Pyrethroid Resistance in *Aedes aegypti* Larvae (Diptera: Culicidae) From Singapore

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ABSTRACT We report the first comprehensive insecticide susceptibility status of *Aedes aegypti* (L.) larvae from Singapore. The study indicated that *Ae. aegypti* is susceptible to temephos, although resistance (RR_{50} = 1.29–4.43-fold) could be developing. Of high concern is the detection of moderate to high resistance to permethrin (RR_{50} = 29–47-fold) and etofenprox (RR_{50} = 14–34-fold). Biolarvicide *Bacillus thuringiensis israelensis* (*Bti*) remains effective. The insecticide susceptibility profile of *Ae. aegypti* larvae was found to be homogenous among the different sites studied across the island city. The addition of synergists piperonyl butoxide, *S,S,S*,-tributyl phosphorotrithioate, and triphenyl phosphate generally failed to enhance the toxicity of the insecticides investigated, suggesting an insignificant role of metabolic-based resistance, and a possible involvement of target site resistance. Further biochemical investigation of specific metabolic enzyme activities suggested that detoxifying enzymes, mono-oxygenases, esterases, glutathione S-transferases, and altered acetylcholinesterases, generally did not contribute to the resistance observed. This study clearly demonstrated that pyrethroid resistance is widespread among *Ae. aegypti* population and lowered susceptibility to organophosphates is developing.

KEY WORDS insecticide resistance, synergist, biochemical assay, Singapore, dengue

Aedes aegypti (L.) is the most important disease vector that lives closely with humans in the urban environment. This principal vector of dengue is responsible for an alarming incidence of dengue cases worldwide. It is estimated that 2.5 billion people are at risk of dengue globally and 50 million infections with 20,000 deaths occur annually (World Health Organization [WHO] 2011). In Singapore, *Ae. aegypti* is the primary dengue vector. Dengue (dengue hemorrhagic fever) was first reported in the 1960s (Lim et al. 1961). A vector control program, established since the 1960s, has brought the disease incidence to a low level for >10 yr along with a reduction of house index from ≈50% in the 1960s to <1% today (Tan 1997, Ooi et al. 2006). However, since the early 1990s, dengue has become a reemerging challenge (Goh 1995). The worst dengue outbreak in 2005 with 14,209 cases (Ang et al. 2007) prompted a revamping of the system, which has since successfully decreased the number of dengue cases to an average of 5,696 yearly.

While source removal remains the mainstay of vector control to deprive the mosquitoes of larval habitats, larvicides are easily available and often used in habitats that cannot be easily accessed or removed (e.g., roof gutters). Temephos sand granules (Abate) have been used for decades, whereas *Bacillus thuringiensis israelensis (Bti*) has become common since 2004.

Ae. aegypti's preference for artificial containers such as water-storage tanks offers us an opportunity to target the mosquitoes at the immature stage. Larviciding, which eliminates vectors at source, is more effective in controlling vector population than fogging (Anonymous 2006). Other advantages include minimal killing of nontarget insects and reduced exposure of public to insecticides. In a sound integrated pest management program, the focus should only shift to larviciding when source reduction is not feasible.

Unfortunately, the indiscriminate and widespread use of different chemical compounds has led to the development of insecticide resistance in many countries, and this has posed a great challenge in vector control management (Penilla et al. 1998, Rivero et al. 2010, Perry et al. 2011). This challenge is further exacerbated by the limited number of insecticide classes and the occurrence of cross-resistance.

Insects have evolved and developed various insecticide resistance mechanisms, two of which play important roles. They are the metabolic-based resistance and target site insensitivity (Brogdon and McAllister 1998, Hemingway and Ranson 2000, Ranson et al. 2011). The former mechanism involves three groups of enzymes, namely, mono-oxygenases (MFOs), es-

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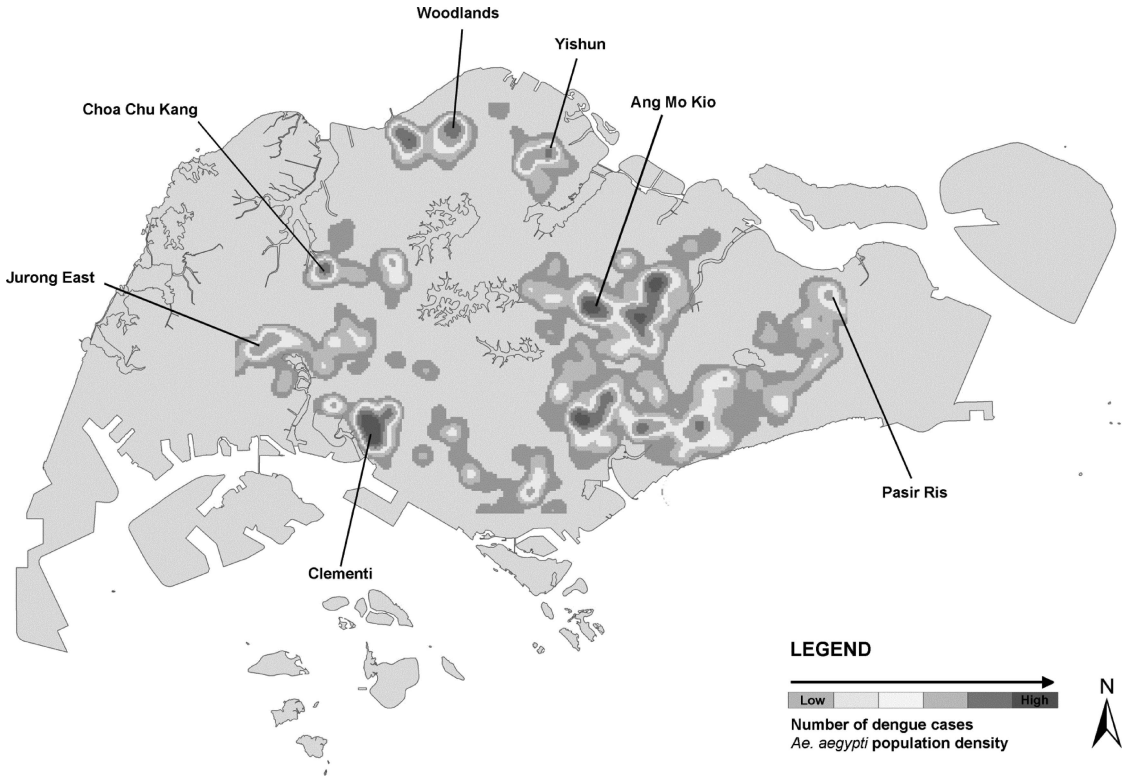

Fig. 1. Map of Singapore showing the sites where populations of *Ae. aegypti* were collected, the distribution of dengue cases, and *Ae. aegypti* breeding from 2001 to 2007.

terases (ESTs), and glutathione S-transferases (GSTs) (Liu et al. 2006), whereas the latter is associated with three target sites, namely, voltage-gated sodium channels, gamma-aminobutyric acid receptors, and acetylcholinesterases (Rivero et al. 2010).

Synergists have been developed to counteract insecticide resistance. However, they are effective only when resistance is due to enzyme-based resistance mechanisms. Synergists are compounds that are nontoxic to the insects when used on their own, but increase the toxicity or effectiveness of insecticides against these pests (Yu 2008). Synergists such as piperonyl butoxide (PBO), *S,S,S,*-tributyl phosphorotrithioate (DEF), and triphenyl phosphate (TPP) are commonly used in combination with insecticides for controlling mosquitoes and other insects (Lorini and Galley 2000).

This report presents the findings of a study to determine the susceptibility status of field populations of *Ae. aegypti* to some common larvicides used in Singapore. Bioassays and biochemical analysis were performed on field populations of *Ae. aegypti* larvae. The effectiveness of synergists on insecticides was also investigated.

Materials and Methods

Experimental Design. The Bora-Bora strain, a reference susceptible strain (F112) that has never been exposed to insecticide, was used to define the diagnostic dose of each insecticide via a baseline assay. Using the diagnostic dose, which is defined as two times (2×) lethal concentration that kills 99% of the reference population tested ($LC_{99} \times 2$), the mortality rate of F2 generation of field-collected mosquitoes was determined. Based on the mortality rate, a range of concentration for each insecticide was prepared and tested against each field strain, to obtain LC_{50} and LC_{99} of each insecticide. In separate experiments, synergists were included to determine their effects on mortality rate at diagnostic rates. Biochemical assays were performed on the same batch of mosquitoes (F1 generation). Supp Fig. 1 [online only] illustrates the experimental design of the study.

Mosquitoes. From January 2010 to March 2011, *Ae. aegypti* was collected using ovitraps from seven dengue-prone public residential areas (government-built flats) in Singapore. These sites (Ang Mo Kio, Jurong East, Yishun, Choa Chu Kang, Clementi, Pasir Ris, and Woodlands) were selected based on the relatively higher number of reported dengue cases and *Ae. aegypti* indoor breeding from 2001 to 2007 (Fig. 1; Table 1). Eggs and mosquitoes from each site were processed and maintained as a single colony. To collect mosquitoes, ovitraps were filled with hay infusion (Lee et al. 2013) and placed along corridors in shaded areas or near potted plants. The traps were replaced weekly and paddles were dried for 1 or 2 wk before

Table 1. Mosquito collection sites in Singapore during 2010–2011

Locations	Collection sites	No. of flats	Coordinates	Collection period
Ang Mo Kio[a]	Ang Mo Kio St 41	5	1° 21′N 103° 51′E	Jan 10–Feb 10
Jurong East[a]	Jurong East St 13	5	1° 20′N 103° 44′E	Feb 10–May 10
Yishun	Yishun St 71	4	1° 25′N 103° 49′E	Jan 10–April 10
Choa Chu Kang	Choa Chu Kang Ave 3	3	1° 22′N 103° 44′E	Feb 10–May 10
Clementi	Clementi Ave 3	6	1° 18′N 103° 45′E	Jan 10–Feb 10
Pasir Ris	Pasir Ris St 21	4	1° 22′N 103° 57′E	Jan 10–April 10
	Pasir Ris Dr 6	5	1° 22′N 103° 57′E	Jan 10–April 10
Woodlands	Woodlands Circle	7	1° 26′N 103° 47′E	May 10–June 10

Ave, Avenue; Dr, Drive; St, Street.
[a] Conduct second collection in first quarter of 2011.

hatching of eggs. All emerged adults were identified to species by morphological characteristics (Rattanarithikul et al. 2010) and those identified as *Ae. aegypti* were maintained at 25 ± 2°C, 75 ± 5% RH, and a photoperiod of 10:14 (L:D) h with provision of 10% sucrose solution. Adult females aged 5–7 d were allowed to blood-feed on a live guinea pig. The use of a live animal for laboratory work was approved by the Institutional Animal Care and Use Committee at the Environmental Health Institute, National Environmental Agency, Singapore. Biochemical assays were performed using larvae F1 progenies that were frozen at −20°C till used in the experiments. Bioassay studies were conducted with F2 progenies. The Bora-Bora strain (F112) was used for comparison.

Insecticides. Technical-grade insecticides (>90% purity) were used: temephos (90%) obtained from Asiatic Agricultural Industries, Singapore; permethrin (97.1%) from Bayer Cropscience, Bangkok, Thailand (state city); etofenprox (95.3%) from Jiangyin Trust, Jiangyin, Jiangsu, China; and *Bti*, VectoBac WG (3,000 ITU/mg, strain H-14), from Sumitomo Chemical, Singapore. To estimate LC_{50} and LC_{99} of each insecticide for each strain, five different concentrations of each insecticide was prepared according to the WHO protocol (WHO 1981a). Ethanol was used as a solvent.

WHO Bioassays. WHO bioassays were carried out in a disposable, transparent, 500-ml plastic cup containing 249 ml of reverse-osmosis water and 1 ml of insecticide solution. A batch of 20–25 late third- to early fourth-instar larvae were randomly picked and transferred to the cup 10–15 min after adding the insecticide solution. The larvae were exposed for 24 h and mortality was recorded. Moribund larvae were considered dead. As for the *Bti* test, the larvae were added directly to 125 ml of *Bti* suspension.

To establish diagnostic dose, Bora-Bora strain was tested on five concentrations of each insecticide in four replicates, to obtain mortality that ranges from 0 to 100% to generate LC_{50} and LC_{99} values according to WHO guidelines (WHO 1981a, 2005). Five controls using 1 ml of 10% ethanol were used per test.

To assess the susceptibility status of field population to each insecticide, larvae were exposed to the diagnostic dose (obtained from susceptibility baseline), as described earlier. Three tests, each performed on different days, were conducted for each insecticide for each of seven field strains. Similarly, five different concentrations for each insecticide were used to determine lethal concentration of the insecticide that kills 50 (LC_{50}) and 99% (LC_{99}) of test population.

Synergism Tests. Synergism tests were undertaken on field mosquitoes to evaluate their effectiveness on detoxification of insecticides. PBO (94.4%) from Endura Fine Chemicals, DEF (97.1%) from Greyhound Chromatography and Allied Chemicals (Birkenhead, Merseyside, UK), and TPP (99%) from Sigma-Aldrich (Singapore) were used. Larvae were exposed to each synergist at varying concentrations to determine the maximum sublethal concentration. Subsequently, the sublethal doses 0.01, 1, and 5 mg/L for DEF, TPP, and PBO, respectively, were used in synergism tests. Synergism tests were performed similarly to the larvae bioassays, except that the insecticide was mixed with the synergist (1:1) before the test. Each synergist was used in conjunction with all chemicals, except *Bti.*

Biochemical Assays. Different enzyme levels in individual larvae of Bora-Bora and field strains were determined according to the WHO procedure, as previously described by Hemingway (1998). Briefly, fourth-instar larvae were individually homogenized in 200 μl of reverse-osmosis water on ice. Twenty-five microliters of homogenate was used for the acetylcholinesterase assay. The remaining homogenate was centrifuged at 14K, 4°C for 30 s, and the supernatant was used as an enzyme source for all other enzyme assays. Ninety-four larvae per strain were analyzed. The assays were performed in a 96-well microplate on ice and the absorbance (optical density [OD] values) was measured on the microtitre plate reader (ELISA system, Sunrise model) with Magellan data analysis software. Enzyme activities were calculated as described further in the text.

AChE Assay. For each sample, 25 μl of insect homogenate was mixed with 145 μl of triton phosphate buffer, following which 10 μl of 0.01 M dithiobis 2-nitrobenzoic acid solution and 25 μl of 0.01 M acetylthiocholine iodide were added to initiate the reaction. Two such reactions were prepared for each sample. While one reaction was allowed to progress, the other was inhibited using 0.05 μl of 0.1 M propoxur. The OD of both reactions was measured at 405 nm after 1 hour of incubation and the activity was expressed as per-

Table 2. Diagnostic dose (mg/L) of different larvicides based on Bora-Bora strain

Larvicides	*n*	LC_{50} (95% CL) (mg/L)	LC_{99} (95% CL) (mg/L)	Diagnostic dose (mg/L)
Temephos	1,856	0.0073 (0.0070–0.0080)	0.0143 (0.0133–0.0156)	0.0286
Permethrin	1,833	0.0015 (0.0010–0.0020)	0.0040 (0.0030–0.0045)	0.0080
Etofenprox	1,867	0.0060 (0.0060–0.0060)	0.0140 (0.0130–0.0150)	0.0280
Bti	1,854	0.1290 (0.0112–0.1493)	0.5703 (0.4463–0.7837)	1.1406

LC: lethal concentrations, CL: confidence limits, diagnostic dose: $LC_{99} \times 2$.

centage insensitive AChE activity after propoxur inhibition (Saelim et al. 2005).

Nonspecific EST Assay. For each sample, 20 μl of supernatant derived from the insect homogenate was mixed with 200 μl of the substrate, 30 mM α-naphthyl acetate. In parallel, the same samples were also incubated with 30 mM β-naphthyl acetate. After 15 min of incubation at room temperature, 50 μl of fast blue stain was then added to each reaction. The OD value was measured at 570 nm 15 min later. The activity against each substrate was calculated from standard curves of absorbance for known concentrations of α-naphthol or β-naphthol. Enzyme activities are expressed as nmole of α-naphthol or β-naphthol/min/mg protein.

GST Assay. In total, 200 μl of 10 mM reduced glutathione and 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) mixture was added to 10 μl of supernatant derived from the insect homogenate. Absorbance was determined at 340 nm after 20 min of incubation. GST activity was calculated following Beer's Law ($A = \epsilon cl$) and reported as mmole of CDNB/min/mg protein. The OD value (A) was transformed to μmole of CDNB conjugates using the extinction coefficient (ϵ) of 4.39 mM^{-1}. The path length (the depth of the buffer solution in the microplate well, l) is 0.6 cm.

MFO Assay. MFO activity was initiated by the addition of 80 μl of 0.625 M potassium phosphate buffer (pH 7.2), 200 μl of 3,3,5,5-tetramethylbenzidine (TMBZ) in methanol solution, and 25 μl of hydrogen peroxide (3%) to 2 μl of supernatant derived from the insect homogenate. The reaction was allowed to oxidize for 2 hours at room temperature before the OD value was read at 650 nm. MFO activity was calculated from standard curve of absorbance for known concentration of cytochrome C (Brogdon et al. 1997). Enzyme activity is expressed as equivalent units of cytochrome P450/min/mg protein.

Protein Assay. Protein concentration was used as a standard correction factor for the analysis of all enzymes activities, to account for size variances among individuals. The protein concentration was calculated and transformed from the bovine serum albumin standard curve using a commercial kit (Bio-Rad, CA). Ten microliters of homogenate was mixed with 300 μl of Bio-Rad dye reagent and incubated for 5 min. The OD was read at 570 nm.

Data Analysis. Two different resistance classifications (mortality percentage and resistance ratio) were used to indicate the *Ae. aegypti* susceptibility status in this study. Mortality percentage was used to assess the effectiveness of synergists to insecticides, whereas resistance ratio provides stronger evidence of resistance detection.

Bioassay results were expressed in mortality percentage, and the susceptibility status of each population was classified according to Davidson and Zahar (Davidson and Zahar 1973). Insects presenting 98–100% mortality are classified as "susceptible," "resistant" insects would have mortality <80%, and an "intermediate" level would be insects with 80–97% mortality. This classification corresponds to incipient resistance for interpreting the mosquito results on 4% dichlorodiphenyltrichloroethane (DDT). Adjustment was done using Abbott's formula (Abbott 1925) when control mortality was between 5 and 20%. Test was rejected if pupation exceeded 10% (WHO 2005).

Resistance ratio (RR_{50}) was calculated by dividing the LC_{50} value of field strains by the corresponding LC_{50} value of susceptible strain. RR_{50} was scaled as follows: $RR_{50} < 1$ (susceptible), $RR_{50} = 1$–10 (low resistance), $RR_{50} = 11$–30 (moderate resistance), $RR_{50} = 31$–100 (high resistance), and $RR_{50} > 100$ (very high resistance) (Khan et al. 2011).

Mortality percentages and enzyme levels were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's tests, respectively. Non-normal data were transformed to arcsine log to stabilize the variance. Two-samples *t*-test or Mann–Whitney nonparametric test was applied to test the mortality differences between Bora-Bora strain and respective field strains. Statistical significance was assumed at $P < 0.05$. Mann–Whitney nonparametric test was used to analyze the effect of synergism on mortality. All analyses and results interpretation were carried out using SPSS (PASW Statistics 19) software.

Results

Larval Susceptibility Status. The diagnostic doses of the insecticides, derived from the Bora-Bora strain, are summarized in Table 2. Permethrin showed the highest toxicity to Bora-Bora strain, with an LC_{50} value of 0.0015 mg/L, whereas *Bti* showed the least toxicity ($LC_{50} = 0.1290$ mg/L).

In total, 86,492 *Ae. aegypti* larvae from seven locations were tested against all insecticides for all tests. When exposed to temephos, the mortality rates of different field strain larvae ranged from 88.62 to 100% (Table 3). Despite the high mortality rate, RR_{50} derived from LC_{50} values of field strains and Bora-Bora strain range from 1.29 to 4.43-fold. These elevated values show that low levels of resistance to temephos

Table 3. Susceptibility status of *Ae. aegypti* larvae to different insecticides and synergists from various locations in Singapore

Insecticides + synergists	Mean % mortality[a] ± SE							
	Bora-Bora	Ang Mo Kio	Jurong East	Yishun	Choa Chu Kang	Clementi	Pasir Ris	Woodlands
Total exposed (*n*)	1,210	1,255	1,209	1,193	1,198	1,201	1,210	1,202
Temephos only	100 ± 0	88.62 ± 6.13	99.58 ± 0.42	100 ± 0	96.67 ± 3.33	97.73 ± 1.68	98.75 ± 1.25	100 ± 0
Temephos + PBO	100 ± 0	99.59 ± 0.41	79.41 ± 11.34	100 ± 0	99.58 ± 0.42	94.98 ± 1.43	100 ± 0	99.18 ± 0.82
Temephos + DEF	100 ± 0	91.81 ± 4.88	54.83 ± 7.96*	93.26 ± 4.98	82.45 ± 10.95	82.84 ± 5.96	67.89 ± 13.57*	91.74 ± 2.69*
Temephos + TPP	100 ± 0	93.70 ± 3.36	32.92 ± 1.10*	93.00 ± 3.93	91.52 ± 3.98	86.20 ± 1.40*	74.57 ± 12.14	91.67 ± 5.07
Temephos + 3 synergists	100 ± 0	76.37 ± 8.84	15.21 ± 6.45*	42.15 ± 1.10*	91.47 ± 3.02	77.55 ± 7.36	13.41 ± 8.51*	74.25 ± 3.09*
Total exposed (*n*)	1,200	1,282	1,198	1,189	1,206	1,190	1,293	1,206
Permethrin only	100 ± 0	0.42 ± 0.42	6.25 ± 3.82	0.83 ± 0.83	4.65 ± 2.91	6.35 ± 3.86	0.84 ± 0.84	3.67 ± 1.86
Permethrin + PBO	100 ± 0	20 ± 7.11*	15 ± 7.11	37.45 ± 4.24*	12.46 ± 11.84	25.49 ± 8.29	22.52 ± 7.27*	0 ± 0
Permethrin + DEF	100 ± 0	1.23 ± 1.23	0 ± 0*	2.16 ± 0.87	3.34 ± 1.50	1.25 ± 0*	0.43 ± 0.43	0.42 ± 0.42
Permethrin + TPP	100 ± 0	2.53 ± 1.27	0.42 ± 0.42	3.38 ± 3.38	0.83 ± 0.41	4.58 ± 1.50	1.23 ± 1.23	0.42 ± 0.42
Permethrin + 3 synergists	100 ± 0	4.95 ± 1.42*	2.50 ± 0	15.13 ± 1.46*	0.81 ± 0.81	14.43 ± 2.44	0.41 ± 0.41	0 ± 0
Total exposed (*n*)	1,207	1,212	1,205	1,210	1,204	1,200	1,205	1,200
Etofenprox only	100 ± 0	3.24 ± 2.13	4.58 ± 2.20	2.50 ± 1.25	12.86 ± 0.81	10 ± 2.17	6.25 ± 2.50	6.67 ± 1.50
Etofenprox + PBO	100 ± 0	22.78 ± 6.29*	2.82 ± 2.23	16.07 ± 8.74*	36.88 ± 7.21*	26.17 ± 5.63	18.75 ± 3.64*	34.17 ± 3.25*
Etofenprox + DEF	100 ± 0	7.56 ± 1.44	0 ± 0	0.42 ± 0.42	7.49 ± 5.64	5.79 ± 2.98	2.92 ± 0.44	2.50 ± 1.91
Etofenprox + TPP	100 ± 0	1.67 ± 0.83	1.67 ± 0.42	1.65 ± 0.82	6.14 ± 3.71	4.14 ± 2.54	1.24 ± 0.71	6.25 ± 1.91
Etofenprox + 3 synergists	100 ± 0	2.53 ± 2.53	1.67 ± 0.42	2.59 ± 1.52	2.50 ± 1.25*	9.10 ± 1.13	3.36 ± 1.68	8.33 ± 5.88
Total exposed (*n*)	240	244	239	244	237	245	242	239
Bti	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

[a] Mean % mortality followed by asterisk symbol denotes rates that were significantly different when compared with Bora-Bora strain ($P < 0.05$, independent *T*-test).

were detected in all locations. Whereas *Ae. aegypti* larvae from most locations showed similarly low resistance to temephos (RR_{50} = 1.29–1.71-fold), the Jurong East and Choa Chu Kang strains showed higher resistance ratios of 4.43 and 2.43, respectively. Based on the disproportionate values of LC_{99} to LC_{50}, we suggest that larvae from these two locations showed heterogeneous response to temephos.

In contrast to temephos, the diagnostic concentrations of permethrin and etofenprox resulted in low mortality rates for all the field mosquito larvae tested (Table 3). Mortality rates affected by permethrin ranged from 0.42 to 6.35%, whereas that of etofenprox ranged from 2.50 to 12.86%. The resistance of field populations against permethrin and etofenprox suggested by the low mortality rate is corroborated by the moderate to high levels of resistance ratios among the field strains (Table 4), which ranged from 29 to 47-fold for permethrin and from 14 to 34-fold for etofenprox.

Table 4. The toxicity of temephos, permethrin, and etofenprox tested against field *Ae. aegypti* larvae collected from seven locations in Singapore

Insecticide	Strain	*n*	LC_{50} (95% CL) (mg/L)	LC_{99} (95% CL) (mg/L)	Slope	χ^2 (df)	RR_{50}
Temephos	Bora-Bora	1,481	0.007 (0.007–0.008)	0.014 (0.012–0.018)	8.293 ± 0.451	2.27 (3)	–
	Ang Mo Kio	1,200	0.009 (0.008–0.009)	0.015 (0.013–0.021)	9.308 ± 0.622	2.09 (3)	1.29
	Jurong East	1,202	0.031 (0.023–0.060)	0.120 (0.061–0.710)	3.940 ± 0.402	1.99 (3)	4.43
	Yishun	1,191	0.010 (0.010–0.011)	0.022 (0.020–0.025)	6.872 ± 0.370	1.04 (3)	1.43
	Choa Chu Kang	1,209	0.017 (0.014–0.023)	0.041 (0.028–0.100)	6.235 ± 0.346	6.53 (3)	2.43
	Clementi	1,205	0.012 (0.011–0.014)	0.029 (0.022–0.048)	6.076 ± 0.298	4.07 (3)	1.71
	Pasir Ris	1,182	0.011 (0.011–0.012)	0.029 (0.026–0.033)	5.719 ± 0.288	0.47 (3)	1.57
	Woodlands	1,202	0.011 (0.009–0.015)	0.020 (0.015–0.056)	8.968 ± 0.559	8.19 (3)	1.57
Permethrin	Bora-Bora	1,479	0.001 (0.001–0.002)	0.004 (0.003–0.007)	5.728 ± 0.265	7.70 (3)	–
	Ang Mo Kio	1,189	0.046 (0.043–0.049)	0.291 (0.235–0.381)	2.898 ± 0.175	0.23 (3)	46.00
	Jurong East	1,201	0.035 (0.033–0.037)	0.181 (0.157–0.216)	3.259 ± 0.168	0.31 (3)	35.00
	Yishun	1,204	0.029 (0.027–0.031)	0.165 (0.142–0.198)	3.059 ± 0.163	0.75 (3)	29.00
	Choa Chu Kang	1,211	0.047 (0.035–0.063)	0.285 (0.153–1.546)	2.964 ± 0.168	7.01 (3)	47.00
	Clementi	1,201	0.032 (0.030–0.034)	0.183 (0.157–0.221)	3.072 ± 0.163	0.96 (3)	32.00
	Pasir Ris	1,197	0.040 (0.038–0.042)	0.176 (0.154–0.206)	3.623 ± 0.184	1.66 (3)	40.00
	Woodlands	1,210	0.042 (0.028–0.057)	0.147 (0.092–0.638)	4.296 ± 0.204	13.45 (3)	42.00
Etofenprox	Bora-Bora	1,493	0.006 (0.006–0.006)	0.014 (0.013–0.015)	6.740 ± 0.357	0.83 (3)	–
	Ang Mo Kio	1,197	0.128 (0.117–0.146)	0.383 (0.292–0.581)	4.896 ± 0.534	0.11 (3)	21.33
	Jurong East	1,197	0.127 (0.114–0.148)	0.815 (0.552–1.438)	2.886 ± 0.279	0.64 (3)	21.16
	Yishun	1,216	0.084 (0.069–0.117)	0.315 (0.182–1.906)	4.050 ± 0.265	6.86 (3)	14.00
	Choa Chu Kang	1,203	0.120 (0.089–0.568)	0.728 (0.267–581.434)	2.976 ± 0.271	6.57 (3)	20.00
	Clementi	1,199	0.119 (0.106–0.139)	1.130 (0.710–2.255)	2.380 ± 0.239	1.59 (3)	19.83
	Pasir Ris	1,199	0.204 (0.128–2.119)	2.129 (0.529–4109.807)	2.282 ± 0.297	2.78 (3)	34.00
	Woodlands	1,199	0.135 (0.119–0.162)	1.084 (0.683–2.168)	2.572 ± 0.265	0.20 (3)	22.50

LC, lethal concentrations; CL, confidence limits; RR_{50}, resistance ratio values are based on LC_{50} levels of the field strain divided by LC_{50} levels of reference strain (Bora-Bora); χ^2, (χ^2) indicates the goodness of fit of the regression line (Throne et al. 1995).

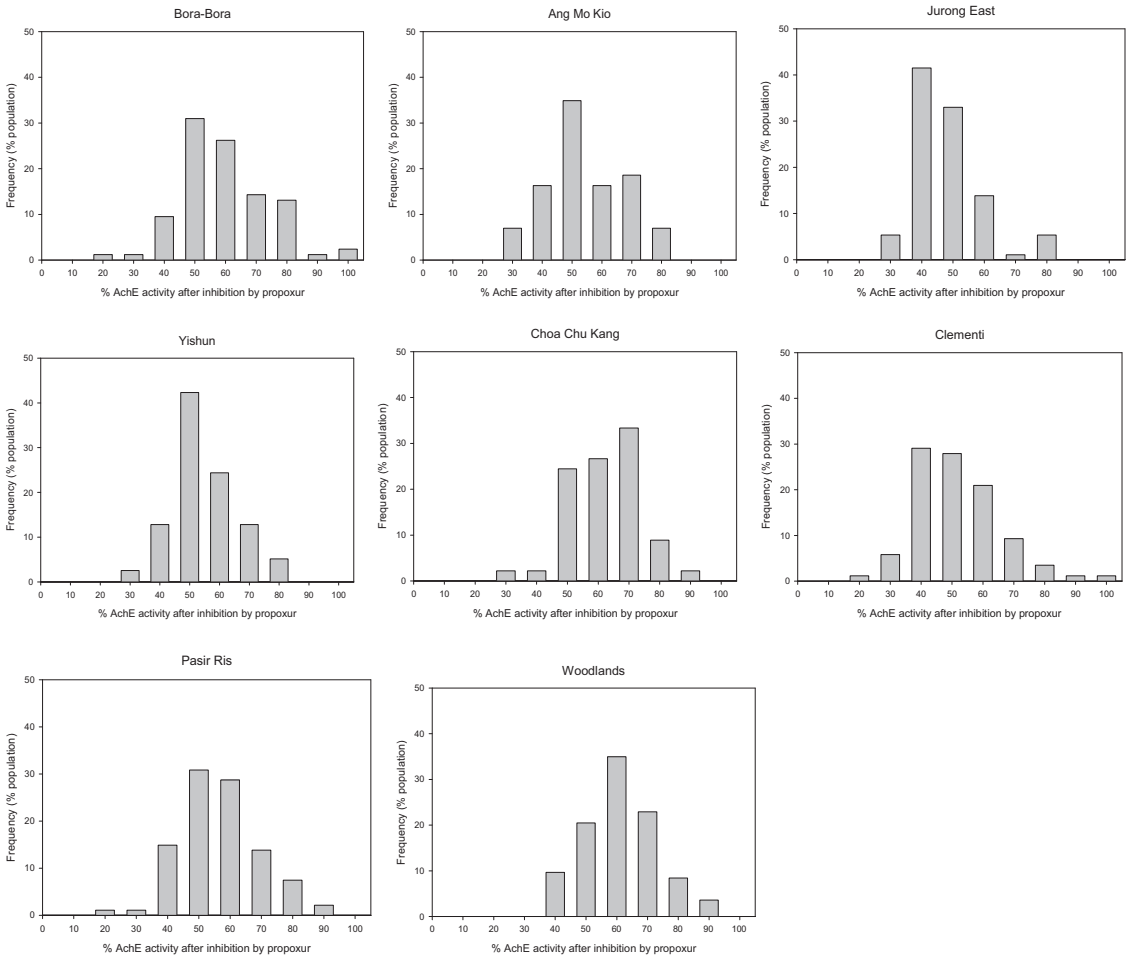

Fig. 2. Altered acetylcholinesterase (AChE) activities in *Ae. aegypti* larval populations in Singapore.

Bti resulted in 100% mortalities among all strains (Table 3). Overall, the mortality rate of larvae was highest with *Bti*, followed by temephos, etofenprox, and permethrin, regardless of location.

Effectiveness of Synergists. To test the hypothesis that synergists can improve the toxic effect of insecticides on mosquitoes, three synergists PBO, DEF, and TPP were applied in conjunction with the insecticides. The mortality rates of the field strains of *Ae. aegypti* larvae, in response to various combination of insecticide and synergist, are shown in Table 3. Treatment with any of the three synergists did not increase the mortality of all *Ae. aegypti* strains to temephos. Instead, lower mortalities were observed in most strains. PBO treatment led to a slight increase in the mortality rate of *Ae. aegypti* effected by permethrin and etofenprox, but the improvements were significant only in some strains (Ang Mo Kio, Yishun, Choa Chu Kang, and Pasir Ris). No significant changes in mortality were found when DEF and TPP treatment were applied in conjunction with permethrin and etofenprox. In summary, the synergists (PBO, DEF, and TPP) did not increase the toxicity of insecticides.

Biochemical Assays. To determine if the observed insecticide resistance of local *Ae. aegypti* population is due to elevated enzyme levels, ≈607 larvae from the different locations were individually assayed for AChE, ESTs, GST, and MFO enzyme activities. All the data from each strain were pooled and the resulting frequency distribution was plotted. The resistance thresholds were fixed as the upper range of each enzyme activity of the Bora-Bora strain (Selvi et al. 2010). The upper range of enzyme activities displayed by the Bora-Bora strain was found to be 19.5, 18.9, 0.04, and 0.23 of reaction rate produced for α-EST, β-EST, GST, and MFO. These values were used as resistance threshold for the respective enzymes. None of the field strains displayed any elevated level of altered AChE activity (Fig. 2). The wide distribution patterns of percentage of altered AChE activity ranged from 16.08 to 94.44% from all populations, indicating heterogeneous populations. In fact, the AChE inhibition rates by propoxur were lower in some field strains. Significant elevation ($P < 0.01$) of α- and β-EST levels were found in all populations (Figs. 3 and 4; Table 5).

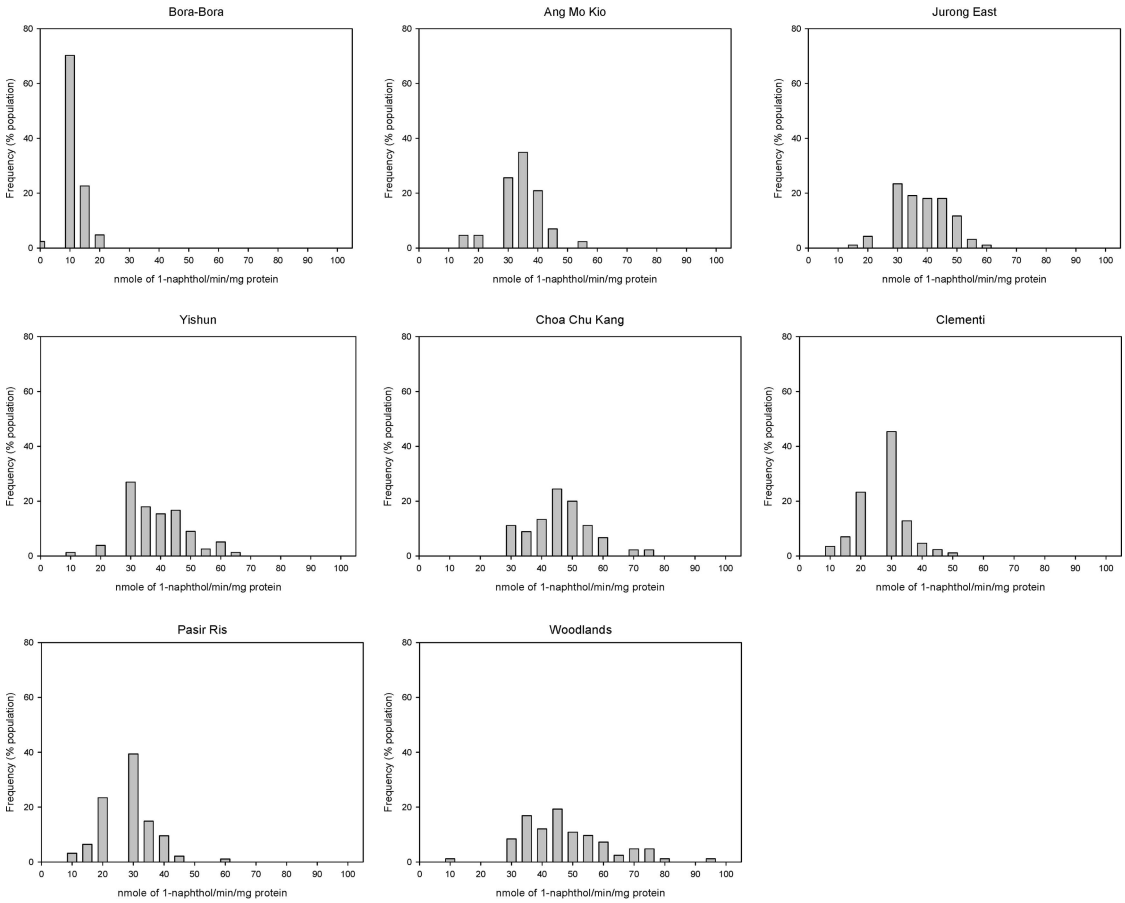

Fig. 3. Esterase activities with α-naphthyl acetate in *Ae. aegypti* larval populations in Singapore.

No elevated GST levels were detected, except in the Clementi strain (Fig. 5).

MFO activity was indirectly determined by measuring the cytochrome P450 content in the body. Larvae from Ang Mo Kio, Jurong East, Yishun, and Woodlands showed significant ($P < 0.01$) higher cytochrome P450 content (6.75–18.33-fold compared with Bora-Bora) (Fig. 6). These populations were heterogeneous toward MFO activity. No correlation ($r_{\alpha\text{-Est}} = 0.668, r_{\beta\text{-Est}} = 0.764$) between resistance level (RR_{50} of temephos) and enzyme activity (ESTs) was found. Similarly, no direct relationship was noted between pyrethroid resistance level and other enzyme activities.

Discussion

Although the diagnostic dose of some insecticides can be obtained from WHO criteria (WHO 1981b, 1998), these proposed doses were based on *Anopheles* mosquitoes for monitoring resistance status of these mosquitoes during malaria outbreaks. Therefore, susceptibility baselines and diagnostic doses of all insecticides tested were established for our local *Ae. aegypti* in this study.

This study clearly demonstrated that larvae from all populations from Singapore are highly resistant to pyrethroids but exhibited low levels of resistance to organophosphates. Despite this, *Ae. aegypti* larvae in Singapore have shown greater tolerance to temephos compared with the past 32 yr. Currently with an LC_{50} of 0.014 mg/L, it is more than two times the 0.00174 mg/L and 0.00612 mg/L in 1979 and 1993, respectively (Liew et al. 1994). Resistance of *Ae. aegypti* to temephos has been reported in other countries such as Brazil (Carvalho Mdo et al. 2004) and Malaysia (Chen et al. 2005). *Ae. aegypti* in Thailand and India have also been shown to have low temephos resistance, ranging from 0.3 to 4.4-fold and 0.33–7.11-fold, respectively (Ponlawat et al. 2005, Tikar et al. 2008).

However, according to WHO guidelines (WHO 2009) for field use, 5 g of the formulated product is required for 50 liters of water in container to ensure toxicity to mosquito larvae (equivalent to 1 mg/L of 1% temephos (Abate) sand granules). This high dosage (≈35-fold of the diagnostic dose) explains why resistant field strains of *Ae. aegypti* in Malaysia could achieve 100% mortality when operational dosage of temephos was used (Chen et al. 2005). It also suggests that temephos would still be an effective larvicide for

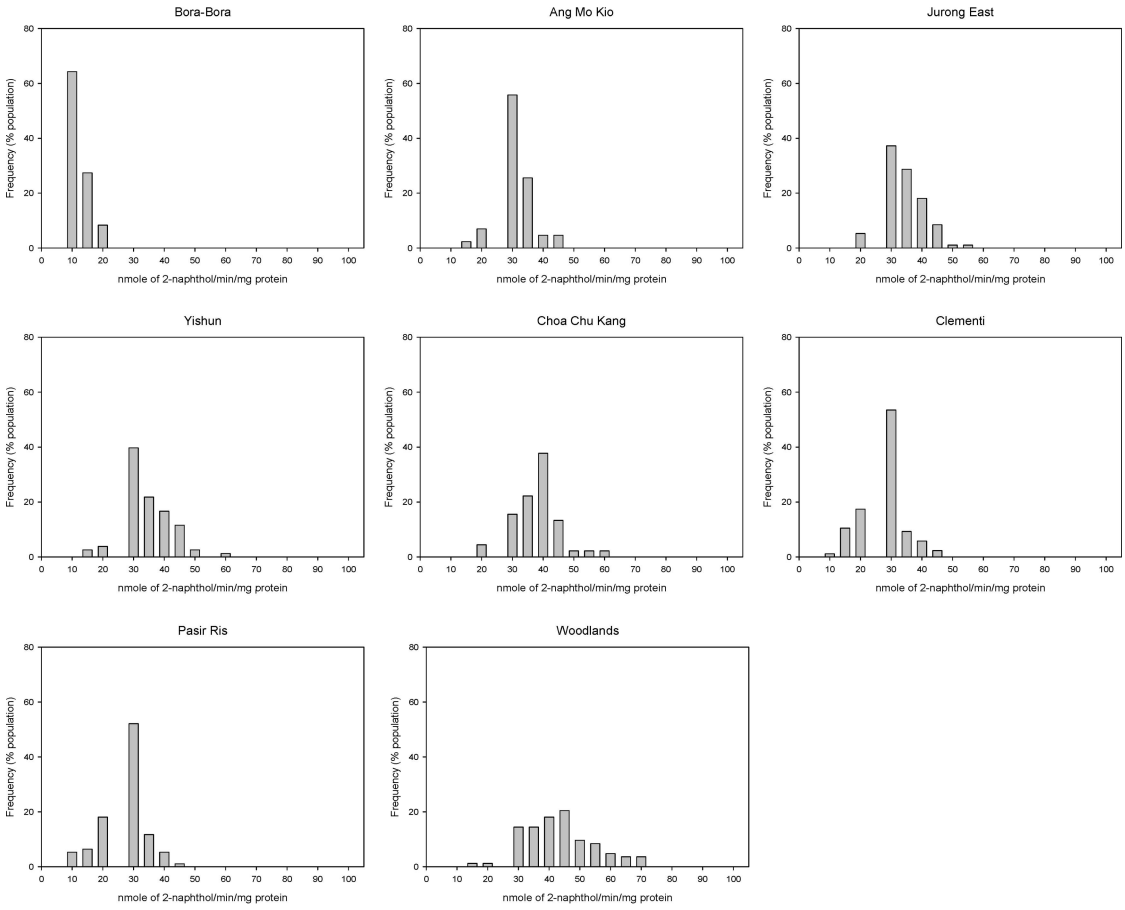

Fig. 4. Esterase activities with β-naphthyl acetate in *Ae. aegypti* larval populations in Singapore.

use in Singapore. Overall, the results suggest that despite efficacy of an insecticide demonstrated in the field, there could be an emerging resistance, which required laboratory monitoring.

Bti has become a popular choice of larvicide in Singapore in the past 10 yr because of its efficacy and low environmental impact (Tan et al. 1998, Chung et al. 2001). Knowledge of possible resistance development of *Bti* is thus important for resistance management strategies. We did not find any resistance toward *Bti*. Although *Bti* can continue to be a choice larvicide, the other considerations such as product's residual effect or persistency should be investigated to maximize the desired outcome. It is clear from the various

Table 5. Mean (± SE) levels of insensitive acetylcholinesterase (iAChE), nonspecific esterases (α- and β-EST), glutathione s-transferase (GST), and mono-oxygenase (MFO) activities of *Ae. aegypti* larvae in Singapore

Strain	*n*	Mean enzyme activities[a] (Mean[b] ± SE)				
		AChE	α-Esterase	β-Esterase	GST	MFO
Bora-Bora	84	54.49 ± 1.54	7.59 ± 0.44	8.77 ± 0.44	0.03 ± 0.00	0.12 ± 0.00
Ang Mo Kio	43	49.91 ± 1.90	31.13 ± 1.22*	27.71 ± 0.94*	0.04 ± 0.00	2.03 ± 0.12*
Jurong East	94	43.26 ± 1.12	35.55 ± 0.93*	31.36 ± 0.67*	0.03 ± 0.00	0.81 ± 0.03*
Yishun	78	49.67 ± 1.20	35.71 ± 1.17*	31.54 ± 0.91*	0.05 ± 0.00	2.20 ± 0.20*
Chua Chu Kang	45	57.51 ± 1.64	43.34 ± 1.52*	35.20 ± 1.17*	0.05 ± 0.00	0.28 ± 0.05*
Clementi	86	46.54 ± 1.55	23.66 ± 0.85*	23.88 ± 0.78*	1.86 ± 0.04*	0.03 ± 0.00
Pasir Ris	94	52.05 ± 1.26	24.50 ± 0.87*	23.62 ± 0.75*	0.04 ± 0.00	0.02 ± 0.00
Woodlands	83	55.85 ± 1.28	45.08 ± 1.62*	41.02 ± 1.27*	0.06 ± 0.00	2.19 ± 0.19*

[a] Enzyme activities expressed as reaction rate of different substrates/min/mg protein.

[b] Means followed by asterisk symbol are significantly higher when compared with Bora-Bora strain ($P < 0.05$, two-samples *t*-test or Mann–Whitney test). AChE: percentage insensitive acetylcholinesterase activity after propoxur inhibition. α-esterase: nmole of 1-naphthol/min/mg protein, β-esterase: nmole of 1-naphthol/min./mg protein. GST: mmole of CDNB/min/mg protein. MFO: nmole equivalent unit cyt P450/min/mg protein.

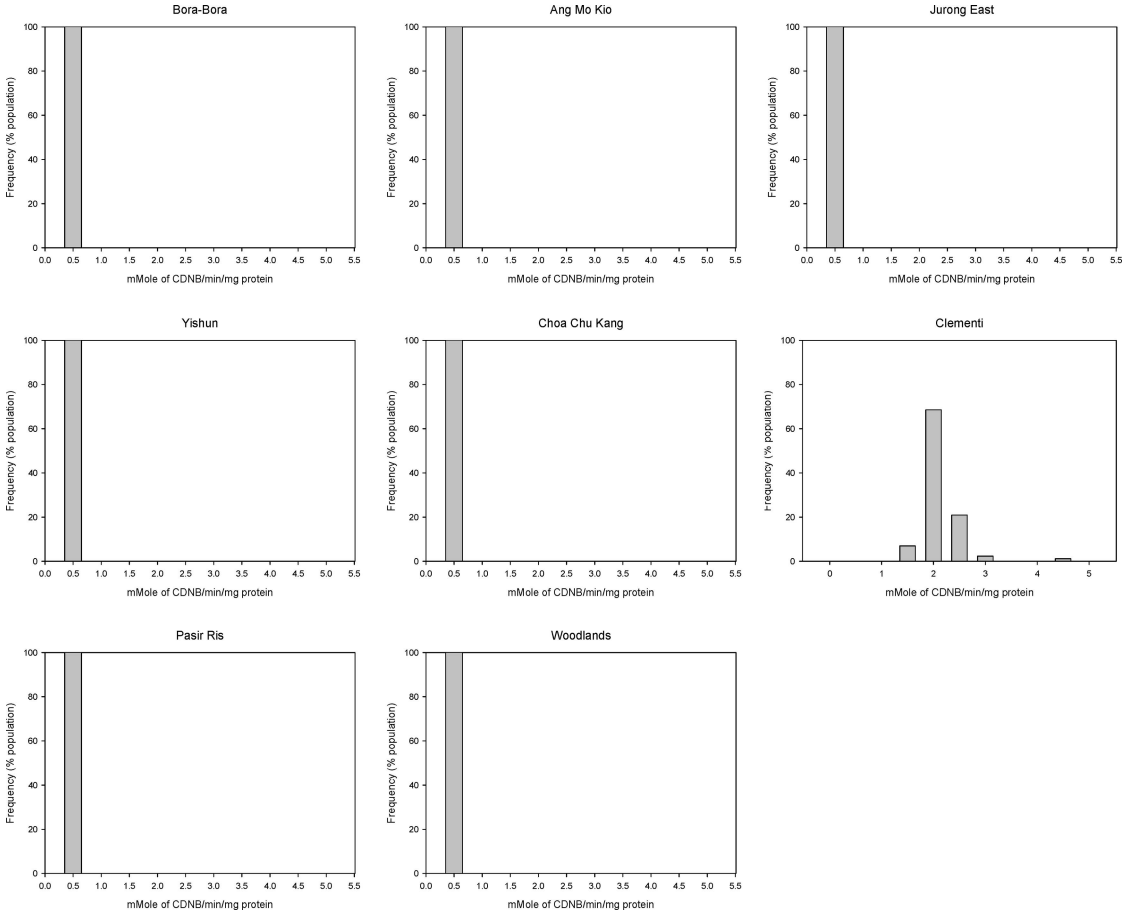

Fig. 5. Glutathione S-transferase (GST) activities in *Ae. aegypti* larval populations in Singapore.

studies that different ways of *Bti* application, either direct treatment or misting, gave different results (Sumanadasa et al. 2011). Even though it was shown to be effective up to 3 months under laboratory conditions (Lima et al. 2005), a local study showed that *Bti* has short residual activity of only 1 week in an industrial area (Sumanadasa et al. 2011). However, in Brazil, *Bti* field studies showed a longer residual effect of up to 5 weeks. Environmental factors such as temperature, sunlight exposure, water condition, and pond sediment can affect the efficacy and residual effect of *Bti* (Ignoffo et al. 1981).

It is interesting to note that the use of pyrethroids as larvicides is generally limited, particularly in Singapore. The observed resistance is thus likely due to the use of adulticides. Pyrethroids were commonly used for fogging in the past and resistance were detected in *Ae. aegypti* (Lai et al. 2001). Since 1990s, the use of pyrethroids for dengue control program has gradually been eliminated. However, pyrethroid remains a common insecticide used by the pest control industry. The resistance observed among the larvae population suggests that the phenotype acquired at the adult stage is displayed by both the mature and immature mosquitoes. However, it should be noted that the resistance profile in larvae may not always be consistent with that in adults, and further adulticide bioassay is necessary to validate this.

Although etofenprox is rarely used in Singapore, the resistance level among the mosquito populations was as high as the commonly used permethrin. This could be due to cross-resistance between the two pyrethroids. This speculation is supported by our findings, which showed that PBO increased the toxicity of etofenprox and permethrin in the same three populations from Ang Mo Kio, Yishun, and Pasir Ris. An additional two populations, Choa Chu Kang and Woodlands, showed improved susceptibility to etofenprox when PBO was used. PBO is known to act by inhibiting MFO, and these findings concur with the biochemical results, which showed that all of these five populations had elevated MFO levels. Together, our findings suggest the involvement of MFO in the resistance of some populations against permethrin and etofenprox. However, the improved susceptibility conferred by PBO in all populations did not lead to high mortality (up to only 37.45%). This further suggests that the resistance may be attributable to more than one mechanism.

Elevated levels of ESTs were detected in all field strains with biochemical assay and may play a role in

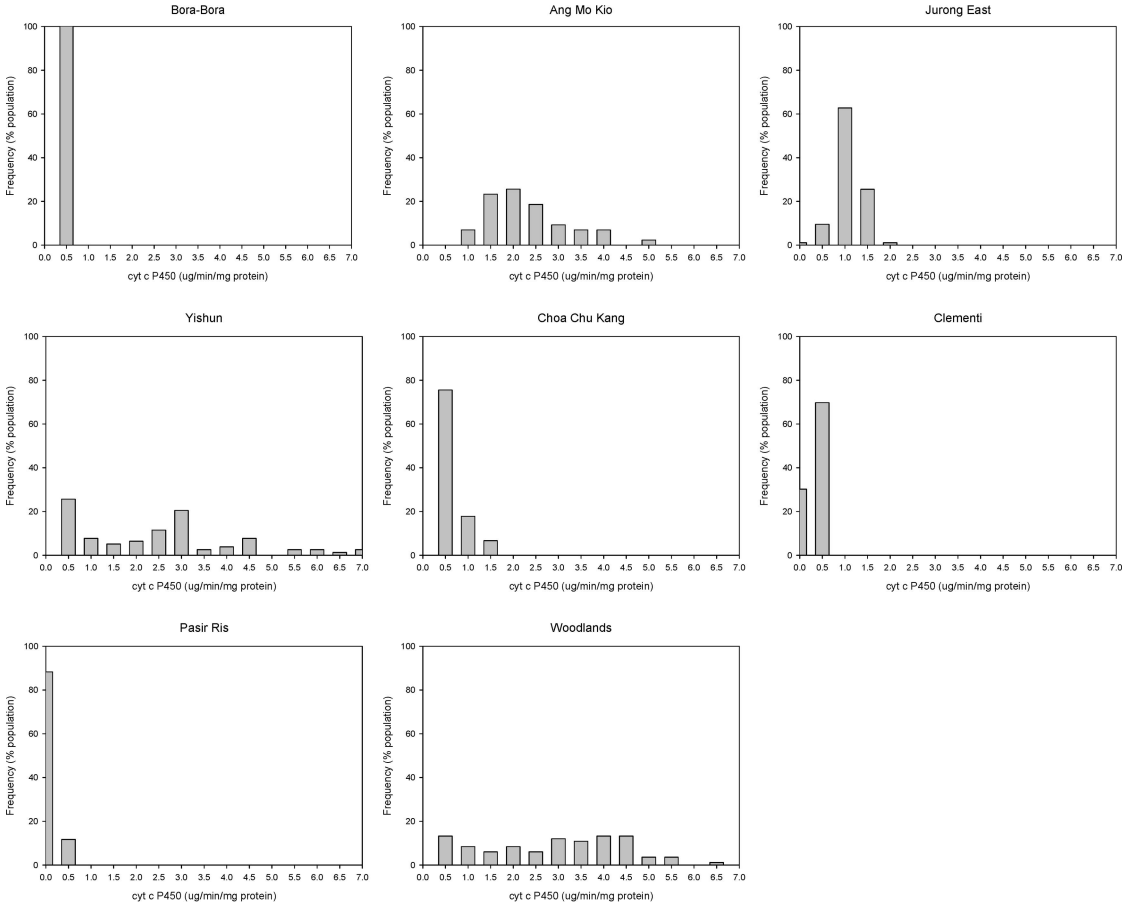

Fig. 6. Mixed-function oxidases (MFO) activities in *Ae. aegypti* larval populations in Singapore.

pyrethroid resistance in *Ae. aegypti* larvae. In addition to oxidative detoxification by MFO, EST-mediated detoxification has been shown to play a predominant role in conferring pyrethroid resistance in mosquitoes (Sahgal et al. 1994, Pethuan et al. 2007, Munhenga et al. 2008, Fonseca-Gonzalez et al. 2011). EST hydrolyzes pyrethroids and organophosphates that contain an ester linkage (Saelim et al. 2005). However, it was surprising that DEF and TPP, which are known to neutralize ESTs, failed to significantly reduce the resistance level of permethrin and etofenprox. High ESTs activities are also associated with organophosphate resistance (Latif et al. 2010), and this could explain the onset of temephos resistance in all populations.

Cuamba et al. (Cuamba et al. 2010) suspected the decreased levels of insensitive AChE in field strains were due to hydrolysis of propoxur by other enzymes such as ESTs; therefore, the propoxur concentrations for AChE inhibition were reduced, as biochemical assays were done on total enzyme extraction on mosquitoes. Only the Clementi strain appeared with higher GST level. Elevated GST level has been suggested to be involved in DDT resistance (Gunasekaran et al. 2011, Polson et al. 2011). DDT-resistant strains of *Anopheles albimanus* in Mexico showed increased levels of GST activity (Penilla et al. 1998). Casimiro et al. (2006) suggested that GST might be involved in pyrethroid resistance in *Anopheles funestus* in Mozambique. Their views on GST as having a secondary role in pyrethroid detoxification are supported by the resistance study on the pyrethroid-resistant strain of *An. funestus* in Mozambique, South Africa (Cuamba et al. 2010).

There is a dearth of published information on the relationship between insecticide resistance level and detoxification enzyme activity. In our study, the lack of correlation between resistance level and enzyme activity does not strengthen the hypothesis that enzyme activities correspond to the insecticide susceptibility status of *Ae. aegypti.* Enzyme activities do not necessarily correlate to toxicological changes (Siegfried and Scott 1992).

Although enzyme elevation could play a role in the permethrin and etofenprox resistance of Singapore *Ae. aegypti*, it is clear from synergist study and biochemical assays that the enzyme elevation cannot account for the high resistance observed. Synergists become ineffective when target site insensitivity mechanism occurs (Xu et al. 2005). Therefore, we proposed to

perform molecular work to investigate the involvement of target site insensitivity mechanism in *Ae. aegypti* populations.

Conclusion. Our studies showed that *Ae. aegypti* in Singapore is considered susceptible to organophosphates. Even though there is evidence of an early development of organophosphate resistance, the impact of the phenotype has not been detected in the field. *Ae. aegypti* from all locations in Singapore displayed high resistance to pyrethroid insecticides. The choice of alternative insecticides is limited and this could become a serious concern.

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