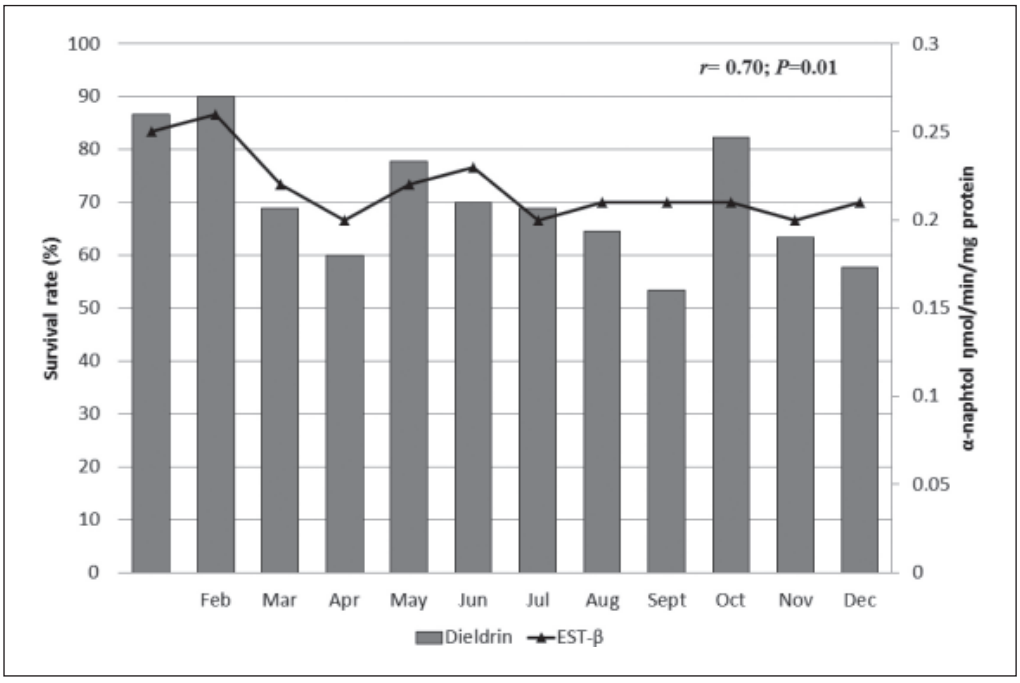

Figure 5. Spearman rank-order correlation between the survival rates of adult *Cx. vishnui* exposed to dieldrin and ESTβ activity

promote the efficacy of insecticides targeted against mosquitoes (Georghiou, 1990). Study conducted in a cotton area and a non-cotton area found that the adult mosquito population within the cotton area was suppressed only mildly by the initial treatments of insecticides and that its density recovered steadily, and by the end of spraying season it had reached a similar level to the non-cotton area, highlighting that the problem of mosquito resistance in agricultural areas where chemical insecticides are heavily used (Georghoiu, 1990). The emergence of insecticide resistance in mosquitoes due to agricultural activities were also reported in El Salcador (Georghiou, 1971), Central America and India (Chapin & Wasserstrom, 1981), Thailand (Overgaard *et al.*, 2005) and Northern Camerron (Muller *et al.*, 2008). In other words, mosquito populations in non-agricultural areas are often more susceptible against insecticides when compared to agricultural areas, although both areas have received equal number of treatments by public health authorities (Georghiou, 1990). However, in our study it was unfortunate that we were unable to get mosquitoes from non-agricultural areas and thus were not able to show the difference.

Field collected adult *Cx. vishnui* in our study exhibited resistance against all insecticides tested and statistical analysis indicated that there was a significant correlation between malathion and permethrin resistance as well as between cyfluthrin and deltamethrin resistance in adult *Cx. vishnui*. Cross-resistance between pyrethroids and organophosphates in mosquitoes has been reported frequently in a number of studies (Bisset *et al.*, 1997; Somboon *et al.*, 2003; Paeporn *et al.*, 2004; Sathantriphop *et al.*, 2006a; Daaboub *et al.*, 2008). A study in Cuba suggested that cross-resistance between pyrethroids and organophosphates may be due to over-production of esterase B in the mosquitoes (Bisset *et al.*, 1997). In studies reported by Sathantriphop *et al.* (2006b) and Low *et al.* (2013), cross-resistance between propoxur and permethrin in *Cx. quinquefasciatus* were reported but the resistance mechanisms have not yet been determined. Inversely, other studies have also reported

the cross-resistance of carbamates and pyrethroids in *Anopheles gambiae and Culex pipiens* and suggest the involvement of mutation of *kdr* and *ace-1*^{R} genes in the mosquitoes that significantly increase the activities in non-specific esterases, glutathione-S-transferases and oxidases (Berticat *et al.*, 2008; Dabire *et al.*, 2012; Koffi *et al.*, 2012). Furthermore, identification of the *ace-1*^{R} resistance gene was also related to organophosphate and carbamate resistance, as observed in *Cx. vishnui* and *Cx. tritaeniorhynchus* (Alout *et al.*, 2007).

Our findings have shown that both adult and larvae of *Cx. vishnui* collected from the study site were multi-insecticides resistant. *Culex quinquefasciatus* has been reported to exhibit multi-insecticides resistant in Thailand (Somboon *et al.*, 2003; Sathantriphop *et al.*, 2006b) and Malaysia Low *et al.* (2013); however, the actual mechanisms contributing to multi-insecticides resistance were not investigated in these studies. In many cases, detoxifying enzymes (NSEs, GSTs and MFOs) play major roles in increasing the survival rate of mosquitoes when exposed to insecticides in standard WHO assays (Nwane *et al.*, 2013). Commonly, MFOs play a major role in detoxifying pyrethroids and organochlorine (Etang *et al.*, 2007; Muller *et al.*, 2008); a minor role in detoxifying organophosphates and carbamates (Hemingway & Ranson, 2000). Glutathione S-transferases are responsible in detoxifying organochlorine and pyrethoids (Grant *et al.*, 1991; Djouaka *et al.*, 2008). Esterases were reported as major detoxifying enzymes for organophosphate insecticides; however, esterases also play a minor role in detoxifying carbamates and pyrethroids (Bisset *et al.*, 1997; Hemingway & Ranson, 2000). Apart from a single enzyme causing multi-insecticides resistance in mosquitoes; several studies mentioned above have reported development of multi-insecticides resistance via interaction between various detoxifying enzymes. A study reported that MFOs and esterases contributed to DDT and lambdacyhalothrin resistance in *An. darling* (Fonseca-Gonzalez *et al.*, 2009). Also, MFOs, esterases and GSTs have been involved in DDT, entofenprox, lambdacyhalothrin, permethrin and fenitrothion resistance in *Ae. aegypti* (Fonseca-Gonzalez *et al.*, 2011).

In the present study, adult *Cx. vishnui* mosquitoes collected from the study site were evaluated for biochemical assays and exhibited dissimilar trend in susceptibility in relation to the three enzymes tested. The occurrence of these may be due to the monthly variation in resistance status of *Cx. vishnui* against various insecticides. Variations in gene frequencies of the Malaysian *Cx. quiqnuefascaitus* have been reported by Lee & Tadano (1994). In that study, the occurrence of time dependent variations was supposedly due to the continuous flow of genetic variations between field mosquito populations, provided by a vast gene pool (Lee *et al.*, 1998).

Results in Table 5 showed that MFO activity in *Cx. vishnui* recorded the highest activity compared to the other enzymes. This may be due to the usage of insecticide formulation containing pyrethoids by farmers to control agricultural pests in the paddy field. By interviewing the rice farmers, it was known that they had applied insecticide and fungicide containing both lambdacyhalothrin (Karate 5EC®) and cypermethrin (Nurelle D505®) in the field (personal communication). Since MFO is usually associated with pyrethroid resistance and this has been reported in *Cx. quinquefasciatus* (Rodriguez *et al.*, 1995), therefore, the application of pesticides containing pyrethoids in the study site may result in the development of high MFO activity.

The biochemical assays have indicated that more than 80% of *Cx. vishnui* strains collected from the study site have lower GST, ESTα and ESTβ activities compared to the reference strain. However, the adult bioassays indicated that *Cx. vishnui* was resistant to all insecticides tested (include organophosphate, organochlorine groups). This occurrence of resistance against

various insecticides might be due to selection pressure against the insecticides, as part of the agricultural practices in the paddy field area; and this continuous pressure led to the development of one or more resistance genes in mosquito. Thus could be due to the active ingredients that share the same target site or mode of action (Liu *et al.*, 2004; Selvi *et al.*, 2005). In the study by Fonseca-Gonzalez *et al.* (2009), *An. darlingi* collected from Choco was resistant against DDT and lambdacyhalothrin; however, DDT has not been used for the last 17 years. Thus, they suggested that this cross-resistance was due to the application of lambdacyhalothrin in the study site, since both insecticides shared the same target site, sodium channels of the central nervous system (Soderlun *et al.*, 1989).

Results showed that there was a positive significant correlation between survival rates of *Cx. vishnui* exposed to fenitrothion, and ESTα and ESTβ activities in *Cx. vishnui*. Esterase α and esterase β have been reported to be associated with organophosphate and fenitrothion resistance in *Cx. quinquefasciatus* (Bracco *et al.*, 1999), *An. albimanus* (Brogdon & Barber, 1990), *Ae. aegypti* (Pethuan *et al.*, 2007) and the beetles, *Oryzaephilus surinamensis* (Lee *et al.*, 2000).

On the other hand, Spearman rank-order analysis indicated that there was a significant correlation between survival rates of *Cx. vishnui* exposed to dieldrin and ESTβ activity. Usually, dieldrin resistance in insect is associated with γ-aminobutyric acid (GABA) receptor mutation (Lee & Kwon, 2011); however, our study revealed that dieldrin resistance was associated with ESTβ. Bisset *et al.* (1997) found that overproduction of ESTβ contributed to pyrethroids resistance in *Cx. quiqnuefasciatus*. Since both pyrethroid and organochlorine share the same target site; therefore, we suggest that the cross-resistance was caused by the over-production of ESTβ.

Spearman rank-order analysis has showed that there were correlations between survival rates of *Cx. vishnui* exposed to lambdacyhalothrin, and GST, ESTα and ESTβ activities. A study conducted by Fonseca-Gonzalez *et al.* (2011), found that *Ae. aegypti* collected from Choco and Putumaya, Colombia were resistant to lambdacyhalothrin due to its extensive usage in malaria and dengue control, and the biochemical assays revealed high levels of non-specific esterases (EST) and GST activities in *Ae. aegypti*. Another study also showed that non-specific esterase and glutathione S- transferase were associated with pyrethroids resistance (Yaicharoen *et al.*, 2005). Another study conducted by Jagadeshwaran & Vijayan (2009), found high enzyme activities of ESTα, ESTβ, GST and MFO in *Ae. aegypti* after selection against deltamethrin for 20 consecutive generations. In the present study, correlations of lambdacyhalothrin with GST, ESTα and ESTβ activities were probably due to the application of lambdacyhalothrin in the study site for a prolonged period of time. Moreover, cypermethrin (pyrethroid) was also used to control pests in paddy field, and continuous use of this insecticide may have contributed to the increase of the enzymes activities in *Cx. vishnui*.

Results of the current study suggest that the insecticides used in agricultural activity led to the development of resistance in *Cx. vishnui* population in Sekinchan, Selangor state. The findings of the current study may assist local authorities by providing an updated baseline data on the resistance of the mosquito which can be used for selecting insecticides and its the application rates for vector control. Therefore, continuous monitoring of mosquitoes susceptibility against commonly used insecticides is a necessity to ensure that the insecticides used are effective. Application of inappropriate insecticides without a proper understanding of the resistance mechanisms in mosquitoes may result in control failure. Hence, early detection of resistance status and knowledge of resistance mechanisms in mosquitoes are

essential steps for effective mosquito control. Further work should be carried out using molecular tools for a much better understanding of the resistance mechanisms involved.

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