The detoxification enzymes activity profile in susceptible Aedes and Culex mosquitoes

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Abstract. The status of susceptible mosquito is important for monitoring and managing insecticide resistance in field population. The purpose of this study is to profile the differential expression level of enzymatic activities of metabolic enzymes exhibited in the mosquito species of Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus from the susceptible strain. The fourth instar larvae of each strain were subjected to biochemical assay. The total protein content and enzymatic activities of Glutathione S-transferase (GST), α-esterase (α-est), β-esterase (β-est) Cytochrome P450 (Cyt P450) and Acetylcholinesterase (AChE) from each mosquito strains were elucidated. Significant difference (P<0.05) was detected between the total protein content between all species of susceptible strain mosquitoes. One-way ANOVA showed that the specific enzymatic activities of GST, α-est and Cyt P450 of all test mosquitoes were significantly different upon comparison with each species (P<0.05). The mean of enzymatic activities of insensitive AChE showed no significant difference upon comparison with each other (P>0.05). The enzymatic activities of β-est shows no significant difference between Ae. aegypti and Cx. quinquefasciatus mosquitoes (P>0.05) but the enzymatic activity of β-est in Ae. albopictus manifested significant difference upon comparison with the enzymatic activities of the other two test species (P<0.05). The results obtained may provide more information about the enzymatic activities of metabolic enzymes in Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus mosquitoes which might be beneficial for public sector for the application of proper vector control measures.

1. Introduction
Mosquitoes are one of the most important groups of insects namely Anopheles, Culex and Aedes which have been deemed as medically important insects due to their ability to be the carrier of numerous arboviruses namely yellow fever, dengue, West Nile virus, Chikungunya, Japanese encephalitis, Zika virus and filariasis [1, 2, 3, 4]. The distribution of mosquitoes and mosquito borne diseases occurs throughout the globe but the types and intensity varies according to many contributing factor such as location, climate and others. Apart from its nuisance biting behavior, the threat it poses caused by its invasive nature has increased the importance to manage and reduce its distribution [5, 6].

Aedes albopictus, Ae. aegypti and Cx. quinquefasciatus mosquitoes are urban oriented insects favoring different breeding areas and serves as disease vectors including in Malaysia [7, 8, 9]. In fact, these species have also been identified to develop resistance to insecticides due to the application of chemical and synthetic insecticides in the vector control measures [7, 10].
Mosquitoes developed various mechanisms in order to survive the exposed toxicity and to protect itself [11]. One of the most common mechanism is metabolic detoxification which predominantly involves metabolic enzymes namely Cyt P450, GST, Esteras and AChE [12, 13]. Various studies have proven the involvement of these enzymes in conferring insecticides resistance [14, 15, 16]. Hence, a study focusing on the enzymatic activity of detoxification enzymes in different species of susceptible mosquitoes (Ae. aegypti, Ae. albopictus, and Cx. quinquefasciatus) is pertinent to have a better knowledge regarding the complex biological process in these insects. On that note, the aim of this study is to profile the differential enzymatic activities (Cyt P450, GST, AChE, α-est and β-est of different susceptible mosquito species (Ae. aegypti, Ae. albopictus, Cx. quinquefasciatus).

2. Materials and method
Susceptible strains of Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus used in this research were provided by the Vector Control Research Unit (VCRU), Universiti Sains Malaysia. The eggs obtained were hatched and reared up to the early 4th instar stage. Differing levels of enzymatic activities of individual 4th instar larvae of susceptible strains of Ae. albopictus, Ae. aegypti and Cx. quinquefasciatus mosquitoes were elucidated according to Hemingway [17], adhering to WHO protocol. Early 4th instar larvae were individually homogenized in 200 μl of season water and placed on ice. Twenty-five microliters of homogenate were used for the AChE assay while the remaining homogenate were centrifuged for 30 seconds at 14000 rpm (4°C). The supernatant was used as the enzyme source for the rest of the biochemical analyses. A total of 100 individual replicates were used per enzymatic assay in a 96-well microplate on ice and the microtiter plate reader (Thermofischer Scientific) with Magellan data analysis software to measure its absorbance (optical density [OD]) values.

2.1. Acetylcholinesterase assay
Briefly, 145 μl of triton phosphate buffer, 10 μl of 0.01 M dithiobis 2-nitrobenzoic acid solution and 25 μl of 0.01 M acetylthiocholine iodide were added to 25 μl of insect homogenate to initiate the reaction. Two reactions were done for each sample whereby one reaction was allowed to progress, and the other reaction was inhibited using 0.05 μl 0.1 M propoxur. The absorbance of the reactions was read at 405 nm after incubation for one hour. The enzymatic activity of insensitive AChE was expressed in nmole of AChE/min/mg upon propoxur inhibition.

2.2. α ester and β esterase assay
Twenty microliter extracted supernatants were added to 200 μl of substrate. For α-esterase assay, 30 mM α-naphthyl acetate were then added to the mixture and 200 μl of 30 mM β-naphthyl acetate were added to the β-esterase assay mixture. After 15 minutes, 50 μl of fast blue stain were mixed with every reaction and the OD was determined at 570 nm. The activity against both substrate was derived from standard curves of absorbance for known concentrations of α-naphthol and β-naphthol. Enzymatic activities were calculated according to Hemingway (1998) and presented as nanomoles (nmole) of α-naphthol or β-naphthol/min/mg protein.

2.3. Glutathione s-transferase assay
A mixture containing 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 200 μl of 10 mM GSH and were mixed to 10 μl of extracted supernatant and OD was determined at 340 nm after incubation period of 20 minutes. The OD value was transformed to μmole of CDNB conjugates by using its extinction coefficient which is 9.6 mM-l. and path length of 0.6cm. The enzymatic activity of GST was calculated by using Beer's Law and presented as μmole of CDNB/min/mg protein.

2.4. Cytochrome P450 titration assay
To initiate the reaction, 80 μl of potassium phosphate buffer, 200 μl of 3,3′,5,5′-tetramethylbenzidine (TMBZ) in methanol solution and 25 μl of hydrogen peroxide were mixed with 2 μl of extracted supernatant. The mixture was incubated for 2 hours and the OD value was measured at 650 nm. The
enzymatic activity of Cyt P450 was elucidated from standard curve of absorbance for known concentration of cytochrome C [18]. Enzyme activity of Cyt P450 was expressed as n mole equivalent units of cytochrome P450/min/mg protein.

2.5. Protein determination
The protein concentration of supernatant derived from all test larvae for each biochemical assays was determined according to [19]. The standard protein used is bovine serum albumin (BSA) to normalize protein concentration activities. Protein concentration was calculated and transformed by using the BSA standard curve. Ten microliters of the extracted homogenate were mixed with 300 μl of Bradford reagent and incubated for 5 minutes before measuring its OD at 595 nm.

2.6. Data analysis
The data obtained after measuring the enzymatic activities were tested for normality and variances homogeneity by using Komolgorov-Smirnov and Levene’s tests. As for comparison of means of the enzymatic activities between different species of mosquito, one-way analysis of variance (ANOVA) for parametric test was conducted. All of the data were analyzed by using SPSS software version 24. The total protein content and enzymatic activities data were presented as mean ± standard error (S.E.) and the overall statistical significance was assumed at P < 0.05.

3. Results and discussions
In this study, the susceptible strains of Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus mosquitoes were used to profile their total protein content, and to determine the enzymatic activities of α-est β-est, Cyt P450, GST and insensitive AChE enzyme activities. Based on the results presented in table 1, the highest significant mean (P<0.05) of total protein content (µg) was found in of Ae. albopictus (3.98±0.09 µg), followed by Ae. aegypti (3.39±0.09 µg), and Cx. quinquefasciatus (3.15±0.06 µg).

For α-est assay, the specific enzymatic activities difference of Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus were statistically significant (P<0.05). In detail, the highest specific enzyme activity (72.94±2.54 n moles of 1 NA/min/mg) was detected in Cx. quinquefasciatus while the lowest specific activity was detected in Ae. aegypti (52.47±2.34 n moles of 1 NA/min/mg). However, in comparison (Ae. aegypti, Cx. quinquefasciatus and Ae. albopictus), β-est specific enzymatic activities in Ae. aegypti (72.76±2.98 n moles of 2 NA/min/mg) and Cx. quinquefasciatus (71.25±2.43 n moles of 2 NA/min/mg) showed no significant difference (P>0.05). However, the specific enzymatic activity of Ae. albopictus (64.71±2.79 n moles of 2 NA/min/mg) showed significant difference in comparison with other mosquito species (P<0.05).

In decreasing order, Ae. albopictus showed, statistically, the highest GST specific activity followed by Ae. aegypti and Cx. quinquefasciatus (1.76±0.08 µ moles/min/mg, 1.31±0.06 µ moles/min/mg, 1.02±0.05 µ moles/min/mg respectively, P<0.05). A similar pattern was observed for the enzymatic activities of Cyt P450 whereby the highest level of cytochrome P450 specific activity (5.53±0.06 n moles/min/mg) was observed also in Ae. albopictus as compared to Ae. aegypti (4.28±0.03 n moles/min/mg) and Cx. quinquefasciatus (4.02±0.08 n moles/min/mg) and showed significant difference (P<0.05).

The highest percentage of AChE inhibition activity was found in Cx. quinquefasciatus (3.64±0.21 n moles/min/mg), followed by Ae. albopictus (3.44±0.25 n moles/min/mg) and Ae. aegypti (3.42±0.19 n moles/min/mg). However, the values obtained were not significantly different when compared with other species (P>0.05).
Insects adapt to different biochemical and physiological mechanisms to withstand internal and external pressure for survival. Generally, one of the main detoxification mechanisms in insects involves the metabolism of insecticides [11]. The goal of this paper is to profile the metabolic enzyme activities in the susceptible VCRU strains of *Ae. aegypti*, *Ae. albopictus* and *Culex quinquefasciatus* mosquitoes. The protein content of all test strains are within a close range (3.15 ± 0.06 - 3.98 ± 0.09), and the existence of protein content shows the natural existence of metabolic enzyme in the system of mosquitoes. [20] reported a correlation between the protein content with the level of metabolic enzymes in *Cx. pipiens*.

The presence and overall pattern of enzymatic activities of all test strain are somewhat similar between the enzymatic activities of α-est and β-est except for the enzymatic activities in the larvae of *Ae. aegypti* with a wide range of α-est and β-est. Esterases have always been known to play a crucial role in conferring pyrethroid, organophosphate and carbamate resistance [14, 21, 22, 23].

For the enzymatic activities of GST, the values are of significant difference upon comparison in the larvae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. The detection and the enzymatic activities of GST are justifiable due to the fact that the elevation in the enzymatic activities of this particular enzyme have frequently been reported as one of the causative factors in the metabolism as well as resistance to insecticides in mosquitoes including in Malaysia [24, 25, 26, 27].

The enzymatic activities of Cyt P450 in the larvae of *Ae. albopictus* appears to be the highest compared to the enzymatic activities in the larvae of *Ae. aegypti* and *Cx. quinquefasciatus* but the fold changes are still minimal (up to 1.38-fold). The fluctuating levels of Cyt P450s in insects, particularly mosquitoes exposed to different insecticides proved the involvement of this rate-limiting enzymes in the metabolism as well as in its defense mechanism [22, 28]. The involvement of Cyt P450 in the defense mechanism of mosquitoes particularly *Aedes* mosquitoes has also been reported in Malaysia [30, 31, 32].

The enzymatic activities of insensitive AChE which have been reported to be involved in the metabolism of insecticides especially organophosphates in all three test mosquitoes similar to one another. As such, there are several studies which emphasizes the role of AChE in the resistance in mosquito species which includes Malaysia [25, 33, 34, 35].

Cumulatively, the results obtained in this study demonstrated the detection of the test metabolic enzymes (GST, α-est, β-est, Cyt P450 and AChE) in a condition whereby the larvae of *Ae. aegypti*, *Ae. albopictus* and *Culex quinquefasciatus* are not exposed to any environmental and xenobiotic pressure. Should the larvae be exposed to insecticides for acute or prolonged duration, the production of enzymes

### Table 1. Mean of specific enzymatic activities (mean±SE) of total protein content, α-esterase (α-est), β-esterase (β-est), glutathione s-transferase (GST), cytochrome P450 (Cyt P450) and acetylcholinesterase (AChE) of susceptible *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes, n=100.

| Mosquito species | Total protein (µg) | α-est (nmoles of 1 NA/min/mg) | β-est (nmoles of 2 NA/min/mg) | GST (µmoles/min/ mg) | Cyt P450 (nmoles/min/mg) | AChE (nmoles/min/mg) |
|-----------------|------------------|-----------------------------|-----------------------------|-------------------|---------------------|---------------------|
| *Ae. aegypti*   | 3.39±0.06a       | 52.47±2.34a                 | 72.76±2.98a                 | 1.31±0.06a        | 4.28±0.03a          | 3.42±0.19a          |
| *Ae. albopictus*| 3.98±0.09b       | 65.19±2.79b                 | 64.71±1.95b                 | 1.76±0.08b        | 5.53±0.06b          | 3.44±0.25a          |
| *Cx. quinquefasciatus* | 3.15±0.06c | 72.94±2.54c                 | 72.25±2.43c                 | 1.02±0.05c        | 4.02±0.08c          | 3.64±0.21a          |

Comparison were made by using 1-way ANOVA between susceptible strain of *Cx. quinquefasciatus*, *Ae. albopictus* and *Ae. aegypti* from the same enzyme only. Mean of total protein content and specific enzyme activity with different letters indicate significant difference at P=0.05.
will be elevated for the survival to withstand the pressure of the insecticides toxicity. Correspondingly, previous studies have demonstrated the alteration of enzymatic activities of Cyt P450, GST, AChE, α-est and β-est and upon induction of mosquito larvae with insecticides [20, 36]. Apart from that, there are numerous studies highlighting the collaboration and roles of more than one enzyme in the metabolism of insecticides in the defense mechanism of insects [12, 23, 37, 38].

4. Conclusion
The biochemical analysis of metabolic enzymes of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus*, from susceptible strain revealed varying levels of the enzymatic activities of AChE, GST, α-est, β-est, and Cyt P450. A more intensive biochemical and protein analysis should be considered in addition to the existing conventional bioassay used in mosquito control measures to enhance the efficacy of insecticides towards the vector. Apart from that, comparison with the susceptible mosquito populations should be made in order to derive a supplementary method to prevent the propagation of insecticide resistance.

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