THE SUSCEPTIBILITY OF *Aedes albopictus* SKUSE POPULATIONS FROM HUMAN DWELLINGS TO MAJOR INSECTICIDES FOR VECTOR CONTROL PROGRAMMES IN AGRICULTURAL AND NON-AGRICULTURAL AREAS

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THE SUSCEPTIBILITY OF *Aedes albopictus* SKUSE POPULATIONS FROM HUMAN DWELLINGS TO MAJOR INSECTICIDES FOR VECTOR CONTROL PROGRAMMES IN AGRICULTURAL AND NON-AGRICULTURAL AREAS

ABSTRACT

Dengue, chikungunya and Zika are important mosquito-borne diseases transmitted by *Aedes aegypti* and *Aedes albopictus*. *Aedes albopictus* was selected to determine its susceptibility against major insecticides used for vector control programmes. The underlying mechanisms of metabolic resistance detected in *Ae. albopictus* populations had been revealed. The efficacy of a synergist, piperonyl butoxide (PBO) which was employed in combination with organochlorines and pyrethroids to enhance the potency of these insecticides had also been assessed. Since chemical controls are applied in both vector control strategies and agricultural pest management, study localities comprising both agricultural and non-agricultural areas were chosen. Initially, ovitrap surveillance was conducted to determine the mosquito species composition in which *Ae. albopictus* was the predominant container-breeder in all study areas. The first offspring generation of *Ae. albopictus* was subjected to both larval and adult mosquito bioassays. The susceptibility of *Ae. albopictus* larvae from dengue prone residential area against fenitrothion, fenthion, temephos, propoxur and permethrin was significantly different than larvae from different types of agricultural areas. Significant difference in the susceptibility of *Ae. albopictus* adults from residential areas with and without the history of fogging activities in comparison with adult mosquitoes from various types of agricultural areas were only observed against fenitrothion, propoxur and bendiocarb. From the enzyme microassays, the significant role of non-specific esterases (EST) in the metabolic resistance was exhibited in all populations of *Ae. albopictus* adults. At larval stage, only α-esterases activity was involved significantly in the metabolic resistance of *Ae. albopictus* from non-agricultural areas. The glutathione-S-transferases (GST) enzyme

was significantly engaged in the metabolic resistance of *Ae. albopictus* adults from paddy cultivation area as well as larvae from almost all types of area. In terms of insensitivity of acetylcholinesterase (AChE), all populations of *Ae. albopictus* adults were still sensitive against propoxur while only larvae from oil palm plantation and fogging-free residential areas were still sensitive to the same carbamate. At larval stage of *Ae. albopictus*, mixed level of resistance against organophosphates and carbamates were associated with significant escalated activities of α-esterases and glutathione-S-transferases. Conversely, for *Ae. albopictus* adults, various levels of resistance against organochlorines, organophosphates and carbamates were correlated with significant increased activities of α-esterases, β-esterases, glutathione-S-transferases and insensitive acetylcholinesterase. Meanwhile, the combination of the synergist piperonyl butoxide (PBO) with dichlorodiphenyltrichloroethane (DDT) of organochlorines improved the susceptibility of several populations of *Ae. albopictus* adults from high resistance to incipient resistance. The pre-exposure of PBO prior to the selection of pyrethroids caused significant reduction of the median knockdown time (KT_{50}) at 30 minutes of the exposure time in all populations of *Ae. albopictus* adults. In summary, since chemical control remains the most preferred and feasible tool of vector control approaches as well as in the agricultural pest management, continuous monitoring actions on the susceptibility of mosquito vectors against these insecticides are crucial to prevent the uncontrolled insecticide resistance development among mosquito populations. The important role of PBO in enhancing the efficacy of insecticides as presented in this study confirmed the utilization of synergist as one of the promising ways to delay and overcome the rise of insecticide resistance in mosquito vectors.

Keywords: *Aedes albopictus*, insecticide resistance, agricultural areas, non-agricultural areas, Malaysia.

TAHAP KERENTANAN POPULASI *Aedes albopictus* SKUSE DARI KEDIAMAN MANUSIA TERHADAP INSEKTISID UTAMA BAGI PROGRAM KAWALAN VEKTOR DI KAWASAN PERTANIAN DAN BUKAN PERTANIAN

ABSTRAK

Denggi, chikungunya dan Zika merupakan penyakit-penyakit bawaan nyamuk yang penting yang disebarkan oleh *Aedes aegypti* and *Aedes albopictus*. *Aedes albopictus* telah dipilih bagi menentukan tahap kerentanannya terhadap insektisid utama yang digunakan untuk program kawalan vektor. Mekanisme yang berperanan dalam kerintangan metabolik yang dikesan dalam populasi *Ae. albopictus* telah dikenalpasti. Keberkesanan sinergis; piperonil butoksida (PBO) yang telah digunapakai bersama dengan organoklorin dan piretroid bagi meningkatkan potensi insektisid tersebut juga telah dinilai. Oleh kerana kawalan secara kimia diaplikasi dalam kedua-dua strategi kawalan vektor dan pengurusan kawalan perosak dalam pertanian, lokaliti-lokaliti kajian yang merangkumi kedua-dua kawasan pertanian dan kawasan bukan pertanian telah dipilih. Sebagai permulaan, kajian taburan menggunakan ovitrap telah dijalankan untuk mengenalpasti komposisi spesies nyamuk yang mana *Ae. albopictus* telah dikenalpasti sebagai spesies nyamuk pra-dominan yang membiak di dalam bekas di semua kawasan kajian. Generasi anak yang pertama bagi *Ae. albopictus* telah digunakan dalam kedua-dua bioasai larva dan nyamuk dewasa. Kerentanan larva *Ae. albopictus* dari kawasan perumahan yang terdedah kepada denggi terhadap fenitrotion, fention, temefos, propoksur dan permetrin adalah berbeza secara bermakna berbanding dengan kerentanan larva dari pelbagai jenis kawasan pertanian. Perbezaan bermakna bagi kerentanan nyamuk dewasa *Ae. albopictus* dari kawasan perumahan dengan dan tanpa sejarah aktiviti semburan kabus berbanding dengan nyamuk dewasa dari pelbagai jenis kawasan pertanian hanya dapat dilihat terhadap fenitrotion, propoksur dan bendiokab. Berdasarkan kepada mikroasai enzim, peranan yang bermakna bagi esterase tidak spesifik (EST) di dalam kerintangan metabolik telah ditemui dalam populasi nyamuk dewasa *Ae. albopictus*. Pada peringkat

larva, hanya aktiviti α-esterase yang terlibat secara bermakna dalam kerintangan metabolik dalam *Ae. albopictus* dari kawasan-kawasan bukan pertanian. Enzim glutation-S-transferase (GST) telah didapati terlibat secara bermakna dalam kerintangan metabolik bagi nyamuk dewasa *Ae. albopictus* dari kawasan penanaman padi dan juga bagi larva dari hampir semua jenis kawasan. Dari segi aktiviti enzim asetilkolinestrase tidak sensitif (AChE), kesemua populasi nyamuk dewasa *Ae. albopictus* masih lagi sensitif terhadap propoksur manakala hanya larva dari kawasan penanaman kelapa sawit dan kawasan perumahan bebas semburan kabus yang masih sensitif terhadap karbamat yang sama. Pada peringkat larva *Ae. albopictus*, pelbagai tahap kerintangan terhadap organofosfat dan karbamat telah dikesan mempunyai perkaitan dengan peningkatan aktiviti yang bermakna bagi enzim α-esterase dan glutation-S-transferase. Sebaliknya, bagi nyamuk dewasa *Ae. albopictus*, pelbagai tahap kerintangan terhadap organoklorin, organofosfat dan karbamat telah dikaitkan dengan peningkatan aktiviti secara bermakna bagi α-esterase, β-esterase, glutation-S-transferase dan asetilkolinesterase tidak sensitif. Sementara itu, kombinasi sinergis; piperonil butoksida (PBO) bersama dengan diklorodifeniltrikloroetan (DDT) yang merupakan organoklorin telah memperbaiki tahap kerentanan beberapa populasi nyamuk dewasa *Ae. albopictus* daripada kerintangan tinggi kepada kerintangan sederhana. Pra-pendedahan PBO sebelum daripada pendedahan kepada piretroid telah menyebabkan penurunan yang bermakna dari segi kadar rebah pertengahan (KT_{50}) semasa minit ke tiga puluh (30) dalam tempoh waktu pendedahan bagi semua populasi nyamuk dewasa *Ae. albopictus*. Secara ringkasnya, memandangkan bahawa kawalan secara kimia masih menjadi kaedah yang paling digemari dan mudah digunapakai dalam pendekatan kawalan vektor dan juga dalam pengurusan perosak pertanian, tindakan pemantauan secara berterusan berkenaan tahap kerentanan vektor nyamuk terhadap insektisid tersebut adalah penting untuk mengelakkan perkembangan kerintangan terhadap insektisid yang tidak terkawal di kalangan populasi nyamuk.

Peranan penting PBO dalam meningkatkan keberkesanan insektisid seperti yang telah dibuktikan dalam kajian ini telah mengesahkan tentang penggunaan sinergis sebagai salah satu jalan penyelesaian yang mampu melambatkan dan mengatasi peningkatan perkembangan kerintangan terhadap insektisid dalam vektor nyamuk.

Kata kunci: *Aedes albopictus*, kerintangan terhadap insektisid, kawasan pertanian, kawasan bukan pertanian, Malaysia.

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"The excellence of knowledge is better than the excellence of worship"

~ Prophet Muhammad S.A.W.; Narrated by Huzaifah Al-Yamani (r.a.) ~

Thank You All.

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α	:	Alpha
β	:	Beta
γ	:	Gamma
ε	:	Extinction coefficient
®	:	Registered trademark
™	:	Trademark
&	:	And
º	:	Degree
ºC	:	Degree Celsius
=	:	Equal to
>	:	Greater than
≥	:	Greater than or equal to
<	:	Less than
≤	:	Less than or equal to
µl	:	Microliter
’	:	Minutes
-	:	Minus / Negative / Absent
/	:	Per
%	:	Percent
+	:	Plus / And / Present
±	:	Plus-minus
x	:	Times
–	:	To
3rd	:	Third

A	:	Absorbance
ACh	:	Acetylcholine
AChE	:	Acetylcholinesterase
ACTHI	:	Acetylthiocholine iodide
Ae.	:	*Aedes*
An.	:	*Anopheles*
Ar.	:	*Armigeres*
ANOVA	:	Analysis of variance
BHC	:	Benzene hexachloride
Bs	:	*Bacillus sphaericus*
Bti	:	*Bacillus thuringiensis israelensis*
C.L.	:	Confidence Limit
c	:	Concentration
CARB	:	Carbamates
CDNB	:	1-chloro-2, 4-dinitrobenzene
CHIKV	:	Chikungunya Virus
cm	:	Centimeter
CPs	:	Cuticular proteins
CSI	:	Chitin synthesis inhibitors
COMBI	:	Community Communication for Behavioural Impact
Cx.	:	*Culex*
CYP	:	Plant / Mammalian cytochrome P450 oxidase
DDT	:	Dichlorodiphenyltrichloroethane
DEET	:	*N,N*-diethyl-*meta*-toluamide
DEF	:	S,S,S-tributyl phophorotrithionate
DEM	:	Diethyl maleate

DEN	:	Dengue
DEN	:	Dengue prone residential areas
DENV	:	Dengue Virus
DF	:	Dengue Fever
df	:	Degree of freedom
DHF	:	Dengue Haemorrhagic Fever
DNA	:	Deoxyribonucleic acid
DSS	:	Dengue Shock Syndrome
DTNB	:	5, 5-dithiobis (2-nitrobenzoic acid)
E	:	East
ELISA	:	Enzyme-Linked Immunosorbent Assay
EST	:	Esterases
et al.	:	Et alia / And others
F0	:	Zero filial / Zero generation / Parental generation
F1	:	First filial / First generation
FBS	:	Fast blue salt
Felda	:	Federal Land Development Authority
FF	:	Fogging-free residential areas
g	:	Gram
GABA	:	γ-aminobutyric acid / Gamma-aminobutyric acid
GSH	:	Glutathione
GST	:	Glutathione-S-transferases
h	:	Hour
H_2O_2	:	Hydrogen peroxide
HCB	:	Hexachlorobenzene
IBM	:	International Business Machines

IGR	:	Insect growth regulators
IMMB	:	Institute of Medical Molecular Biotechnology
IMR	:	Institute for Medical Research
IRAC	:	Insecticide Resistance Action Committee
IRS	:	Indoor residual spraying
kdr	:	Knockdown resistance
KH_2PO_4	:	Potassium phosphate
Kg.	:	Kampung
KT_{50}	:	Knockdown time of insecticide which cause 50% knockdown in bioassay
KT_{95}	:	Knockdown time of insecticide which cause 95% knockdown in bioassay
L	:	Liter
l	:	Length
LACU	:	Laboratory Animal Care Unit
LC_{50}	:	Lethal concentration of insecticide which cause 50% mortality in bioassay
LC_{95}	:	Lethal concentration of insecticide which cause 95% mortality in bioassay
LC_{99}	:	Lethal concentration of insecticide which cause 99% mortality in bioassay
LT_{50}	:	Lethal time of insecticide which cause 50% knockdown in bioassay
LT_{95}	:	Lethal time of insecticide which cause 95% knockdown in bioassay
M	:	Molarity

M	:	Probable resistance / Moderate resistance / Incipient resistance / Tolerance
m	:	Meter
MFO	:	Mixed function oxidases
mg	:	Milligram
mg/L	:	Milligram per liter
min	:	Minutes
ml	:	Milliliter
mM	:	Millimolar
mmoles	:	Millimoles
MO	:	Missouri
MOE	:	Ministry of Education
MOH	:	Ministry of Health
N	:	North
N.D.	:	Not Determined
Na_2HPO_4	:	Sodium phosphate
nm	:	Nanometer
nmoles	:	Nanomoles
No.	:	Number
OC	:	Organochlorines
OI	:	Ovitrap Index
OP	:	Oil palm plantations
OP	:	Organophosphates
P	:	Probability (statistic)
pH	:	Potential of hydrogen
PBO	:	Piperonyl butoxide

PD	:	Paddy cultivation areas
PSMO	:	Polysubstrate monooxygenases
PY	:	Pyrethroids
Ph.D	:	Doctor of Philosophy
R	:	Resistant / Highly resistant
r	:	Correlation coefficient
RB	:	Rubber estates
RDL	:	Resistant to dieldrin
R.H.	:	Relative humidity
RR	:	Resistance Ratio
RR	:	Homozygous resistance
RS	:	Heterozygous
RVF	:	Rift Valley Fever
S	:	Susceptible
S.E.	:	Standard Error
SDS	:	Sodium dodecyl sulphate
SIT	:	Sterile Insect Technique
SLAB	:	Academic Staff Bumiputera Training Scheme
sp.	:	Species
SPSS	:	Statistical Package for the Social Sciences
SR	:	Synergistic Ratio
SS	:	Homozygous susceptible
St.	:	Saint
TMBZ	:	3,3'5,5'-tetramethylbenzidine
TPP	:	Triphenyl phosphate
Tx.	:	*Toxorhynchites*

ULV	:	Ultra low volume
UiTM	:	Universiti Teknologi MARA
UM	:	University of Malaya
USA	:	United States of America
USM	:	Universiti Sains Malaysia
VCRU	:	Vector Control Research Unit
VGSC	:	Voltage-gated sodium channel
VSSC	:	Voltage sensitive sodium channel
WHO	:	World Health Organization
YF	:	Yellow Fever
YFV	:	Yellow Fever Virus
ZIKV	:	Zika Virus

CHAPTER 1: INTRODUCTION

1.1 Background of Research

Mosquitoes are haematophagous arthropods that are well-studied by researchers across the globe due to their medical importance. *Aedes* mosquitoes are one of the most crucial species as they involve in the spread of many potentially life-threatening vector-borne diseases. *Aedes* mosquitoes act as the principal vectors of dengue fever (DF), dengue haemorrhagic fever (DHF), Zika, yellow fever (YF), chikungunya, filariasis and Rift Valley Fever (RVF) (Dedkhad et al., 2018; Heinisch et al., 2018; Nyasembe et al., 2018).

The prominent competency of mosquitoes in transmitting numerous parasites and pathogens to mankind has triggered the necessity of devising effective mosquito control approaches. Vector control strategies include environmental methods such as source reduction and habitat manipulation, mechanical methods like the use of polystyrene beads, biological methods such as the exploitation of botanicals and natural enemy of vectors as well as chemical methods like larviciding, adulticiding, the use of insecticide-treated bednets and chemical repellents (World Health Organization, 2012a). Furthermore, health education and legislation enforcement are also crucial in order to intensify knowledge, create awareness and steer behavioural change of general public in combatting the dispersal of mosquito vectors.

Nevertheless, there are many obstacles in conducting most of these control approaches such as the lack of appropriate skills among public health personnel and communities, labour-intensive, time-consuming, highly priced and limited natural sources. Hence, the chemical control using insecticides continues as the most preferred mosquito control tool in many countries including Malaysia (Caputo et al., 2016; Bkhache et al., 2019; Hashim et al., 2018; Tmimi et al., 2018).

Insecticides used in vector control strategies are categorized under several classes namely organochlorines, organophosphates, carbamates, neonicotinoids and pyrethroids (Abreu-Villaca & Levin, 2017). Each of these insecticides possesses either similar or different modes of action and target sites. They are widely utilized as larvicides and/or adulticides to eliminate larvae, adult mosquitoes or both stages. For instance, in Malaysia, pyrethroids like deltamethrin are frequently utilized in the residual spraying and space spraying which target on adult mosquitoes (Rozilawati et al., 2005). On the contrary, malathion and temephos of organophosphates are regularly employed as an adulticide and larvicide, respectively (Vythilingam et al., 1992; Seleena et al., 2001; Teng & Singh, 2001; Chen et al., 2013a). Moreover, control activities using chemical compounds are not only being conducted in public health, but also in the agricultural sector worldwide (Nicolopoulou-Stamati et al., 2016) including Malaysia to suppress crop pest infestations. In fact, many pesticides used in crop pest management possess akin modes of action and target sites as public health insecticides as they belong to similar chemical classes.

Nonetheless, indiscriminate, persistent and inappropriate use of insecticides in vector control activities has prompted the insecticide resistance development among mosquito populations (Sarkar et al., 2018) which subsequently led to control failures. Literally, the exploitation of pesticides in agriculture has also been proven to affect the susceptibility level of mosquitoes against public health insecticides of the same classes. Findings on this scenario have been reported from different types of agricultural areas within several countries namely Greece, Tanzania, Thailand, China and Iran which encompassed mosquito genera of *Aedes*, *Anopheles* and *Culex* (Fotakis et al., 2017; Mbepera et al., 2017; Sumarnrote et al., 2017; Yang et al., 2017; Ghorbani et al., 2018). On the other hand, such information among local mosquito vectors especially *Aedes* mosquitoes are still lacking.

Previous studies on susceptibility status of mosquitoes against public health insecticides in Malaysia comprised of mosquitoes collected from residential areas either in urban, suburban, rural or remote areas but none of them has yet to include any human dwellings within agricultural areas. Consequently, little information is known on the susceptibility status of local vectors within agricultural areas against public health insecticides which will make the vector control activities within these areas if needed in the future become challenging. Hence, this study was performed to fill up the knowledge gap by determining the insecticide susceptibility status of mosquito vectors originated from human habitations within agricultural and non-agricultural areas. Those agricultural areas selected were free from any public health control activities and solely exposed to constant use of pesticides of agriculture. In contrast, non-agricultural areas chosen for this study consisted of residential areas with no history of fogging activities ever conducted by the health department or local authorities as well as residential areas with records of frequent fogging activities due to previously reported dengue cases. Additionally, the underlying mechanisms of insecticide resistance presented in all mosquito populations collected for this study were also investigated.

This study was initiated with the ovitrap surveillance in order to identify the mosquito species available in each study area. Eventually, *Aedes albopictus* was selected as the main focus of this study as only this mosquito species was present in all study areas selected instead of *Ae. aegypti* which is the primary vector of dengue in Malaysia (Chin et al., 2017) but had been successfully captured only in few study areas of this research work. The role of *Ae. albopictus* in the distribution of several mosquito-borne diseases locally is still undeniable and should not be left aside as it has been identified as the major vector of chikungunya in Malaysia (Rozilawati et al., 2011) and retains a significant ecological plasticity that allows it to adapt swiftly to diverse types of breeding habitats which aggravates the control efforts (Wan-Norafikah et al., 2018).

In this research work, bioassays were carried out on both matured larvae and adults of *Ae. albopictus* from all study areas in order to reveal their susceptibility level against common larvicides and adulticides at WHO recommended diagnostic doses and also at independent diagnostic doses of larvicides established from the reference strain. These results were then be supported by the conduct of biochemical assays and the synergism study to detect and understand the underlying metabolic mechanisms of resistance occurred in individual insects. The resistance mechanisms in survivors of insecticide bioassays could only be verified by performing the synergist bioassays and biochemical or molecular tools, or both (World Health Organization, 2016b). These procedures would be able to confirm whether the resistance development detected in selected mosquito populations is caused by either the metabolic activities or gene mutations, or both.

In performing the chemical control of mosquitoes, insecticides could be applied either alone or in combination with a synergist. A synergist such as piperonyl butoxide (PBO) is an emulsifiable organic compound that intensifies the potency of insecticides but does not has any insecticidal effect on its own (Dadzie et al., 2017). PBO is commonly used in combination with pyrethroids due to its capability to inhibit the action of mixed function oxidases (MFO) (Cisse et al., 2017) which consequently persists the usefulness of insecticides. Hence, the study of synergism effects on adults of *Ae. albopictus* had also been carried out in this research work by combining PBO with not only pyrethroids, but also with organochlorines since resistance mechanisms of both classes involve the same target site where the knockdown resistance (*kdr*) mutations arise (Casimiro et al., 2006). Overall, this research work has not only revealed the susceptibility status of selected *Ae. albopictus* populations against common larvicides and adulticides as well as the underlying metabolic mechanisms of resistance involved, but also embraced one of the promising ways in delaying and combatting the insecticide

resistance development in mosquito vectors using the combination of synergist and insecticides.

1.2 Problem Statement

Various types of agricultural areas use different types and doses of pesticides depending on operators, pests and the seriousness of the pest attack in those agricultural areas. However, certain agricultural pesticides utilized have similar targets and modes of action with insecticides used in vector control activities which could cause insecticide resistance development among mosquito vectors. Hence, it is believed that mosquito vectors within those agricultural areas have developed resistance against vector control insecticides due to unintentional exposures of pesticides used in agriculture. Unfortunately, no such information on mosquito vectors of Malaysia is yet to be reported.

1.3 Research Questions

This study was concerned with the following research questions:

1. What mosquito species are present within human dwellings of different types of agricultural and non-agricultural areas?
2. Does the unintentional pesticide exposure of agricultural pest management influence the susceptibility of *Aedes albopictus* larvae and adults against insecticides used in vector control programmes?
3. What are the underlying mechanisms of insecticide resistance present in *Aedes albopictus* populations collected?
4. How effective is the use of the synergist; piperonyl butoxide (PBO) in enhancing the potency of organochlorine and pyrethroid adulticides?

1.4 Objectives of Study

It is crucial to understand the underlying mechanisms of insecticide resistance occurring among mosquito vectors prior to the implementation of appropriate, reliable and effective vector control strategies. Thus, this study was performed based on the following general and specific objectives :

General Objective :

1. To determine the impact of indirect agricultural pesticide exposure on the resistance occurrence against vector control insecticides among *Aedes albopictus* mosquitoes.

Specific Objectives :

1. To determine the mosquito populations present within human dwellings of different types of agricultural and non-agricultural areas.
2. To discover the susceptibility status of *Aedes albopictus* larvae collected from human dwellings within different types of agricultural and non-agricultural areas against common larvicides used in vector control activities at WHO recommended diagnostic doses.
3. To reveal the susceptibility status of *Aedes albopictus* larvae collected from human dwellings within different types of agricultural and non-agricultural areas against common larvicides used in vector control activities at independent diagnostic doses established from the reference strain of *Aedes albopictus*.

4. To ascertain the susceptibility status of *Aedes albopictus* adults collected from human dwellings within different types of agricultural and non-agricultural areas against common adulticides utilized in vector control activities.
5. To detect any occurrence of cross resistance between insecticides of the same and different classes among larvae and adults of *Aedes albopictus* collected.
6. To correlate the occurrence of vector control insecticides resistance among *Aedes albopictus* populations collected with associated detoxification enzymes.
7. To evaluate the efficacy of the synergist; piperonyl butoxide (PBO) in enhancing the potency of organochlorine and pyrethroid adulticides.

A schematic flowchart of this research work is illustrated in Figure 1.1.

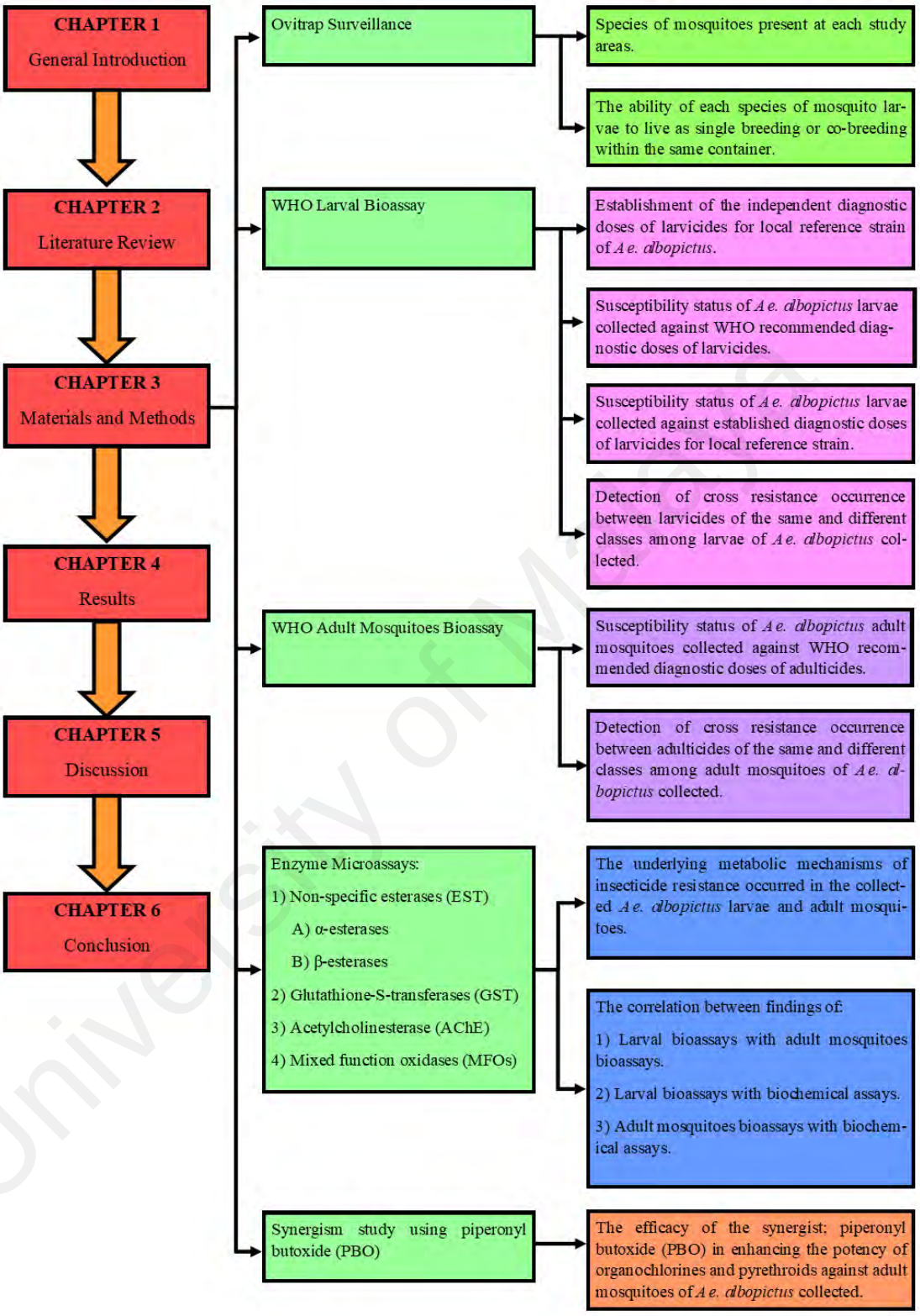

Figure 1.1: A schematic flowchart of "The susceptibility of *Aedes albopictus* Skuse populations from human dwellings to major insecticides for vector control programmes in agricultural and non-agricultural areas".

CHAPTER 2: LITERATURE REVIEW

2.1 Mosquitoes

Mosquitoes have a strong relationship with humans. Besides being a nuisance biting insect, the spreading of many mosquito-borne diseases across the world has become a massive burden on human populations. Hence, countless research work have been carried out globally for centuries until today in order to combat the dispersal of these diseases by mosquitoes.

2.1.1 Classification of Mosquitoes

Mosquitoes are arthropods that are almost globally distributed. There are more than 3500 mosquito species placed in the class Insecta, order Diptera and family Culicidae (Jeffery et al., 2012). These mosquitoes are grouped into 113 genera under subfamilies Culicinae, Anophelinae and Toxorhynchitinae (Rao & Rai, 1990). They dominate a diversity of habitats, covering from as low as 1,250 m below sea level and up to the altitude of 3,600 m (Jeffery et al., 2012).

Mosquitoes that have public health importance belong to the genera *Aedes*, *Anopheles*, *Culex*, *Mansonia*, *Psorophora*, *Haemagogus* and *Sabethes* (Service, 2012). These mosquitoes are capable in spreading diseases like dengue, yellow fever, chikungunya, Zika, malaria, filariasis and Japanese encephalitis (Saleeza et al., 2013). These vector mosquitoes transmit viruses and parasites during their blood feeding on humans and other animals (Hantosh et al., 2012). However, other non-vector mosquitoes are still a nuisance to mankind due to their biting behaviour (Service, 2012).

2.1.2 *Aedes* Mosquitoes

Aedes mosquitoes are categorized under the subfamily Culicinae of the family Culicidae (Service, 2012). Out of approximately 500 distinguished species of *Aedes* genus (Abu Hassan & Yap, 2003), *Aedes aegypti* and *Aedes albopictus* are the most important vector species in global public health (Dickens et al., 2018).

This study emphasized *Aedes albopictus* as one of the principal vectors of dengue, yellow fever, chikungunya and Zika in Malaysia. The other primary vector of these diseases, *Aedes aegypti*, was not covered in this study due to difficulties in obtaining a sufficient amount of samples from ovitrap surveillance conducted in all study areas. However, since both *Ae. aegypti* and *Ae. albopictus* are closely related to one another, the biology and morphological characteristics of *Ae. aegypti* are also briefly discussed in this chapter for further understanding on the common differences between these two species.

2.1.2.1 Biology of *Aedes aegypti* (Linnaeus)

Aedes aegypti (Linnaeus) is believed to be originated from the forests of tropical Sub-Saharan Africa (Mousson et al., 2005). The presence of *Ae. aegypti* in Malaysia had been primarily reported in 1908 (Leicester, 1908; Ho & Vythilingam, 1980). By 1990, *Ae. aegypti* populations had entirely dispersed in Peninsular Malaysia (Lee & Hishamudin, 1990).

Aedes aegypti is anthropophilic and also a predominant indoor breeder (Chareonviriyaphap et al., 2013; Noor Afizah et al., 2018). Hence, *Ae. aegypti* usually breeds inside and within the immediate surroundings of human habitations and prefers to blood feed and rest indoors (Koou et al., 2014b).

Aedes aegypti larvae are normally found in domestic containers indoors and outdoors that are meant to store water for household use especially drinking (Service, 2012).

Aedes aegypti larvae also breed in man-made containers like jar, drums, buckets, bath tubs, flower pots and roof gutters (Vythilingam, 2016).

The distribution of *Ae. aegypti* is worldwide (Gloria-Soria et al., 2018). Among different geographical backgrounds, *Ae. aegypti* is more prevalent in neighbourhoods in urban settings (Kamgang et al., 2017).

2.1.2.2 Biology of *Aedes albopictus* Skuse

Aedes albopictus Skuse is known as the Asian tiger mosquito. *Aedes albopictus* populations are distributed globally but this mosquito species is known to be indigenous in tropical Asia including Malaysia (Rozilawati et al., 2007).

Aedes albopictus is an aggressive biter and feeds on many hosts including humans, domestic and wild animals (Chareonviriyaphap et al., 2013). As an invasive mosquito, *Aedes albopictus* prefers to feed and rest outdoors (Rahim et al., 2018).

Aedes albopictus is also a dominant outdoor breeder (Noor Afizah et al., 2018). However, few local studies had also reported on its presence indoors (Dieng et al., 2010; Wan Norafikah et al., 2011). In fact, the overlapping distribution of *Ae. aegypti* and *Ae. albopictus* within the same breeding receptacles indoors and outdoors have been reported by many researchers (Wan-Norafikah et al., 2012; Roslan et al., 2013; Guo et al., 2016; Hashim et al., 2018).

Aedes albopictus demonstrates preferences to natural breeding grounds that can hold water such as tree holes, leaf axils, bamboo internodes as well as outdoor artificial receptacles with greater amount of organic compounds than tolerated by *Ae. aegypti* (Chareonviriyaphap et al., 2013; Vythilingam, 2016).

Aedes albopictus is previously recognized as a forest and rural species (Hawley, 1988) but later studies had indicated its wide dispersal in urban and suburban areas as

well (Rozilawati et al., 2015). *Aedes albopictus* is also closely associated with high dense vegetation (Higa et al., 2010).

Aedes albopictus is a highly competent mosquito species which results in rapid dispersion into different ecology of many countries (Ayllon et al., 2018). Intrinsic factors like great ecological plasticity, lower anthropophily of adult mosquitoes and resilient competitive aptitude as well as extrinsic aspects including globalization, lack of surveillance and ineffective control activities have facilitated the expansion of *Ae. albopictus* across the globe and thereby lessen its possibility of being exposed to insecticides (Kawada et al., 2010). Other than that, the geographical expansion of *Ae. albopictus* grows rapidly and widely as its eggs could withstand desiccation and could also tolerate with diapause which is an adaptation to lower temperatures (Suter et al., 2017). In fact, for several localities, rather than *Ae. aegypti*, *Ae. albopictus* has been incriminated as the principal vector of important mosquito-borne diseases due to massive invasion of this mosquito species in these areas. For instance, *Ae. albopictus* has been replacing *Ae. aegypti* as the major vector of dengue and chikungunya in Mayotte (Pocquet et al., 2014). Furthermore, instead of *Ae. aegypti* which can be found only in Hainan Province, *Ae. albopictus* which is dominating the mainland of China has been incriminated as the main dengue vector in China (Yiguan et al., 2017).

2.1.2.3 Morphological Characteristics of *Aedes aegypti* and *Aedes albopictus*

Despite various developmental stages throughout a single life cycle, the mosquito species identification by morphological method is usually being performed either at late third instar or early fourth instar of the larval stage, or at adult stage. Several morphological characteristics are pinpointed to distinguish *Aedes* from other mosquito genus. At larval stage, *Aedes* larva possesses an abdomen whereby a short barrel-shaped siphon with one hair or subventral tuft on each of its side is attached at the end of the

abdomen (Service, 2012). On the other hand, *Aedes* which is small to medium-sized adult mosquito with approximately 4 to 6 mm is normally black to dark in colour (Division of Medical Entomology, 2000a). The banded legs and abdomen of *Aedes* adult mosquito are adorned with distinctive patterns of white spots, patches or lines of scales (Division of Medical Entomology, 2000a). The female *Aedes* adult mosquito has a pointed abdomen at its tip and its pair of palps is shorter than one third of its black proboscis (Division of Medical Entomology, 2000b). The most prominent morphological characteristic to distinguish between *Ae. aegypti* and *Ae. albopictus* adults is the difference in the pattern of the silvery white stripe on their mesonotum (Division of Medical Entomology, 2000a; 2000b) (Plate 3.1).

Since *Ae. aegypti* and *Ae. albopictus* are sympatric species (Sumruayphol et al., 2016), it is crucial for researchers to be able to distinguish between these two species. The differences in morphological characteristics between *Ae. aegypti* and *Ae. albopictus* for the stage of larva and adult mosquito are illustrated in Table 2.1.

Plate 3.1: *Aedes aegypti* (left side) and *Aedes albopictus* (right side) female adults.

Table 2.1: The morphological characteristics of larva and adult mosquito of *Aedes aegypti* and *Aedes albopictus* (Division of Medical Entomology, 2000a; 2000b).

Developmental stage	**External structures**	***Aedes aegypti***	***Aedes albopictus***
Larva	Abdomen	Comb of 8 – 12 teeth with well-developed lateral denticles on the eighth segment.	Comb with 8 – 12 large strong teeth without lateral denticles on the eighth segment.
	Thorax	Bases of pleural hairs on mesothorax and metathorax are large and ending in a single point.	Bases of pleural hairs on mesothorax and metathorax are small and ending in several points.
Adult mosquito	Thorax	Dark brown with typical lyre-shaped marking with silvery white scales on the mesonotum.	Dark brown with a one longitudinal medium silvery white narrow stripe on the mesonotum.
		Broad flat scales on the scutellum.	Broad flat scales on the scutellum.
	Legs	Narrow white bands at the bases of tarsi on the fore and mid pairs of legs.	Narrow white bands on the fore and and mid tarsi.
		Five broad white basal bands on the hind pair of legs.	Broad white bands on the hind tarsi.
		Last segment is almost or entirely white in colour.	Fifth segment is entirely white in colour.
		No white scales or spots on all tibiae.	No dots of white scales on all tibiae.
	Abdomen	Dark in colour with white basal bands laterally and on the segment dorsum.	White basal bands on the dorsum and laterally on the abdominal segments.
	Head	Two dots of white scales on clypeus which is the segment above the proboscis.	No dots of white scales on clypeus which is the segment above the proboscis.

2.1.2.4 Life Cycle of *Aedes* Mosquitoes

Similar to other mosquito species, *Aedes* mosquitoes develop through a holometabolous life cycle (Farnesi et al., 2012). This complete metamorphosis comprises of four life stages namely eggs, larvae, pupae and adults (Kauffman et al., 2017) (Figure 2.1). The immature stages of eggs, larvae and pupae grow in aquatic ecology (Yang et al., 2011). *Aedes* mosquitoes require clear water but not necessarily clean water as their breeding sites (Chen et al., 2009a). The entire life cycle of *Aedes* at ambient temperature generally takes about 10 to 12 days (Service, 2012). For *Ae. aegypti* and *Ae. albopictus*, the egg hatching, larval period and pupal phase are within 1 to 48 hours at ambient temperature, 6 to 8 days and 1 to 2 days, respectively (Lee, 2000).

Eggs of *Aedes* are black and oval in shape with about 0.5 mm long (Christophers, 1960). These eggs are laid singly on moist medium just above the water line (Service, 2012). The egg laying of *Aedes* is not deterred by partial shade as *Aedes* immatures had also been found in partly covered containers (Lee, 2000). *Aedes* eggs are able to withstand desiccation (Brown et al., 2017). Eggs of *Aedes* are also easily being dispersed by humans during transportation of containers or materials that can hold water (Vythilingam, 2016).

The hatching process of *Aedes* eggs is initiated by the reduction of dissolved oxygen concentration in the surrounding aquatic environment due to biotic actions in the flooded habitat (Zheng et al., 2015). This condition will indirectly stimulate the rupture of the water-resistant barrier which allows the water entrance while the larvae swell out from the shells (Weissman-Strum & Kindler, 1963). Egg hatching rates of *Aedes* are different between one another even among the same egg batch or those exposed to similar ecological conditions (Gillet, 1955; Fischer et al., 2011). This scenario ruins the

effectiveness of the larvicidal activities as only hatched larvae can be destroyed by the larvicides while the unhatched eggs are still viable by the end of the operations.

Aedes larvae dwell in water and arise at the water surface occasionally to breathe through their siphon tubes (Kauffman et al., 2017). *Aedes* larvae undergo four instars (Valzania et al. 2018), whereby the subsequent instar is larger in size than the former. These larvae shed their exoskeleton while molting (Bara et al., 2013). *Aedes* larvae feed voraciously on microorganisms, fine detritus, algae and organic particles (Schaper & Hernandez-Chavarria, 2006; Garcia-Sanchez et al., 2017). The whole larval stage period depends on several factors like temperature, nutrition and crowding (Kauffman et al., 2017).

Active mosquito pupae emerge upon the cuticle shedding of the fourth instar larvae (Bar & Andrew, 2013). Mosquito pupae are comma-shaped with the head and thorax are merged forming the cephalothorax (Ha et al., 2017). These pupae breathe through a pair of trumpet positioned on top of the cephalothorax (Reid, 1963). Mosquito pupae do not require any feeding throughout the pupal stage and only depend on reserved energy from the previous larval stages (Awasthi et al., 2012). These pupae possess all adult organs but at the state of incomplete development (Goma, 1966).

Upon full development of adult mosquitoes in the pupae cuticle, the adult mosquitoes emerge from the pupal case using air pressure while floating at the water surface (Nasci & Miller, 1996). The newly emerged adult mosquitoes rest on the water surface for a while to dry themselves and let their body parts to harden (Nasci & Miller, 1996). The male adult mosquitoes usually emerge earlier than female adult mosquitoes (Wang et al., 2017). The frequency of wing-beats by female adult mosquitoes are detected by the male adult mosquitoes using their plumose antennae (Arthur et al., 2014). Adult mosquitoes mate after several days of their emergence (Oliva et al., 2011). During copulation, the male mosquitoes transfer sperm and seminal fluid proteins to the female

mosquitoes (Helinski & Harrington, 2011). Single mating is the most common in mosquito populations as the initial mating will significantly lessen the tendency of female mosquitoes to be inseminated again by other male mosquitoes (Klowden, 1999). After mating, sperm are stored and nourished in the spermathecae of the female adult mosquitoes for their entire lifespan (Degner & Harrington, 2016).

Both male and female adult mosquitoes feed on natural sugar sources particularly floral nectars or other plant juices for their nutrition and energy (Gu et al., 2011). Only female adult mosquitoes bite humans as the blood meal provides a highly nutritive protein for the production and development of their eggs (Kim et al., 2011). Both *Ae. aegypti* and *Ae. albopictus* bite during day time especially at dawn and dusk with their preferences towards humans but *Ae. albopictus* is more likely a generalist feeder (Vythilingam, 2016). The flight range of *Aedes* adults are generally about 100 metres (Hiscox et al., 2013). According to Lee (2000), the flight range of both *Ae. aegypti* and *Ae. albopictus* adults are approximately 200 metres.

Upon egg maturation, gravid female adult mosquitoes instigate oviposition searching flights to seek suitable breeding sites (Day, 2016). Once the egg laying is completed, these female adult mosquitoes initiate the hunting of the next blood meal in order to deposit another set of eggs. The time interval between two consecutive blood meals starting from taking a blood meal until depositing a batch of eggs is referred as the gonotrophic cycle (Lardeux et al., 2008). Several gonotrophic cycles are repeated by female adult mosquitoes throughout their lifetime but the duration of each gonotrophic cycle relies on temperature (Paaijmans & Thomas, 2011). Furthermore, several blood feeding within one gonotrophic cycle of female adult mosquitoes may also happen whereby this scenario will escalate the risk of disease transmission due to recurrent contact with hosts (Farjana & Tuno, 2013). The whole life cycle is reiterated until the adult mosquito perishes (Rozendaal, 1997).

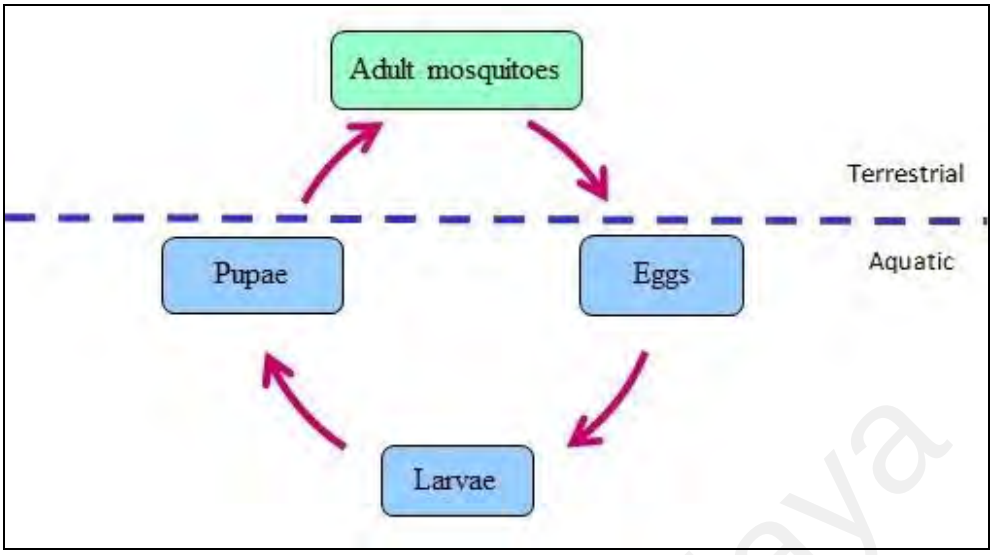

Figure 2.1: Life cycle of *Aedes* mosquitoes.

2.1.2.5 Medical Importance of *Aedes* Mosquitoes

Mosquitoes are the most important insects due to their competencies in transmitting variety of bacteria, viruses and parasites that cause diseases to mankind, livestock and wildlife. These pathogens are transmitted by female mosquitoes during blood-feeding (Lee et al., 2016). The capacity of mosquitoes in transmitting life threatening diseases relies on their perseverance, ecology, nutrition and also the existence of diverse and dynamic microbiota in their midgut (Brady et al., 2014; Porretta et al., 2016; Yadav et al., 2016).

Mosquito-borne diseases cause massive human illnesses and deaths (Carvalho & Moreira, 2017). Dengue fever (DF), dengue haemorrhagic fever (DHF), yellow fever (YF), chikungunya and Zika are life-threatening diseases for humans which are transmitted by *Aedes aegypti* and *Ae. albopictus* (Alvarez Costa et al., 2017; Mordecai et al., 2017). *Aedes aegypti* is the primary vector of these diseases (Gonzales et al., 2017). However, in situations where *Ae. aegypti* is rare or absent, *Ae. albopictus* has become the main vector (Delatte et al., 2010). As such, *Ae. albopictus* acted as the

initial dengue and chikungunya vectors in Hawaii and Gabon, respectively (Effler et al., 2005; Pages et al., 2009).

2.1.2.5.1 *Aedes* Mosquitoes as a Vector of Dengue and Dengue Haemorrhagic Fever (DHF)

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are public health concerns worldwide. Both diseases are transmitted by *Ae. aegypti* and *Ae. albopictus* (Soto-Garita et al., 2016). Other *Aedes* species that may transmit these diseases include *Ae. polynesiensis*, *Ae. pseudoscutellaris*, *Ae. scutellaris*, *Ae. rotumae* and *Ae. horrescens* (Prakash et al., 2001).

DF and DHF are caused by a re-emerging dengue virus (DENV) of the genus *Flavivirus* within the family Flaviviridae. There are four distinct serotypes (DEN-1 to DEN-4) of dengue virus known (Guillaumot, 2005). DF is typically a self-limiting disease (Pacsa et al., 2002). DF can be either asymptomatic or symptomatic (Olivera-Botello et al., 2016). Clinical symptoms of DF which vary among different age of patients include mild febrile illness or high-grade fever, intense headache, myalgia, arthralgia, retro-orbital eye pain, photophobia and generalized body ache (World Health Organization, 2012b). DHF and dengue shock syndrome (DSS) are the more severe form of dengue infection (Suleman et al., 2017). DHF is characterized by major clinical manifestations comprising of high fever, haemorrhagic manifestations, hepatomegaly and circulatory failure (World Health Organization, 2012b). Clinical features of DHF resemble DF with thrombocytopenia and evidence of plasma leakage (Guerdan, 2010).

Millions of people are infected with DF and DHF each year globally (Ray et al., 2017). According to World Health Organization (2011a), 50 million dengue infections are estimated to occur throughout the world each year with two fifths of the world's population particularly in tropical and subtropical regions are at risk. DF and DHF are

endemic in more than 100 nations where both Southeast Asia and Western Pacific regions are the most seriously involved (World Health Organization, 2011a). The first DHF outbreak was reported from the Philippines in 1953 which was followed by another DHF outbreak in Thailand in 1958 (Chareonsook et al., 1999; Kalayanarooj, 2011).

In Malaysia, the first report of DF and DHF was recorded in 1902 and 1962 from Penang, respectively (Skae, 1902; Rudnick et al., 1965). A major dengue epidemic was documented in 1973 (Mudin, 2015). Since then, DF has become endemic in Malaysia with major outbreaks in a 4-year cycle (Lam, 1993). DF and DHF continue as the main cause of morbidity and mortality in Malaysia with dengue-related deaths involved mainly adult patients (Cheah et al., 2014; Mohd-Zaki et al., 2014; Woon et al., 2016). For the year of 2018, from January until 22nd December 2018, the cumulative total of reported dengue cases in Malaysia was 78,066 cases with 140 deaths (Ministry of Health Malaysia, 2018).

2.1.2.5.2 *Aedes* Mosquitoes as a Vector of Yellow Fever

Yellow fever (YF) is a zoonotic disease caused by the yellow fever virus (YFV) from genus Flavivirus of the family Flaviviridae (Holanda et al., 2017). Symptoms of YF infection include sudden onset of fever, backache, headache, nausea, general muscle pain and vomiting (World Health Organization, 1998a). Jaundice, vomiting, haemorrhagic features, albuminuria and oliguria may occur in patients undergoing toxic phase of YF infection (World Health Organization, 1998a).

Aedes aegypti is the most responsible vector in the urban cycle of YF transmission which is also the cause of major YF outbreaks in Africa (Chen & Lu, 2016). On the other hand, *Ae. africanus*, *Ae. bromeliae*, *Haemagogus* sp., *Sabethes* sp. and other *Ae*des species act as vectors in the sylvatic cycle of YF transmission where monkeys serve as

the primary host and humans act as the accidental host (World Health Organization, 1998a; Miyaji et al., 2017). In addition, YF transmission occurred in rural areas particularly at the edges of forests in Africa is triggered by an intermediate transmission cycle where *Aedes* sp. serve as the vector while both humans and non-human primates act as the host (World Health Organization, 1998a).

It is estimated that 200,000 cases of YF infection with 30,000 deaths occurred each year globally (World Health Organization, 1992a). YF is endemic in tropical and sub-tropical Africa and South America (Kongsgaard et al., 2017). No YFV has ever been detected in Asia so far (Watson and Klimstra, 2017). However, traveller-associated cases of YF had been recorded in China for the first time in 2016 (Li et al., 2016a).

Specific antiviral treatment for YF infection is unavailable but YF vaccination using 17D strain discovered by Max Theiler and his colleagues is the most effective and safe preventive measure (Tan and Pettigrew, 2017). A single dose of YF vaccine which has been proved to provide a long-term immune protection is recommended by World Health Organization (2013).

Malaysia is currently free from any yellow fever cases. Nevertheless, the Malaysian government has outlined a preventive measure to minimize the risk of YF transmission in the country by requiring all Malaysians going to or through those YF endemic areas or countries as well as visitors coming from or through those YF endemic areas or countries to show the YF vaccination certificate. Only YF vaccination certificate issued upon receiving vaccination that is approved by World Health Organization (2018) and administered at an approved YF Vaccinating Centre within 10 days after vaccination until 10 years of duration is valid.

2.1.2.5.3 *Aedes* Mosquitoes as a Vector of Chikungunya

The causative agent of chikungunya is a re-emerging chikungunya virus (CHIKV) which is an alphavirus of the family Togaviridae (Chua et al., 2017). CHIKV was isolated for the first time in Tanzania in 1952 (Seyedi et al., 2016). Three genotypes of CHIKV are discovered so far consisting of West Africa, East/Central/South African (ECSA), and Asian genotypes (Chiam et al., 2015).

Aedes aegypti and *Ae. albopictus* play an important role as the vector of CHIKV infection (Vythilingam et al., 2016). As such, *Ae. aegypti* involved as the vector during chikungunya outbreaks in the Caribbean region, whereas *Ae. albopictus* was determined as the chikungunya vector during outbreaks in Johor, Malaysia (Rozilawati et al., 2011; Morrison, 2014).

Chikungunya infection is characterized by clinical features like high fever, headache, rashes, arthralgia, myalgia, and fatigue (Simarmata et al., 2016; Luksic et al., 2017). Similar clinical presentations of dengue and chikungunya may cause misdiagnosis of the co-infection status. Hence, the actual number of chikungunya cases may also be underestimated (Morrison, 2014).

Chikungunya cases were detected mostly in Africa and Asia (Sam & Abubakar, 2006). In Malaysia, the first report of chikungunya infection was recorded in 1998 until 1999 in Klang, Selangor (Lam et al., 2001). The second chikungunya outbreaks in Malaysia were reported in Perak in 2006, followed by related outbreaks in 2008 and 2009 that initially occurred in Johor before consequently spread to other parts of the country (Chua, 2010).

2.1.2.5.4 *Aedes* Mosquitoes as a Vector of Zika Virus

Zika virus (ZIKV) is a flavivirus of the family Flaviviridae (Mehrjardi et al., 2017). ZIKV was first discovered in 1947 from serum of a sentinel monkey in Zika Forest in Uganda (Weinbren & Williams, 1958). Infection of ZIKV in human was initially documented in 1954 in Nigeria (Macnamara, 1954).

The first ZIKV infection outbreak outside Africa and Asia was recorded in 2007 in Pacific Island of Yap in the Federated States of Micronesia (Vorou, 2016). In Malaysia, ZIKV was isolated for the first time in 1969 from *Ae. aegypti* (Marchette et al., 1969). The first three cases of ZIKV infection in human in Malaysia was reported in August and September 2016 (Salehuddin et al., 2017). No ZIKV infection has been reported in Malaysia for the year of 2017 until 1st April 2017 (Ministry of Health Malaysia, 2017).

Aedes aegypti is the principal vector of ZIKV but *Culex quinquefasciatus* and other *Aedes* species are also capable as ZIKV vectors (Hart et al., 2017). For instance, *Ae. albopictus* was incriminated as a vector of ZIKV infection in Gabon in 2007 (Grard et al., 2014).

ZIKV infection is usually asymptomatic (Sam et al., 2016). However, symptoms like mild dengue-related illness, fever, joint pain, maculopapular rash, myalgia, headache, arthralgia and conjunctivitis may also occur (Yaren et al., 2017). Serious ZIKV infection is often associated with rising cases of congenital microcephaly in babies of infected mothers and Guillain-Barre syndrome in adults (Yi et al., 2017). No vaccine and specific treatment is available for ZIKV infection so far (Al Ali & Al Ali, 2016).

2.1.3 Control of Mosquitoes

Prevention of many mosquito-borne diseases relies mostly on vector control measures since there is still no specific and effective treatment and vaccines available for these diseases (Banumathi et al., 2017). In general, control of mosquito vectors is comprised of several categories namely environmental management, mechanical control, community participation, biological control, genetic control and chemical control. Most of these methods could be applied in the control of different diseases indicating their usefulness when more than one disease co-occur in the same environment.

2.1.3.1 Environmental Management, Mechanical Control of Mosquitoes and Community Participation

Environmental management includes the environmental modification or manipulation to prevent or minimize the vector breeding in both natural and artificial habitats which will then reduce the human-vector contact (World Health Organization, 1992b). Even though the use of insecticides has now becomes the most selected control method for mosquito populations, the frequency of insecticide applications and the concentrations of insecticides used could be diminished by the reduction of mosquito populations through other vector control approaches including the environmental management. Various methods of environmental management have been suggested to be implemented such as source reduction to eliminate potential breeding sites, irrigation management as well as regular and proper waste disposal (World Health Organization, 2012a).

Source reduction is the most effective environmental control method. Source reduction is practicable in reducing mosquito density since mosquito larvae are confined only to water bodies and thus, they are easier to be controlled as compared to adult

mosquitoes (World Health Organization, 2012a). However, it is tough to be conducted since it requires the involvement and time of many trained health personnel (Unlu et al., 2016). Hence, active and continuous participation of residents is crucial to ensure the success and effectiveness of the source reduction campaigns conducted (Johnson et al., 2017).

Meanwhile, the distribution of mosquito populations could also be controlled by mechanical strategies. The mechanical control of mosquito breeding includes house-proofing by screen installation on windows and doors, employment of mosquito trapping devices and placement of polystyrene beads on water surface in water receptacles (World Health Organization, 2012a). Numerous mosquito trapping devices have been designed and invented with various specifications and attractants but with similar aim of reducing the population of mosquito vectors. For instance, an electrical mosquito trapping device named as "Mosquito Killing System" (MKS) had been produced to trap and destroy nuisance adult mosquitoes outdoors (Wan-Norafikah et al., 2017a). In contrast, the "Mosquito Larval Trapping Device" (MLTD) which has been introduced and used by the Kuala Lumpur City Hall, Malaysia for surveillance and control activities attracts gravid adult mosquitoes to oviposit in it (Azil et al., 2014). The succeeding mosquito generation is then being trapped within the MLTD which will eventually perish in the device.

The mosquito control by environmental management and mechanical tools usually require high empowerment and commitment of community members. Hence, it is essential to ensure that all community members are provided with sufficient information on mosquito biology and the seriousness of the spread of mosquito-borne diseases in order to create awareness among them. The public awareness and education programmes on the importance of source reduction and personal protection in

minimizing the man-mosquito contact are among the recommended approaches proposed by World Health Organization (2012a).

In Malaysia, the media campaign for dengue prevention and control has been carried out by the Ministry of Health Malaysia via television, advertising spots and newspaper insertions to encourage the community members to set aside ten minutes per week for searching and discarding mosquito breeding receptacles both indoors and outdoors. Moreover, 'gotong royong' activities and local Community Communication for Behavioural Impact (COMBI) Dengue have been performed throughout the nation constantly (Ministry of Health Malaysia, 2011). Nevertheless, all these efforts have not yet showed much improvement in the reduction of dengue cases in Malaysia due to the lack of community awareness and engagement.

2.1.3.2 Biological Control of Mosquitoes

Biological control is an environmentally friendly approach in which natural enemies or predators are utilized to control the populations of mosquito immatures in the aquatic environments (Kumar et al., 2008). The predator-prey interaction is the main concept in biological control. These natural predators of mosquito larvae include larvivorous fishes, fungi, bacteria, cyclopoid copepods and dragonflies as well as larvae of other mosquito species (Benelli et al., 2016).

The use of larvivorous fishes is one of the alternative methods in controlling mosquito larvae in small and monitored natural habitats (Barik et al., 2018). In Malaysia, Saleeza et al. (2014) exhibited the potential use of the guppy *Poecilia reticulata* in the control of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae. On the other hand, Zuharah et al. (2016) displayed significant reduction of *Aedes* larvae with the presence of the predatory fish of *Hampala macrolepidota* and its kairomone remnant that could be detected by these mosquito larvae. In India, the mosquito fish,

Gambusia affinis displayed high predation level against several mosquito immatures such as *An. stephensi* and *Ae. albopictus* (Subramaniam et al., 2015). Furthermore, Chala et al. (2016) showed a high number of *An. arabiensis* immatures consumed by the African catfish, *Clarias gariepinus* while Barik et al. (2018) reported on the predatory potential of the tiger barb, *Puntius tetrazona* and the rosy tetra, *Hyphessobrycon rosaceus* as the larvivorous fishes for the control of *Cx. vishnui* larvae.

Besides that, the application of entomopathogenic fungi in mosquito control which involves only insect's cuticular external contact to trigger the fungal infections among mosquito vectors (Kamareddine, 2012) has also been highlighted by researchers. For instance, the effectiveness of the entomopathogenic fungus, *Metarhizium anisopliae* in controlling different developmental stages of *Ae. aegypti* have been widely stated (Falvo et al., 2018). Different susceptibility level was also demonstrated by *Ae. aegypti* and *Cx. quinquefasciatus* larvae against entomopathogenic fungus, *Metarhizium brunneum* (Alkhaibari et al., 2018).

Many biological control studies of mosquito vectors have been carried out using microbial agents like *Bacillus thuringiensis israelensis* and also *Lysinibacillus sphaericus* which was formerly known as *Bacillus sphaericus* (Bs). Both *Bacillus thuringiensis israelensis* (Bti) and *Lysinibacillus sphaericus* are gram-positive bacteria that produce the insecticidal crystal proteins during the sporulation (Saiful et al., 2012). These crystals are comprised of one or more toxins that are environmentally safe and biodegradable (Chen et al., 2009b; Saiful et al., 2012). Bti toxins have been proved to be specifically and greatly effective against mosquito larvae (Lee et al., 2008). In a one year study of Bti application in a dengue area in Selangor, Malaysia, the populations of both *Ae. aegypti* and *Ae. albopictus* were significantly suppressed (Tan et al., 2012). In Cambodia, the population of *Ae. aegypti* pupae and adults were significantly suppressed for three months upon the single application of Bti (Setha et al., 2016). Significant

density reduction was also observed for mosquito larvae and pupae in the majority of the Bti-treated breeding habitats in India (Uragayala et al., 2018). The promising efficacy of Bti in controlling *Ae. aegypti* populations in Lao PDR was also exhibited through the laboratory and field studies by Marcombe et al. (2018).

Resistance to Bti has been reported in the laboratory against some mosquito species including *Ae. aegypti* (Gharib & Szalay-Marzso, 1986; Goldman et al., 1986). However, no incidence of *Aedes* resistance against Bti has been encountered in the field populations thus far. The susceptibility level of *Ae. aegypti* larvae from Bti-treated and non-treated localities in Selangor, Malaysia remained uniform even after eighteen Bti treatments for Bti-treated area throughout seven months of study (Loke et al., 2010). Additionally, Suter et al. (2017) reported on similar susceptibility level against Bti among *Ae. aegypti* and *Ae. albopictus* collected from the non-treated and Bti-treated areas of Swiss-Italian border implicating that there was no development of resistance against Bti in these mosquito populations. Nevertheless, resistance development against *Lysinibacillus sphaericus* among mosquito vectors has been described by several researchers. As reported by Yu et al. (2017), even though the oviposition rate of *An. dirus* in China had been suppressed using a sub-lethal dose of *Lysinibacillus sphaericus*, similar dose could also confer resistance among this mosquito population. Resistance against *Lysinibacillus sphaericus* had been demonstrated among *Cx. pipiens* collected from California as well (Su et al., 2018).

Other than that, the employment of predacious copepods, *Mesocyclops*, had significantly eliminated *Aedes* populations in Vietnam (Nam et al., 2012). In fact, the combination of the copepod; *Mesocyclops* and the bacterium, Bti exhibited significant reduction of *Ae. albopictus* larval densities in comparison to the application of either *Mesocyclops* or Bti alone in Brazil (Silva et al., 2015). However, lower effectiveness on

the use of *Mesocyclops* was displayed in Cambodia due to polluted water source, lacking of colonizing training and poor community acceptance (Hustedt et al., 2017).

Meanwhile, the predatory mosquito larvae of *Toxorhynchites* sp. have been considered as an ideal natural biocontrol agent for larvae of other mosquito species especially *Aedes*. Both male and female adults of *Toxorhynchites* sp. feed exclusively on plant nectar and do not require a blood meal for egg development (Nordin et al., 2013; Mohamad & Zuharah, 2014; Donald et al., 2018). Furthermore, *Toxorhynchites* female adults possess the ability in seeking out the aquatic habitats with positive larval breeding of mosquito vectors especially those that are not accessible to insecticide treatment (Collins & Blackwell, 2000; Ong, 2016). Mixed breeding of *Toxorhynchites* larvae with *Ae. aegypti* or *Ae. albopictus* larvae had been found in water jars, rubber tires, tins and also ovitraps (Yasuno & Tonn, 1970; Trpis, 1973; Nyamah et al., 2011).

In Malaysia, two laboratory studies and one field study had demonstrated the significant role of *Tx. splendens* larvae in controlling both *Ae. aegypti* and *Ae. albopictus* larval populations (Nyamah et al., 2011; Mohamad & Zuharah, 2014; Zuharah et al., 2015). The Subang Jaya Municipal Council in Selangor, Malaysia had even conducted several field release attempts of *Toxorhynchites* adults into two dengue prone localities starting from 2011 until 2013 (Nathan, 2013; Sen, 2014). Nevertheless, although the outcomes were promising as observed in the significant reduction of reported dengue cases in both study areas (Nathan, 2013; Sen, 2014), more inclusive field studies are still needed to prove the suitability and efficacy of this biological control method before it could be implemented in a large scale locally. This is because the main vector of dengue *Ae. aegypti* is highly urbanised while *Toxorhynchites* sp. is actually a forest mosquito which is unlikely to survive in urban ecosystems (Collins & Blackwell, 2000; Higa, 2011; Babulal & Ying, 2019). Moreover, the three times longer of *Toxorhynchites* larval development time as compared to their prey and also the egg

laying behaviour by *Toxorhynchites* gravid female adults into different breeding habitats from the target species had led to failure in controlling the prey immature populations (Collins & Blackwell, 2000).

In general, despite harmless effects against the environment, the mosquito control using biological control agents however requires manual introduction of these agents to the mosquito larval habitats and consistent monitoring in order to achieve a significant level of the predation effectiveness (Collins & Blackwell, 2000). Hence, sufficient educational programmes for public are essential to guide them on the appropriate use of water receptacles and correct maintenance of larvivorous organisms cultured in these water containers.

2.1.3.3 Genetic Modification of Mosquitoes

Mosquito control using genetic modification methods are progressively developing throughout the years. The Sterile Insect Technique (SIT) is a traditional method which involves the mass rearing, sterilization and open field release of high numbers of sterile male insects (Seirin Lee et al., 2013). These radiation-sterilised males are released in the field to mate with wild females to produce progeny that are not viable (Yakob et al., 2017). Promising results from SIT trials had been reported involving agricultural pests such as the New World screwworm (*Cochliomyia hominivorax*), the Mediterranean fruit fly (*Ceratitis capitata*) and the pink bollworm (*Pectinophora gossypiella*) in the United States (Alphey et al., 2010). However, a large-scale SIT trial had never succeeded against mosquitoes due to damaging and deleterious effects of radiation on mosquitoes which also reduced their mating competitiveness in the field (Lee et al., 2009; Lacroix et al., 2012).

The Release of Insects carrying a Dominant Lethal gene (RIDL) is an enhancement approach of the SIT by which a dominant lethal transgene is inserted into the mosquito (Alphey et al., 2013). Without tetracycline as a suppressor in the mosquitoes' diet, the RIDL system is expressed (Nordin et al., 2013) which eventually causes lethality of the offspring at larval or pupal stage (Bargielowski et al., 2011). Since RIDL method allows the setting of the effect to occur at any developmental stage of the mosquito, the RIDL larvae that survived up to pupal stage will indirectly act as a natural control tool through the nutrient restraint (Yakob et al., 2017). *Aedes aegypti* RIDL strain males that were released and tested in Cayman Islands demonstrated potential outcomes in mosquito control through population suppression (Harris et al., 2012). Similar RIDL strain of *Ae. aegypti* males had also been field released in Brazil and Malaysia (Lacroix et al., 2012; Carvalho et al., 2015).

Although both SIT and RIDL are considered as eco-friendly and species-specific (Hoang et al., 2016; Qsim *et al.*, 2017), these methods are still considered as a provisional methods of suppressing the mosquito population (Yakob et al., 2017). Moreover, for sterile-release approaches of mosquito control, mating competitiveness of the sterile males released into the environment is the main concern before further aspects could be evaluated (Lee et al., 2013).

Recently, the release of self-sustaining *Wolbachia*-infected *Aedes* mosquitoes offers a potential approach in preventing the spread of arboviruses in mosquitoes. *Wolbachia* is an endosymbiotic intracellular bacterium that naturally infects various arthropods including about 28% of mosquito species namely *Ae. albopictus*, *Cx. quinquefasciatus* and *Cx. pipiens* (Amuzu et al., 2018). However, for certain insects like *Ae. aegypti* that are not naturally infected with *Wolbachia*, the transinfection of *Wolbachia* from any natural hosts such as *Drosophila melanogaster* or *Ae. albopictus* using the microinjection technique is required (van den Hurk, 2018). *Wolbachia* infection could

act as one of the mosquito control strategy named as Incompatible Insect Technique (IIT) (Benelli et al., 2017). In *Wolbachia*-based IIT, the cytoplasmic incompatibility (CI) is presented when infected female insects produce viable progeny but uninfected females do not have viable progeny upon mating with infected males (Yakob et al., 2017). The former scenario allows *Wolbachia* to propagate within the mosquito population (Amuzu et al., 2018). On the other hand, the latter scenario reduces mosquito oviposition rates, disrupts mosquito reproduction, diminishes the ability of mosquito in transmitting arboviruses and shortens the mosquito lifespan by several days (Amuzu et al., 2018; Xue et al., 2018). Furthermore, the ability of *Wolbachia* to be maternally transmitted facilitates the dispersal of *Wolbachia* into target mosquito populations (Yakob et al., 2017; van den Hurk, 2018). The earliest field releases of *Wolbachia*-infected *Ae. aegypti* males that were carried out in northern Queensland, Australia had successfully invaded the natural populations of the same mosquito species (Hoffmann et al., 2011). In Peninsular Malaysia, the widespread of *Wolbachia*-infected *Ae. albopictus* females and males in five localities as reported by Noor Afizah et al. (2015a) provides a positive prospect in discovering the potential advantages of *Wolbachia* infection for local mosquito control.

2.1.3.4 Physical and Chemical Barriers for Personal Protection

Personal protection strategies are the initial line of defense for an individual or the household against mosquito biting. Physical barriers for personal protection include wearing long-sleeved shirts and long pants as well as the utilization of bed nets (Lalani et al., 2016; LaRocque & Ryan, 2016). In fact, the use of physical barriers has even been improved with the addition of chemical substances to enhance the efficacy of these physical protective tools in preventing humans from the mosquito bites.

Chemical barriers are widely applied in miscellaneous ways. Topical skin repellents made from either synthetic chemicals like *N,N*-diethyl-*meta*-toluamide (DEET) or plant chemical compounds provide different level of individual protection against adult mosquitoes (Bedini et al., 2018). Furthermore, the use of clothing and bed nets impregnated with insecticides particularly pyrethroids are among the most utilized methods for individual and household protection in malaria control (Crawshaw et al., 2017; Marcombe et al., 2017). Additionally, chemical substances are also utilized in the form of coils, mats and liquid vaporisers to protect personnel and household from indoor and outdoor mosquito biting (Laksham et al., 2016; Amelia-Yap et al., 2018; Tangena et al., 2018).

2.1.3.5 Chemical Control of Mosquitoes

Among all mosquito control methods outlined by World Health Organization, the employment of different classes of insecticides remains the most preferred vector control strategy worldwide (Auteri et al., 2018). Chemical insecticides utilized for mosquito control are applied in various ways depending on the ecology and behaviour of the targeted mosquitoes. Chemical control measures of mosquitoes include larviciding activity which involves insecticidal treatment of mosquito breeding habitats as well as space spraying, residual spraying, insecticide-treated bed nets and assorted forms of chemical repellents that are targeting the adult mosquitoes (World Health Organization, 2012a).

The use of larvicides such as temephos in larviciding activity of natural and artificial breeding grounds has been practiced in several countries including Malaysia and Cambodia (Chen et al., 2013a; Boyer et al., 2018). Meanwhile, adulticides like malathion of organophosphates and also a wide range of pyrethroids are employed in the space spraying and residual spraying activities. For example, malathion is the

adulticide of choice for dengue control in Malaysia, Sri Lanka and United States of America (Nazni et al., 1998; Karunaratne et al., 2013; Muturi, 2013). On the other hand, pyrethroids are utilized in the space spraying and indoor or outdoor residual spraying operations conducted in Malaysia, Thailand, China and Peru (Wan-Norafikah et al., 2013a; Gao et al., 2018; Gunning et al., 2018; Son-un et al., 2018).

Besides that, bed nets treated with pyrethroids like deltamethrin are widely used for malaria control (Parker et al., 2017). Moreover, pyrethroids are also commercially produced as mosquito coils, electric mats and vaporizers as well as household aerosol sprays (Li et al., 2016b).

Other than that, a group of insecticides named as insect growth regulators (IGR) could also be applied in water holding containers to control mosquito larvae. Juvenile hormone analogues like methoprene and pyriproxyfen are IGR that inhibit the emergence of adult mosquitoes while chitin synthesis inhibitors (CSI) like diflubenzuron, cyromazine and novaluron are IGRs that impede the chitin synthesis during ecdysis of all developmental stages of mosquito immatures (Lau et al., 2015). Several trials had demonstrated the potential use of IGR in the control of *Aedes* populations (Abad-Franch et al., 2017; Suman et al., 2018).

The selection of proper chemical control tool, the active ingredient of the insecticide itself and also the preparation of insecticide solution will influence the efficacy of vector control activities conducted (Aziz et al., 2014; Benelli & Beier, 2017). Hence, it is crucial for the Health Department and local authorities to obtain adequate background information of the targeted locality and the ecology of local vectors through mosquito surveillance prior to the selection of the most reliable insecticide and its application method.

2.2 Insecticides

In general, pesticides are classified into four major groups namely insecticides, fungicides, herbicides and rodenticides (Aktar et al., 2009; Martin-Reina et al., 2017). Insecticides which are the main focus of the present study are capable of exterminating insects including mosquitoes through direct dermal contact, oral as well as respiratory entry (Kim et al., 2017). Insecticides are usually neurotoxicants which affect the target insects by poisoning their nervous system (Casida, 2009; Martin-Reina et al., 2017).

2.2.1 Classification of Insecticides

Four main classes of neurotoxic insecticides that have been applied in vector control strategies until now including organochlorines, organophosphates, carbamates and pyrethroids (Moyes et al., 2017).

2.2.1.1 Organochlorines

Organochlorines are synthetic insecticides that are classified under the group of chlorinated hydrocarbon derivatives (Jayaraj et al., 2016). Organochlorines which had been extensively used as insecticides in the 1950s and 1960s are divided into three groups consisting of DDT and its analogues, cyclodienes like aldrin, dieldrin, endrin, chlordane, heptachlor and endosulfan as well as hexachlorobenzene (HCB) such as lindane (Aprea et al., 2002). Organochlorines are neurotoxins and chemically stable in the environment (Karami-Mohajeri & Abdollahi, 2010). The use of organochlorines was banned in many countries since 1970s (Sexton et al., 2013). However, due to their persistence, bioaccumulation and high toxicity in the environment (Gao et al., 2008), organochlorine compounds are still being detected in the ecology until now (Singh & Singh, 2017).

DDT or dichlorodiphenyltrichloroethane was the first synthetic insecticide discovered and had been massively utilized for the agricultural crop management and control of vector-borne diseases throughout the world since 1940s (Mansouri et al., 2016). DDT prevents the activation of the voltage-gated sodium channels resulting in an uncontrolled neuronal firing that consequently triggers crucial muscle spasms and later leading to paralysis and death of insect (Rossi et al., 2017). DDT has been widely utilized due to its high insecticidal effectiveness and economical value (Han & Currell, 2016).

Meanwhile, cyclodienes including dieldrin are inhibitors of γ-aminobutyric acid (GABA) receptors in the central nervous system (Walker, 2003). Dieldrin has been used in Malaysia since 1980 before being banned in 1994 but resistance against dieldrin has still been detected in local mosquito vectors due to its high environmental persistence (Low et al., 2015).

2.2.1.2 Organophosphates

Organophosphates include esters, amides, or thiol derivatives of either phosphoric acid or thiophosphoric acid (Fulton & Key, 2001). Organophosphates inhibit acetylcholinesterase activity in the cholinergic synapses in the central nervous system (Walker, 2003). Insecticides of this group possess high toxicity on insects but rapidly degrade in the environment (Zhu et al., 2009). Organophosphates are utilized for space treatment and indoor residual spraying (IRS) of mosquito control (Edi et al., 2014).

Organophosphates that are frequently used in public health worldwide include malathion, fenitrothion, fenthion, temephos, chlorpyrifos and bromophos. Temephos is usually applied in larviciding of mosquito larval breeding grounds while malathion is used in space spraying activities to control adult mosquito populations (Raghavendra et al., 2011). Fenitrothion has both larvicidal and adulticidal effects but it is more

commonly used in the space treatment for the control of adult mosquito populations such as in Colombia and Benin (Sulaiman et al., 1999, Maestre-Serrano et al., 2014; Gnanguenon et al., 2015). Fenthion is employed as a larvicide in India and Sri Lanka (Mukhopadhyay et al., 2006; Jayasundara & Pathiratne, 2008) while in Latin American countries and Malaysia, fenthion is utilized in space treatment and indoor residual spraying to combat adult mosquitoes (Rodriguez et al., 2007; Ong, 2016). Additionally, chlorpyrifos was applied in the control of mosquito larvae and adult mosquito populations in Iran and Mexico, respectively (Vatandoost et al., 2005; Lopez et al., 2014).

2.2.1.3 Carbamates

Carbamates were primarily introduced in the 1950s (Vale & Lotti, 2015). Carbamates which are derived from carbamic acid are inhibitors of acetylcholinesterase (AChE) activity (Dhouib et al., 2016). The inhibition of AChE enzymes leads to the accumulation of excessive acetylcholine at nerve terminals and synapses in the central nervous system (King & Aaron, 2015). The toxicity effects of carbamates are more reversible and less severe as compared to similar mechanism of organophosphates (Barr & Buckley, 2011). Additionally, carbamates have relatively low mammalian toxicity (Tha-in et al., 2013).

Both propoxur and bendiocarb are among the most frequently utilized carbamates globally. In certain countries such as Ethiopia and Sudan, both propoxur and bendiocarb are being applied for indoor residual spraying of malaria control programmes (Abraham et al., 2017; Ismail et al., 2018a). On the contrary, in Malaysia, propoxur and bendiocarb have never been applied in the local vector control programmes (Rong et al., 2012). Propoxur was originally meant for wall residual application but it had been widely used for space spraying activities (H. L. Lee, personal communication, August

12, 2019). The use of propoxur as the household aerosol to eliminate indoor mosquitoes in Malaysia started since early 1970s and was stopped in 1990s (Low et al., 2013), following the new rule of the Malaysian Pesticide Board that allowed only pyrethroids to be utilized as the household insecticides onwards (H. L. Lee, personal communication, August 12, 2019).

2.2.1.4 Pyrethroids

Pyrethrins are natural compounds originated from the extraction of pyrethrum flowers namely *Chrysanthemum cinerariaefolium* (Linnaeus) (Muzinic & Zeljezic, 2018). Despite their remarkable effectiveness, pyrethrins are very unstable and rapidly degraded upon the exposure of light and air (Casida, 1980). Hence, pyrethroids which are synthetic organic analogues have been derived from the natural pyrethrins but with modified molecular structures for greater stability and enhanced insecticidal activity (Chrustek et al., 2018; Tang et al., 2018).

Pyrethroids are generally divided into two groups based on the presence or absence of the α-cyano group, their action and their produced behavioural changes (Chrustek et al., 2018). The type I pyrethroids that are lacking of the α-cyano group include allethrin, bifenthrin, *d*-phenothrin, permethrin, resmethrin and tetramethrin as well as their associated analogues (Marettova et al., 2017). On the other hand, the type II pyrethroids possess the α-cyano group consist of cypermethrin, deltamethrin, lambdacyhalothrin, cyhalothrin, cyfluthrin, etofenprox, bifenthrin, fenvalerate, and other various analogues (Anadon et al., 2009). The first group has a great knockdown effect but low killing action, whereas the second group possesses great killing action and are also highly photostable that enable them to be used outdoors including in the agricultural sector (Kawada et al., 2009). Moreover, pyrethroids classified as type I are less toxic to mammals as compared to type II pyrethroids (Marettova et al., 2017).

Pyrethroids are neurotoxins that open of the voltage sensitive sodium channel (VSSC) in insects which is the target site of pyrethroids is extended by the toxic effect of pyrethroids (Smith et al., 2016). At the moment, synthetic pyrethroids are the most preferred insecticides to be used in public health as compared to other insecticide classes due to their rapid knockdown and killing effects upon insects even at low concentrations with relatively minimum toxicity effects to humans and mammals (Chareonviriyaphap et al., 2013). However, the most worrying issue is that the resistance occurrence against one particular pyrethroid could actually cause cross resistance to other pyrethroids of either killing agents or knockdown agents (Kawada et al., 2009). Furthermore, although the use of DDT of organochlorines has been banned in many countries for decades, DDT resistance in mosquitoes is still being reported (Kawada et al., 2014). Hence, the cross resistance between pyrethroids and DDT is also likely to be expected due to similar mode of action possessed by these insecticides (Du et al., 2016a).

2.2.2 Insecticide Resistance in Mosquitoes

Numerous chemical insecticides of different classes have been applied worldwide for decades. In public health, insecticides are employed to combat medically important insects especially mosquitoes in order to control the spread of vector-borne diseases among human populations. On the other hand, pesticides are utilized in agricultural sector to eliminate crop pests in order to maintain the quality of crops and increase the yields. Nevertheless, the selection pressure due to extensive and persistent use of chemical insecticides in both sectors has triggered the insecticide resistance development among insects particularly mosquitoes which eventually hampers the vector control strategies.

Insecticide resistance is defined as "the ability of mosquitoes to survive exposure to a standard insecticide dose which is due to their physiological and behavioural adaptation" (World Health Organization, 2016b). The occurrence of insecticide resistance among various species of mosquito vectors has been continuously reported by researchers throughout the world. Furthermore, the incidence of cross resistance among mosquito populations has worsened the mosquito control efforts. Cross resistance arises due to resistance to one insecticide by a mechanism which also confers resistance to insecticides of the other class, even though the insect population has never been exposed to the latter insecticides (World Health Organization, 2016b). In other words, it is possible for an insect with multi insecticide resistance to demonstrate more than one resistance mechanisms triggered by any detoxification enzymes and/or target site modifications.

2.2.2.1 Insecticide Resistance in *Aedes* Mosquitoes Reported Worldwide

Development of resistance among *Aedes* mosquitoes against various insecticides has been described worldwide. However, the insecticide resistance status among *Ae. albopictus* populations is still being less documented as compared to among *Ae. aegypti* populations. In Indonesia, *Ae. aegypti* of Bandung-West Java strain developed resistance to permethrin and deltamethrin while Palembang-South Sumatera strain was moderately resistant to permethrin only (Ahmad et al., 2007). On the other hand, DDT resistance was discovered among *Ae. albopictus* larvae from China as well as among adult mosquitoes of the same species from Cameroon and Sri Lanka (Vontas et al., 2012).

Five strains of *Ae. albopictus* adults in India also showed various degrees of resistance against DDT while two of them were moderately resistant to permethrin and deltamethrin (Kushwah et al., 2015a). The knockdown resistance (*kdr*) gene was not

detected in any of these populations which confirmed the involvement of detoxification enzymes activities in the resistance mechanisms of these mosquito populations. However, the *kdr* gene was significantly detected in *Ae. aegypti* from Delhi verifying its role in DDT and deltamethrin resistance occurred in the population (Kushwah et al., 2015b).

Other than that, *Ae. aegypti* adults from Chiang Mai, Thailand developed resistance to deltamethrin with the mean mortality percentage of 62.8% (Plernsub et al., 2016). Incipient resistance to permethrin and deltamethrin was also reported among *Ae. albopictus* adults collected from Peniscola, Spain which was linked to increased activities of mixed function oxidases and glutathione-S-transferases detected in the mosquito population (Bengoa et al., 2017).

Furthermore, *Ae. aegypti* larvae from French West Indies displayed high resistance to temephos at 8.9 to 33.1 fold and low resistance to malathion at 1.7 to 4.4 fold while adult mosquitoes of the same mosquito species were moderately resistant to deltamethrin at 8.0 to 28.1 fold (Goindin et al., 2017). Insecticide resistance development in these mosquitoes was due to the overexpression of knockdown resistance (*kdr*) gene, glutathione-S-transferases gene (*GSTe2*), carboxylesterase gene (*CCEae3a*) and P450 genes.

In Madeira Island, *Ae. aegypti* adults were resistant to fenitrothion, bendiocarb, permethrin and cyfluthrin. However, increased mortality rates were achieved using the synergists which also indirectly confirmed the involvement of detoxification enzymes in the resistance development of these mosquitoes (Seixas et al., 2017). The role of elevated level of detoxification enzyme activities particularly esterases, mixed function oxidases and glutathione-S-transferases in the resistance mechanisms of *Ae. aegypti* was also documented in Brazil whereby two wild strains of this mosquito species were

found to be highly resistant to larvicide temephos and adulticide deltamethrin with resistance ratios of more than ten fold (Viana-Medeiros et al., 2017).

Meanwhile, *Ae. aegypti* adults collected from Jeddah and Makkah, Saudi Arabia exhibited high resistance to permethrin, deltamethrin and bendiocarb (Al Nazawi et al., 2017). Interestingly, in Florida, the occurrence of permethrin resistance in *Ae. albopictus* adult mosquito populations found to be very low (RR < 1.6) although *Ae. aegypti* adults from similar study sites developed resistance to permethrin with resistance ratios ranging from 6 to 61 fold (Estep et al., 2018). Besides that, *Ae. albopictus* collected from five localities in China also developed resistance to permethrin, deltamethrin and lambdacyhalothrin in which *kdr* mutant allele of F1534S was shown to be significantly associated with pyrethroid resistance in the tested populations (Gao et al., 2018).

2.2.2.2 Insecticide Resistance in *Aedes* Mosquitoes Reported in Malaysia

The rapid progression of urbanization has prompted the propagation of man-made mosquito breeding habitats which subsequently promoted the spread of mosquito-borne diseases (Nazni et al., 2005). Similar to many other developing countries, chemical control using insecticides remains one of the major vector control strategies in Malaysia (Ong & Jaal, 2015). Massive use of larvicides and adulticides led to insecticide resistance development in mosquitoes including among local mosquito vectors (Husna Zulkrnin et al., 2018).

Many insecticide resistance studies conducted in Malaysia have been focusing on *Ae. aegypti* instead of *Ae. albopictus* considering that the former species has been incriminated as the principal vector of important *Aedes*-borne diseases in this country. Insecticide resistance development among local mosquito vectors particularly *Aedes* has been reported since as early as 1970s. Thomas (1970) had revealed on increased

resistance of *Ae. aegypti* larvae to DDT and dieldrin by 9 fold and 11 fold, respectively as well as escalated resistance of *Ae. albopictus* larvae against the same larvicides by 1.7 to 2.7 fold. Since then, many more insecticide resistance studies employing local mosquito vectors including *Aedes* had been performed and documented from time to time.

In 2012, propoxur and bendiocarb resistance were reported for the first time in local *Ae. aegypti* populations which were collected from Shah Alam, Selangor (Rong et al., 2012). On the other hand, *Ae. albopictus* adults from Tanjung Sepat, Selangor, Malaysia demonstrated high resistance to two organophosphates and two carbamates (Chen et al., 2013b). Furthermore, four populations of *Ae. albopictus* populations originated from Kuala Lumpur exhibited permethrin resistance development with resistance ratios ranging from 1.90 to 2.15 and from 1.22 to 1.30 at larval stage and adult stage, respectively (Wan-Norafikah et al., 2013b).

Meanwhile, in Bagan Dalam, Penang, Hasan et al. (2015) revealed lambdacyhalothrin resistance in *Ae. aegypti* and *Ae. albopictus* adults. Two years later, high permethrin resistance had been noticed in *Ae. aegypti* captured from dengue endemic localities of West Malaysia (Rosilawati et al., 2017). As for East Malaysia, either incipient resistance or high resistance was displayed among *Ae. albopictus* larvae from Sabah against selected organochlorines and organophosphates at WHO recommended doses (Elia-Amira et al., 2018).

2.2.3 Effects of Pesticides Use in Agriculture on Insecticide Resistance Development in Mosquitoes

Chemical compounds are not only being used in public health for vector control strategies but also in agriculture. Application of pesticides is still crucial in agricultural sector as it provides significant effects in the control of pests, weeds and plant diseases

in such an economical way (Lim et al., 2012). Variety of pesticides are massively utilized in agriculture to enhance crop yields and eradicate crop pests (Sutris et al., 2016a). Nevertheless, agricultural pest management using pesticides also acts as an indirect selection pressure which causes the insecticide resistance development in mosquito vectors (Chouaibou et al., 2016). Moreover, pesticides that are sprayed on crops to control pests and minimize the crop losses contaminate the water bodies within and near to agricultural sites by the wind or rain (Ahmad et al., 2008) whereby these water bodies also serve as breeding habitats of mosquito immatures. Many previous studies reported on the use of pesticides especially DDT, organophosphates and pyrethroids in crop pest management which had been accumulating in natural and artificial larval breeding receptacles around agricultural areas (Aizoun et al., 2013). In sub-Saharan Africa, insecticide resistance has been frequently reported in *An. gambiae s.l.* populations in which their larvae were discovered from breeding habitats near to agricultural areas with regular usage of pesticides to control agricultural pests (Mathias et al., 2011). According to Luc et al. (2016), soils from agricultural areas with history of agrochemicals exposures decreased the rates of pupation and emergence of *An. gambiae*.

There are several chemical compounds that are commonly applied in various types of agricultural areas. Dieldrin is exploited as pests contact poison in tea, vegetable and cotton plantations while chlorpyrifos is applied in a spray form to control pest insects of rice, vegetables, fruits and ornamental plants (Leong et al., 2007). Chlorpyrifos and cypermethrin that are regularly utilized in the tropical vegetable cultivation system could be strongly absorbed by soil particles which will delay their degradation (Chai et al., 2009a). In fact, pesticides like chlorpyrifos are also applied at high doses in between the planting seasons (Chai et al., 2009b). Therefore, there is a high possibility that these insecticides could contaminate nearby natural water bodies. In Malaysia, chlorpyrifos is

employed in eradicating pests in oil palm plantations and vegetable farms (Halimah et al., 2016). Organophosphates are also commonly applied in the paddy cultivation areas (How et al., 2014).

Other than that, pyrethroids are another group of pesticides of choice in agricultural sector. For instance, pyrethroids were widely applied in the farming of cotton in Malawi (Mzilahowa et al., 2016). A knowledge-attitude-practice (KAP) study conducted by Chouaibou et al. (2016) in Cote d'Ivoire found that herbicides and insecticides as the most common pesticides utilized in rice fields and vegetable farms. Among all classes of chemical compounds, pyrethroids were the most employed insecticides in both types of agricultural areas but adult mosquitoes from these areas developed resistance against deltamethrin, DDT and bendiocarb.

Resistance development among mosquito vectors captured from different types of agricultural areas against public health insecticides has been documented throughout the world for many years. Most of these reports involved insecticide resistance occurrence in *Anopheles* mosquitoes from African region. *Anopheles* mosquitoes had gained the greatest concern of researchers in African countries due to the fact that malaria infection is still widespread in this continent in which several *Anopheles* species act as the main vectors.

Resistance to DDT and permethrin was discovered among *An. gambiae* and *Cx. quinquefasciatus* collected from urban vegetable farms in Benin with regular application of pyrethroids (Corbel et al., 2007). Laboratory experiments showed that the selection pressure of the herbicide glyphosate or commercially known as Roundup had caused a significant escalation of permethrin resistance in *Ae. aegypti* larvae (Riaz et al., 2009).

Insecticide resistance detection among mosquito vectors collected from cotton growing areas and paddy cultivation areas were the most frequently reported by researchers. High resistance to chlorpyrifos was observed among *Ae. albopictus*

collected from three cotton cultivation areas in Punjab, Pakistan with resistance ratios of more than one hundred fold (Khan et al., 2011). Resistance against DDT and permethrin discovered among *An. gambiae* originated from cotton fields in Northern Benin confirmed the association of the use of both insecticides for cotton crop protection with the resistance development within the mosquito population tested (Yadouleton et al., 2011). In Mali, *An. gambiae* from three cotton growing areas developed resistance against fenitrothion and bendiocarb whereby both insecticides were applied only for agriculture pests control and not for public health operations (Cisse et al., 2015). The mutation of G119S *ace-1*^{R} was also identified in *An. gambiae* captured from a Soumousso village situated within the cotton farms in Burkina Faso (Badolo et al., 2015).

Meanwhile, continuous selection pressure from agrochemicals applied to rice fields in Korea had triggered pyrethroid resistance development among *Cx. tritaeniorhynchus* (Yoo et al., 2013). *Anopheles sinensis* of South Korean rice fields were also less susceptible to several organophosphates and pyrethroids (Chang et al., 2014). Additionally, moderate deltamethrin resistance was demonstrated among *An. gambiae* larvae and adults collected from Kilimanjaro region in Tanzania that was covered by rice fields, sugar cane and coffee plantations with massive pesticides application (Nkya et al., 2014). Besides that, cross resistance between DDT and pyrethroids particularly permethrin and lambdacyhalothrin were demonstrated among *An. gambiae* captured from rubber estates in Cameroon (Bigoga et al., 2012).

To the best of my knowledge, there is no inclusive documented literature on insecticide resistance occurrence among mosquito vectors originated from any types of agricultural areas in Malaysia for now. Hence, the present study was aimed to focus on this issue in order to provide basic information on the susceptibility status of important mosquito vectors particularly *Ae. albopictus* from human dwellings within agricultural

areas that may facilitate the Ministry of Health Malaysia, the local municipal councils and the Department of Agriculture Malaysia to co-regulate the application of insecticides in both public health and agricultural sector in the near future.

2.2.4 Underlying Mechanisms of Insecticide Resistance in Mosquitoes

Extensive and persistent use of insecticides in public health for vector control as well as in the agricultural sector for crop pest management has lead to the resistance development among mosquito vectors and other insects. Insecticide resistance in insects including mosquitoes is conferred by one or more underlying mechanisms that include cuticle alteration causing reduced penetration of the insecticide, behavioural adaptations, elevated level of detoxification enzymes and mutations of the target site (Kasai et al., 2014). To date, both metabolic resistance and target site resistance are widely studied across the globe while the pathways of reduced penetration resistance and behavioural resistance are still less understood.

2.2.4.1 Reduced Penetration Resistance

The cuticle and exoskeleton of insects including the mosquito are comprised of cuticular proteins (CPs), lipids and chitin (Vannini et al., 2014). The permeability of insect cuticle is influenced by the composition and various roles of hydrocarbons (Stinziano et al., 2015). The mechanisms involved in reduced cuticular penetration include the greater expression of detoxification enzymes in the integument, the escalated numbers of binding proteins and surface lipids, the segregation of insecticides, the thickening of cuticle, or a combination of certain or all of these mechanisms (Lilly et al., 2016). In reduced cuticular penetration resistance, the insecticide penetration through the insect cuticle becomes slower due to modification and thus, decreases the

number of insecticide molecules that could successfully entered into the insect (Nkya et al., 2013).

Few researchers had reported on the potential role of reduced penetration mechanism in the resistance development detected in several mosquito species. An over-expression of two cuticular genes was demonstrated in two resistant populations in *An. gambiae* from Southern Benin and Nigeria indicating the possible involvement of reduced penetration resistance in these mosquitoes (Djouaka et al., 2008; Awolola et al., 2009). Meanwhile, thickened cuticle was observed in *An. funestus* from southern Africa that was resistant to bendiocarb (Ibrahim et al., 2016) while Balabanidou et al. (2016) in Greece had also shown that the occurrence of high resistance against pyrethroids among *An. gambiae* was induced by the thickening of the epicuticular layer in these mosquitoes.

At the moment, the underlying mechanism of reduced penetration resistance is still poorly explored. However, reduced penetration resistance is known to frequently work together with other mechanisms (Kasai et al., 2014). Further investigations are needed to comprehend the actual pathways involved in reduced penetration resistance.

2.2.4.2 Behavioural Resistance

Behavioural resistance refers to any changes in insect behaviour due to prolonged exposure to insecticides which allow these insects to escape from lethal effects of those insecticides [Insecticide Resistance Action Committee (IRAC), 2011]. Behavioural modifications could be with or without treated materials such as any chemical or control tool (Pennetier et al., 2009). Behavioural resistance includes behavioural adaptations such as peak biting time behavioural shifts as well as changing preferences for resting and feeding indoors or outdoors (Sokhna et al., 2013; Ambrose et al., 2014).

Behavioural resistance to insecticides is still scantily understood and difficult to measure (Mathias et al., 2011; Parker et al., 2015).

Changes in mosquito behavioural responses may significantly increase the possibility of vector-borne disease transmission (Sougoufara et al., 2014). In other words, mosquito control activities are now being impeded by the persistent development of behavioural resistance (Le et al., 2014; Menger et al., 2015). Therefore, behavioural resistance has caused an enormous task for current mosquito control activities (Muema et al., 2017). With the emergence of behavioural resistance in mosquitoes, it is essential to focus on other bionomic vulnerabilities such as in the immature stages or during mating or feeding of mosquitoes to suppress the disease transmission (Russell et al., 2013).

2.2.4.3 Metabolic Resistance

Metabolic resistance occurs when a proliferated amount of detoxification enzymes binds to the target insecticide molecule and activate the breakdown reactions to metabolize the insecticide (Horstmann & Sonneck, 2016). This scenario causes the enhancement of the insect detoxifying capacity (Macoris et al., 2018) which eventually instigates the insecticide resistance development (Liu, 2015). Mixed function oxidases (MFO), glutathione-S-transferases (GST) and non-specific esterases (EST) are primary detoxification enzymes that are typically associated with insecticide metabolic resistance in mosquito vectors (Li et al., 2018).

2.2.4.3.1 Non-specific Esterases (EST)

The esterases have been reported to play an important role in the resistance to organophosphates, carbamates and pyrethroids (Chouaibou et al., 2014). The esterases-mediated resistance involves either gene amplification, upregulation, mutations of coding sequence or a combination of these genetic mechanisms (Li et al., 2007). These

mechanisms allow the esterases to efficiently hydrolyse ester bonds present in many insecticides and also trigger the overproduction of esterases causing resistance development against these insecticides (Montella et al., 2012). Two major loci that are closely linked and involve in the overproduction of esterases are *Est-2* or also known as *esterase β* and *Est-3* or also recognized as *esterase α* (Raymond et al., 1998). Significant elevated esterase activities causing resistance development against different insecticides were demonstrated in several mosquito species such as in *Ae. aegypti* (Leong et al., 2018), *Ae. albopictus* (Li et al., 2018), *Cx. pipiens* (Ferrari, 2015), *An. stephensi* (Gorouhi et al., 2018) and *An. culicifacies* (Kona et al., 2018).

2.2.4.3.2 Mixed Function Oxidases (MFO)

Mixed function oxidases (MFO) are also known as microsomal monooxygenases, cytochrome P450 or polysubstrate monooxygenases (PSMO) (Brattsten, 1988). Mixed function oxidases are an important superfamily of enzymes that are involved in metabolic resistance in insects (Hao et al., 2014). Mixed function oxidases metabolize xenobiotics including insecticides and regulate the concentration and amount of endogenous substances like hormones and fatty acids (Smith et al., 2016). Although mixed function oxidases may potentially involve the detoxification of all four classes of public health insecticides, their close association with pyrethroid resistance is the most notably (Brooke et al., 2001).

In *Ae. aegypti*, the P450 genes from *CYP6*, *CYP9* and *CYP4* subfamilies were found to be overtranscribed indicating their roles in the metabolic resistance against organophosphates and pyrethroids (Marcombe et al., 2009). On the other hand, the overtranscription of the P450 gene *CYP6P12* was highly correlated with the pyrethroid resistance in *Ae. albopictus* while *CYP6N3* was constantly overexpressed in DDT and carbamate resistant populations of the same mosquito species (Ishak et al., 2016). In *Cx.*

quinquefasciatus, the overexpression of other P450 genes such as *CYP6AA7*, *CYP9J40*, *CYP9J45*, *CYP4H34* and *CYP9M10* has been significantly correlated to pyrethroid resistance (Delannay et al., 2018). Meanwhile, the P450 gene *CYP6Z1* was highly expressed in the permethrin resistant *An. coluzzii* (Main et al., 2018).

2.2.4.3.3 Glutathione-S-transferases (GST)

Glutathione-S-transferases are dimeric multifunctional enzymes that are involved in the detoxification of xenobiotic substances including insecticides (Prapanthadara et al., 2000). Glutathione-S-transferases mainly catalyze the conjugation of the tripeptide reduced glutathione to electrophilic centres of lipophilic compounds in order to increase their water solubility and facilitate excretion from the cell (Hemingway & Ranson, 2000; Vontas et al., 2001).

Glutathione-S-transferases are divided into three main groups namely cytosolic, mitochondrial and microsomal (Oakley, 2005). The majority of GST are cytosolic (Lumjuan et al., 2005). Among several classes of cytosolic GST discovered in insects, the Delta and Epsilon classes are the main classes that are involved in the insecticide detoxification causing the metabolic resistance development (Ding et al., 2005). Elevated levels of GST activity have been linked to organophosphates, organochlorines and pyrethroids resistance in mosquitoes (Che-Mendoza et al., 2009). For example, elevated levels of Epsilon GST activity particularly *AaGSTe2* and *GSTe2* had been revealed to confer DDT resistance in *Ae. aegypti* and *An. gambiae*, respectively (Ortelli et al., 2003; Lumjuan et al., 2005).

2.2.4.4 Altered Target Site Resistance

Target site resistance in mosquitoes involves mutations in the voltage-gated sodium channel, acetylcholinesterase (AChE) and γ-aminobutyric acid (GABA) receptor genes (Hemingway et al., 2004). The occurrence of target site modifications is due to changes of specific amino acid at a point or few important positions of target proteins which limit the binding of the insecticides products (Cui et al., 2006a; Tmimi et al., 2018).

2.2.4.4.1 Mutation of GABA Receptors

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter which reduces neuronal excitability in both mammals and invertebrates (Jiang et al., 2017). The GABA receptor subunit termed as resistant to dieldrin (RDL) is one of the cys-loop ligand-gated ion channel superfamily (Taylor-Wells et al., 2018). RDL encompasses an N-terminal extracellular domain in which GABA binding takes place, the typical cys-loop pattern comprising two disulphide bond-forming cysteines detached by thirteen amino acids, and also four transmembrane domains (Nys et al., 2013). The second transmembrane domain lines the ion channel in which the alanine to serine or glycine mutations at the *Rdl* gene occurs leading to resistance against cyclodiene insecticides like dieldrin and also fipronil (Taylor-Wells et al., 2015). Although the utilization of dieldrin of organochlorines is banned for mosquito control, previous dieldrin exposure could have caused the occurrence of γ-amino butyric acid (GABA) mutation that would persist within the mosquito populations and have the possibility of conferring the cross resistance against other insecticides with the same target site (Nardini et al., 2017).

The *Rdl* gene mutation in dengue vector was initially reported by Thompson et al. in 1993 involving *Ae. aegypti* population. In 2005, alanine to glycine and alanine to serine substitutions in *Rdl* gene were discovered in *An. gambiae* that was resistant to dieldrin (Du et al., 2005). The presence of *Rdl* gene mutation was also reported in *Cx.*

quinquefasciatus and *Ae. albopictus* originated from La Reunion Island (Tantely et al., 2010). The alanine to serine (A296S) mutation in *Rdl* gene was displayed in dieldrin-resistant *An. funestus* from Burkina Faso and Cameroon whereby this mutation was accompanied by another mutation of V327I occurred in the large intracellular loop between the third and fourth transmembrane domain (Wondji et al., 2011). In Indonesia, the mutation of *Rdl* 302S gene was expressed in *An. vagus*, *An. aconitus*, *An. barbirostris*, *An. sundaicus* and *An. nigerrimus* while the *Rdl* 302G gene mutation was demonstrated only in *An. farauti* (Asih et al., 2012). The alanine to glycine (A296G) and threonine to methionine (T345M) in *Rdl* gene were detected in dieldrin-resistant *An. gambiae* (Taylor-Wells et al., 2015). The A302S *Rdl* gene mutation in *Ae. albopictus* was also revealed for the first time in Malaysia by Low et al. (2015). Moreover, the 296S, 327I and 345S mutations in *Rdl* gene demonstrated in *An. sinensis* from Guangxi, China indicated multiple insecticide resistance development in these mosquito populations (Yang et al., 2017).

2.2.4.4.2 Insensitive Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is the enzyme target of organophosphate and carbamate insecticides which are the competitive inhibitors of acetylcholine (ACh) (Tmimi et al., 2018). After binding to AChE, the organophosphate or carbamate insecticide terminates nerve impulses through hydrolysis of ACh neurotransmitters in the cholinergic synapses of the central nervous system (Liu et al., 2005). Consequently, the ACh maintains active while the nervous influx is sustained (Tmimi et al., 2018). The accumulated ACh causes overstimulation leading to the neurotransmission blockage and finally the death of the mosquito or any other insect (Engdahl et al., 2015).

In some insects, two forms of acetylcholinesterase enzymes have been encountered which are the synaptic and non-synaptic proteins whereby their sensitivity level against

organophosphates and carbamates are different (Karunaratne et al., 2013). These two acetylcholinesterase enzymes are known as AChE1 and AChE2 which are encoded by *ace-1* and *ace-2* genes, respectively (Tmimi et al., 2018). While AChE1 enzyme encoded by *ace-1* gene is associated to resistance mechanisms of organophosphates and carbamates, the *ace-2* gene that encodes the AChE2 enzyme is more related to the sex factor (Alout et al., 2016). However, according to Weill et al. (2002), the *ace-1* gene involves in the resistance mechanisms of Culicidae insects including mosquitoes while *ace-2* gene confers resistance in Brachyceran Drosophilidae and Muscidae insects. The organophosphate and carbamate resistance is caused by the substitution of glycine to serine for amino acid residue 119 at the target site of acetylcholinesterase (*ace-1*) (Poupardin et al., 2014). In mosquitoes, the *ace-1* mutation can steer to high carbamate resistance but low organophosphate resistance (Fodjo et al., 2018).

In Alabama, USA, elevated level of AChE in two field strains of *Cx. quinquefasciatus* demonstrated its significant role in the development of chlorpyrifos resistance in these field populations (Liu et al., 2005). A high frequency of *ace-1* G119S mutation was displayed in *Ae. aegypti* population from Tamil Nadu which was resistant to temephos (Muthusamy & Shivakumar, 2015). On the other hand, the mutation of N485I *ace-1* in *An. funestus* from southern Africa had been found to be correlated with bendiocarb resistance (Ibrahim et al., 2016). Furthermore, low frequencies of *ace-1* G119S and F290V mutations were demonstrated in four populations of *Cx. pipiens* from Greece (Fotakis et al., 2017). In French West Indies, *ace-1* mutation G119S had been discovered in *Cx. quinquefasciatus* populations that were resistant to malathion and temephos (Delannay et al., 2018). A low frequency of *ace-1* G119S mutation has also been found in Moroccan *Cx. pipiens* especially those originated from urban settings (Bkhache et al., 2019).

2.2.4.4.3 Knockdown Resistance (*kdr*) in the Voltage-Gated Sodium Channel

The voltage-gated sodium channel (VGSC) or voltage sensitive sodium channel (VSSC) which is present in cells of the central and peripheral nervous system is the target site of pyrethroids and DDT of organochlorines which share a similar mode of action (Silva et al., 2014). Voltage-gated sodium channels are important for the instigation and proliferation of action potentials in the nervous system and also other excitable cells (Du et al., 2016b). The sodium channels of insects consist of four homologous domains (I – IV) with each of these domains possessing six α-helical transmembrane segments (S1 – S6) connected by loops (Du et al., 2013). The S1 – S4 segments of each domain constitute the four independent voltage-sensing domains while the S5 – S6 segments and also the loop connecting them act as the pore-forming domains (Silva et al., 2014).

The occurrence of the knockdown resistance (*kdr*) is due to single point mutations in the VSSC or VGSC encoded by the *Vssc* gene with most of them positioned in the transmembrane segments IIS5, IIS6 and IIIS6 (Auteri et al., 2018). In *An. gambiae*, although the knockdown resistance (*kdr*) is conferred by the substitution of leucine to phenylalanine (L1014F) causes West *kdr* while the substitution of leucine to serine (L1014S) causes East *kdr* (Djouaka et al., 2008), current studies have reported on *kdr*-West gene detected in East African mosquito vectors and vice versa resulting the control efforts becoming more challenging (Fodjo et al., 2018). Moreover, the L1014F *kdr* mutation was also discovered in *Cx. quinquefasciatus* and *Cx. pipiens* (Yanola et al., 2015; Bkhache et al., 2016).

Meanwhile, in several strains of *Ae. aegypti* worldwide, mutations at the voltage-gated sodium channel (VGSC) gene (I1011M/V, V1016G/I, F1534C and S989P) which are associated with pyrethroids and/or DDT resistance have been reported (Brengues et al., 2003; Harris et al., 2010; Saavedra-Rodriguez et al., 2007; Kawada et al., 2014). In

Malaysia, Ishak et al. (2015) detected the F1534C and the V1016G mutations in *Ae. aegypti* field strains but significant correlation between F1534C genotypes and pyrethroid resistance was only demonstrated in Penang strain. In contrast, no *kdr* mutation was detected in any field strains of *Ae. albopictus* collected from the same localities in Malaysia. Additionally, another local study by Rasli et al. (2018) showed the potential role of the V1023G mutation alone or in combination with the S996P mutation that could confer pyrethroid resistance in *Ae. aegypti*. As for *Ae. albopictus*, the F1434C *kdr* mutation has so far been detected only in Singapore and China (Kasai et al., 2011; Chen et al., 2016).

2.2.4.5 WHO Susceptibility Tests for Insecticide Resistance Detection in Mosquitoes

The World Health Organization (WHO) susceptibility tests are the most frequently used assays for resistance detection in insects worldwide. The WHO susceptibility tests are direct response-to-exposure tests which measure the mortality of mosquitoes or other insects upon exposure to a known standard concentration of insecticides (World Health Organization, 2016b). The WHO susceptibility tests are the first line of resistance detection (Gnankine et al., 2013). The monitoring of insecticide resistance in field populations of mosquitoes is essential to determine the levels, underlying mechanisms and geographical dispersal of resistance prior to the selection of appropriate insecticides to control these mosquito populations (World Health Organization, 2016b).

The WHO susceptibility tests not only include the susceptibility tests for larvae and adults, but also include the synergist bioassays. The synergist-insecticide bioassay is recommended to be performed to measure the effect of pre-exposure to a synergist on the insecticide resistance expression (World Health Organization, 2016b). By

performing the synergist bioassays, the role of the detected detoxification enzymes in the resistance development in the mosquito populations could be confirmed (Verhaeghen et al., 2009).

Despite standard protocols in discovering insecticide resistance in mosquitoes, WHO susceptibility tests do not offer information on the underlying resistance mechanisms and gene profile of the populations that are linked to the insecticide resistance occurrence (Brogdon, 1989). Moreover, although the WHO susceptibility test kits are user-friendly and supported with the WHO guidelines of recommended diagnostic dosages specifically for important mosquito species, the need of the large number of mosquito samples with only several insecticide solutions or impregnated papers available for testings limit the dependability of these methods (Lee & Tadano, 1994). Hence, although the standard WHO susceptibility tests remain as the recommended principal method in detecting resistance in insects, supplementary test approaches such as biochemical assays and molecular methods are highly recommended (World Health Organization, 2016b).

2.2.4.6 Biochemical Methods for Insecticide Resistance Detection in Mosquitoes

Several limitations of WHO susceptibility tests have driven towards the development of biochemical assays. Biochemical assays reveal the elevated activities of detoxification enzymes in the tested populations and thus, signifying the importance and involvement of metabolic resistance (Seixas et al., 2017; Li et al., 2018). Changes in the frequencies of resistance genes in field samples upon different insecticide exposure could also be measured using biochemical assays (World Health Organization, 1998b). Biochemical techniques should be performed simultaneously with the WHO larval and

adult mosquito bioassays in order to obtain precise information on the susceptibility status among mosquito vectors (Chen et al., 2013a).

In biochemical assays, individuals with elevated enzyme activities could be easily detected (Nunes et al., 2016). Any individual insect that carries a mutant resistance allele could also be revealed by biochemical assays (Chen et al., 2013a). Additionally, multiple resistance within an individual insect could be detected using the same individual that is being subjected to several biochemical assays (Brogdon, 1989). With the capacity of being performed on individual samples, biochemical techniques permit a more sensitive resistance detection (Matowo et al., 2010).

Nevertheless, biochemical approaches still lack sensitivity and specificity (Bingham et al., 2011). Results of biochemical assays could not completely correlate with phenotypic resistance or act as a consistent indicator of metabolic resistance since not all detoxification enzymes involved in the phenotypic resistance could be detected together by biochemical assays (Matowo et al., 2010). In other words, there is no direct linkage between the measurement of enzyme activities with the resistance phenotype (Seixas et al., 2017). Therefore, it is crucial to ensure that the outcomes of WHO susceptibility tests and biochemical assays are to be further confirmed and established using other advanced strategies such as molecular approaches whenever possible and affordable.

2.3 The Use of Synergist in the Insecticide Resistance Management

Among numerous control interventions of mosquito vectors proposed by World Health Organization (2012a), chemical control using insecticides is the most frequently chosen method to be implemented. Nevertheless, the persistent use and over-reliance of insecticides have obviously initiated the development of insecticide resistance among mosquito vectors. Hence, there is a need to treasure any new or obtainable tools or

substances that could improve the outcomes of vector control strategies particularly the chemical control. One of the promising approaches is the complementary use of substances known as synergists with insecticides. Synergists are used to enhance the efficacy of insecticides in order to increase mortality of targeted insects but they have no toxicity against these insects by themselves (Barbosa & Hastings, 2012). Examples of synergists include piperonyl butoxide (PBO), S,S,S-tributyl phophorotrithionate (DEF), triphenyl phosphate (TPP) and diethyl maleate (DEM). In this research work, only PBO was selected and tested since only this synergist has been commercially marketed and used in combination with pyrethroids worldwide.

2.3.1 The Use of Piperonyl Butoxide (PBO) as a Synergist in Combination with Pyrethroids

Piperonyl butoxide (PBO) with a chemical name of 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole is classified as a chemical under methylenedioxyphenyl group with its molecular formula of $C_{19}H_{30}O_5$ and 338.4 g/mol molecular weight (Canyon et al., 2010). Piperonyl butoxide is an analogue of the sesame oil-derived compound (Gross et al., 2017). Piperonyl butoxide is classified by World Health Organization as "unlikely to present acute hazard in normal use" which could cause only minimal eye and skin irritation as well as degrades rapidly in the mammalian metabolism and in the environment (World Health Organization, 2011b). Piperonyl butoxide is an effective synergist of insecticide due to its interaction with mixed function oxidases and also a great diagnostic tool to determine any involvement of mixed function oxidases in insecticide resistance in mosquito populations (Hodgson & Levi, 1998). Piperonyl butoxide inhibits the oxidation activity of mixed function oxidases so that the active ingredients of pyrethroids could stay accessible and the toxic effects on targeted insects are intensified (Klaasen et al., 1986).

Susceptibility studies using PBO to discover the underlying mechanisms of resistance occurred in different mosquito species and whether or not the utilization of PBO in combination with pyrethroids or any other insecticide classes could enhance the efficacy of these insecticides were conducted throughout the world. The effectiveness of PBO as a synergist prior to the exposure of the insecticide has been reported as early as 1961 by Fox & Garcia-Moll. The absence of synergism effect upon the utilization of PBO would suggest the role of metabolic enzymes other than oxidases and also the non-metabolic mechanisms in the resistance occurred in the tested mosquito populations. For instance, lack of synergism observed in the use of PBO in combination with fenitrothion and propoxur, respectively had demonstrated that oxidases were not involved in the resistance in *An. atroparvus* from Spain (Hemingway & Davidson, 1983). Furthermore, the use of PBO prior to insecticide exposures proved the solitary role of *kdr* mutation in pyrethroid resistance in *Ae. aegypti* from two states of Venezuela (Mazzarri & Georghiou, 1995).

Additionally, the combination of PBO with deltamethrin has suppressed the LC_{50} value to 21-fold lower than the exposure to deltamethrin alone in *Ae. aegypti* Mysore strain (Vijayan et al., 2007). In Riyadh, more than 90% resistance suppression was demonstrated in three strains of *Culex pipiens* after the exposure to PBO + pyrethroids (Al-Sarar, 2010). A year later, Somwang et al. (2011) reported that both *Aedes aegypti* susceptible and resistant strains from Chiang Mai, Thailand that were exposed to PBO + permethrin showed 2.27- and 3.03-fold of reduction in LC_{50} values. Eventually, in 2014, permethrin resistance was detected in *Ae. aegypti* Puerto Rico strain at 73-fold. However, the use of PBO prior to permethrin selection had partially reduced its resistance level against permethrin to 15-fold (Reid et al., 2014). At the same time, permethrin resistance was also detected in *Ae. aegypti* Singapore strain of adult mosquitoes with resistance ratio (RR_{50}) of 35-fold (Kasai et al., 2014). However, after

the pre-exposure of PBO prior to permethrin, the resistance ratio reduced to 11-fold with synergistic ratio (SR_{50}) of 5.3. Furthermore, the use of PBO in combination with deltamethrin had also significantly increased the susceptibility of *Ae. aegypti* Jeddah strain to deltamethrin (Al Nazawi et al., 2017).

Very limited synergism studies involving PBO and *Ae. albopictus* were reported so far. In Malaysia, the utilization of PBO in combination with permethrin has confirmed the role of oxidases in the permethrin resistance in *Ae. albopictus* from different study areas (Nazni et al., 2000; Wan-Norafikah et al., 2013b). Conversely, incomplete mortality was exhibited among *Ae. albopictus* exposed to PBO prior to DDT indicating the contribution of other metabolic enzymes or target site mutations in the DDT resistance in this mosquito population (Ishak et al., 2015).

CHAPTER 3: METHODOLOGY

3.1 Mosquito Samples

In view of the fact that very few *Ae. aegypti* were successfully captured during the ovitrap surveillance study, it was quite challenging to breed its impending generation in the insectarium. Hence, only *Ae. albopictus* populations (F0) collected during the ovitrap surveillance were further bred in the insectarium to produce their offspring (F1) which were utilized in all subsequent studies after the ovitrap surveillance study.

In general, two strains of *Ae. albopictus* were employed for all studies: the laboratory strain and field strains. *Ae. albopictus* laboratory strain (F69) acted as a reference strain in all studies. This reference strain was initially collected from Selangor, Malaysia and had been maintained in the insectarium of the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia for more than ten years. *Ae. albopictus* laboratory strain is free from any past exposure of insecticides. Meanwhile, *Ae. albopictus* field strains were obtained from fifteen different study areas through the ovitrap surveillance study. Microscopically and morphologically identified *Ae. albopictus* field strains larvae collected during the ovitrap surveillance were separately reared to adulthood which was known as F0. These *Ae. albopictus* field strains (F0) were then further colonized to produce their progenies (F1) that were employed in all subsequent studies after the ovitrap surveillance study. Only the first generation (F1) of all *Ae. albopictus* field strains were used in all subsequent studies so that the representation of these mosquitoes as field strain samples were maintained. For all strains, only late third (3^{rd}) instar larvae and 3-5 days old sucrose-fed adult female mosquitoes of *Ae. albopictus* were utilized in respective testings.

3.2 Colonisation of *Aedes albopictus*

All strains of *Ae. albopictus* utilized in this study were reared according to the Standard Operating Procedure of Medical Entomology Unit, IMR (ISO/IEC 17025) which was prepared by the Medical Entomology Unit, IMR (2000). These colonies were bred simultaneously in a designated room in the insectarium. They were handled in the same manner through all manipulations and free from any insecticide exposure. The temperature of the insectarium was maintained at 27 ± 2 ºC and 75 ± 10% relative humidity (R.H.).

All *Ae. albopictus* adults of all strains were separately maintained in cages measuring 32 cm x 32 cm x 32 cm that were made of wood, covered with fine mosquito netting and fixed with glue. They were daily fed with 10% sucrose solution mixed with vitamin B complex that was provided using lint cloth. Mice were supplied as their blood meal once a week. These mice were restrained gently in a modified small wire mesh trap and left overnight in mosquito cages before being removed on the next morning. Female *Aedes* mosquitoes took about three (3) to six (6) days after blood meal to oviposit.

Aedes albopictus female adults were allowed to oviposit in small, round, black-coated plastic containers measuring 4 cm in depth and 7 cm in diameter which contained dechlorinated water. Contents of these oviposition containers were daily and individually strained using funnels lined with Whatman No. 1 filter papers. These *Ae. albopictus* eggs were then air-dried at room temperature before being kept in well saturated, sealed and labeled plastic bags and stored in plastic containers. All *Ae. albopictus* eggs were kept until use but not exceeding six (6) months.

For egg hatching, the dried filter paper with eggs on it was soaked into dechlorinated water in a plastic tray measuring 25.5 cm in width x 30.5 cm in length x 5 cm in depth. A commercially available liver powder (Difco™ Liver; Becton, Dickinson and Company; France) and small pieces of partially-cooked cow liver were added into the

same plastic tray as larval food. Eggs of *Ae. albopictus* hatched within 1 to 24 hours after the steeping of filter paper attached with these eggs into the dechlorinated water. *Aedes albopictus* larvae spend about 12 to 15 days before they pupated. *Aedes albopictus* pupae were hand-picked using a disposable pipette and transferred into a new plastic container with its lid being modified with tiny air holes that was kept covered to avoid egg laying of other mosquitoes in the vicinity in this container. The pupal period of *Ae. albopictus* takes about 2 to 3 days. Newly emerged *Ae. albopictus* adults in modified plastic containers were then introduced and released into adult cages of their respective strains.

3.2.1 Standard Food Preparation for *Aedes* Larvae and Adults

Different types of food were supplied to different stages of *Aedes* mosquitoes regardless of their strains and generation. All mosquito food was either purchased from the supplier or self-prepared in accordance with the Standard Operating Procedure of Medical Entomology Unit, IMR (ISO/IEC 17025) which comprised of:

(a) 10% sucrose solution

A 10% sucrose solution which is mimicking the plant nectar was prepared for adult mosquitoes to gain their energy. 100 g sucrose was added into 1 L dechlorinated water. Approximately 5-10 g vitamin B complex (1%) was then put into the sucrose solution and mixed.

(b) Cow Liver Powder

A finely ground cow liver powder was supplied as the food for the first and second instar larvae. This cow liver powder was purchased commercially from the supplier (Difco™ Liver; Becton, Dickinson & Company; France).

(c) Partially-cooked Cow Liver

Partially-cooked cow liver served as the food for the third and fourth instar larvae. It was made by cooking the fresh cow liver using the microwave for a minute. This cow liver was then cut into small pieces before being introduced into the mosquito larval colonies.

3.3 Ovitrap Surveillance of *Aedes* Mosquitoes in Selected Agricultural and Non-agricultural Areas in Peninsular Malaysia

3.3.1 Description of Study Areas

Ovitrap surveillance was carried out in fifteen study areas within Peninsular Malaysia. In general, study areas selected for this study include human dwellings within agricultural areas of oil palm plantations, rubber estates, and rice cultivation areas as well as human dwellings within non-agricultural areas which consisted of fogging-free and dengue prone residential areas which acted as negative and positive controls for this study. Oil palm plantations, rubber estates and rice cultivation areas were chosen for this study as they were the top most widely planted industrial crops in Malaysia (Department of Agriculture Peninsular Malaysia, 2015). Three (3) study areas were selected for each type of agricultural, fogging-free and dengue prone areas, respectively (Plate 3.2). These study areas covered different regions of Peninsular Malaysia; the northern region, the southern region, the eastern region, and the central region. Agricultural areas selected were ensured to have no reported cases of mosquito-borne diseases such as dengue and chikungunya which made them free from any vector control activities but with consistent use of pesticides for crops management. Meanwhile, for the selection of fogging-free and dengue prone residential areas, a cross-checking had been done with the Ministry of Health Malaysia in order to verify whether or not that these study areas had any record of reported cases of dengue. Any history of

chemical control strategies that had ever been carried out in these non-agricultural areas had also been investigated and verified with the Ministry of Health Malaysia and the Department of Health of local authorities. The geographical and ecological description of all study areas are provided in Table 3.1.

In the present study, only the results of ovitrap surveillance were discussed individually for each study locality. Meanwhile, for the rest of bioassays and enzyme microassays, those findings were discussed according to different types of area whereby each study area selected was clustered into the respective type of area.

Plate 3.2: Selected agricultural and non-agricultural areas in Peninsular Malaysia: (a) Kota Tinggi OP (b) Klang OP (c) Temerloh OP (d) Kuala Selangor PD (e) Kulim PD (f) Kuala Pilah PD (g) Sungai Buloh RB (h) Temerloh RB (i) Kota Tinggi RB (j) Shah Alam FF (k) Padang Serai FF (l) Temerloh FF (m) Kota Tinggi DEN (n) Shah Alam DEN (o) Cheras DEN.

Table 3.1: Geographical and ecological description of study areas.

State	District	Study areas	Geographical description	Ecological description
Agricultural area : Oil palm plantations				
Johor	Kota Tinggi	University of Malaya Oil Palm Research Plantation, Jementah **(Kota Tinggi OP)**	• Coordinate: 02˚01.727'N, 103˚51.924'E • Elevation: 28 m	• An area managed by UM Plantations Sdn Bhd and Boustead Estates Agency Sdn Bhd which consists of a research complex, an administration office and single storey staff quarters within oil palm plantation. • Trees, shrubs, ornamental plants and heavy vegetation could be observed around human dwellings as well as the administration building and the research complex. • Well-built and well-managed water supply system, drainage system and waste management. • Trimonthly use of the herbicide in the oil palm plantation which was made from an organophosphorus compound but it does not inhibit cholinesterase activity since it is not an organophosphate ester that is widely used in insecticides.
Selangor	Klang	Jalan Paip Kiri, Meru **(Klang OP)**	• Coordinate: 03˚09.201'N, 101˚27.535'E • Elevation: 5 m	• A small residential area consisting of single storey terraced houses located next to an oil palm plantation. • Trees, shrubs, decorative plants and dense vegetation could be observed within the area. • Proper water supply system, drainage system and waste management. • Persistent use of the herbicide in the oil palm plantation which was made from an organophosphorus compound but it does not inhibit cholinesterase activity since it is not an organophosphate ester that is widely used in insecticides.
Pahang	Temerloh	Taman Paya Pulai **(Temerloh OP)**	• Coordinate: 03˚27.642'N, 102˚28.098'E • Elevation: 42 m	• A small and matured residential area comprising of single storey terraced houses and located next to an oil palm plantation. • Shrubs, ornamental plants and dense vegetation could be seen around the area. • Appropriate water supply system, drainage system and waste management. • Frequent use of the herbicide in the oil palm plantation which was made from an organophosphorus compound but it does not inhibit cholinesterase activity since it is not an organophosphate ester that is widely used in insecticides.

Table 3.1, continued.

State	District	Study areas	Geographical description	Ecological description
Agricultural area : Rice cultivation areas				
Selangor	Kuala Selangor	Parit 3, Ban 3, Tanjung Karang **(Kuala Selangor PD)**	• Coordinate: 03˚29.770'N, 101˚09.288'E • Elevation: -25 m	• A rural area with wooden- and brick-made houses scattered along small roads in between rice cultivation fields. • Trees, ornamental plants, vegetation, cash crops and heaps of coconut shells could be seen within compounds of many houses. • Artificial containers such as plastic containers, plastic pails and livestock water tanks could be found inside and outside some houses. • Piped water supply is available but the system to each house is self-built by the home owner. • Improper drainage system and waste management. • Continuous use of herbicides, insecticides and fungicides in rice cultivation fields for pest management.
Kedah	Kulim	Kg. Terat Batu, Mukim Sidam Kanan **(Kulim PD)**	• Coordinate: 05˚32.741'N, 100˚32.350'E • Elevation: 9 m	• A rural area with wooden- and brick-made houses scattered within rice cultivation fields. • Trees, ornamental plants, moderate vegetation, shrubs and cash crops could be seen within the area. • Artificial containers such as plastic containers, plastic water tanks and livestock water tanks could be found inside and outside some houses. • Piped water supply is available but the system to each house is self-built by the home owner. • Inappropriate drainage system and waste management. • Constant use of herbicides, insecticides and fungicides in rice cultivation fields for pest management.
Negeri Sembilan	Kuala Pilah	Kg. Padang Lebar Terachi, Tanjong Ipoh **(Kuala Pilah PD)**	• Coordinate: 02˚44.520'N, 102˚07.787'E • Elevation: 81 m	• A rural area with brick- and wooden-made traditional style Malay village houses built on stilts which scattered within small rice cultivation fields. • Most of rice cultivation fields are located at the valley floors, near to the foot of hills. • Trees, decorative plants, moderate vegetation, shrubs and cash crops could be seen around the area. • Heaps of coconut shells and artificial receptacles such as plastic containers and livestock water tanks could be found inside and outside some houses. • Piped water supply is available but the system to each house is self-built by the home owner. • Inappropriate drainage system and waste management. • Regular use of herbicides, insecticides and fungicides in rice cultivation fields for pest management.

Kg. = Kampung

Table 3.1, continued.

State	District	Study areas	Geographical description	Ecological description
Agricultural area : Rubber estates				
Selangor	Sungai Buloh	Sungai Pelong **(Sungai Buloh RB)**	• Coordinate: 03˚12.549'N, 101˚32.436'E • Elevation: 39 m	• A village area with wooden- and brick-made houses scattered along small roads surrounding a rubber estate. • Large trees, decorative plants, high vegetation, shrubs and bushes could be seen within the area. • Artificial habitats such as plastic containers and paint buckets could be found within the compound of some houses. • Piped water supply is offered but the system to each house is self-built by the home owner. • Appropriate drainage system and waste management. • Frequent use of herbicides, insecticides and fungicides in rubber estate for pest management.
Pahang	Temerloh	Taman Jaya 8 **(Temerloh RB)**	• Coordinate: 03˚27.423'N, 102˚27.638'E • Elevation: 43 m	• A residential area consisting of single storey semi-detached houses located next to rubber estates. • Trees, shrubs, decorative plants and moderate vegetation could be seen within the area. • Proper water supply system, drainage system and waste management. • Regular use of herbicides, insecticides and fungicides in rubber estate for pest management.
Johor	Kota Tinggi	Malaysian Rubber Board, Desaru **(Kota Tinggi RB)**	• Coordinate: 01˚33.844'N, 104˚14.267'E • Elevation: 23 m	• An area managed by Malaysian Rubber Board which comprises of an administration office, working sheds and double storey semi-detached staff quarters situated next to rubber estates. • Trees, shrubs, decorative plants and moderate vegetation could be seen around human dwellings as well as the administration building and working sheds. • Appropriate water supply system, drainage system and waste management. • Constant use of herbicides, insecticides and fungicides in rubber estate for pest management.

Table 3.1, continued.

State	District	Study areas	Geographical description	Ecological description
Non-agricultural area : Fogging-free residential areas				
Selangor	Shah Alam	Alam Nusantara, Setia Alam **(Shah Alam FF)**	• Coordinate: 03˚06.692'N, 101˚28.134'E • Elevation: 34 m	• A new residential area comprising of double storey terraced houses and recreation parks. • The environment is generally clean and well-managed. • Young trees and shrubs were planted around recreation parks. • Ornamental plants could be seen placed at the car garage of many houses. • Proper water supply system, drainage system and waste management. • No chemical control strategies had been conducted by the Department of Health or local authority as there were no reported dengue incidents from the area until the time that this study was carried out.
Kedah	Padang Serai	Taman Serai Wangi, Mukim Kulim **(Padang Serai FF)**	• Coordinate: 05˚31.301'N, 100˚32.673'E • Elevation: 3 m	• A matured residential area consisting of single storey terraced houses, shophouses, and other community facilities. • Big trees, shrubs, ornamental plants and dense vegetation could be observed within the area. • Appropriate water supply system, drainage system and waste management. • No chemical control activities had been performed by the Department of Health or local authority as there were no reported dengue cases from the area till the time that this study was conducted.
Pahang	Temerloh	Taman Seberang Temerloh **(Temerloh FF)**	• Coordinate: 03˚26.985'N, • 102˚26.743'E • Elevation: 19 m	• A new residential area containing of single storey semi-detached houses, recreation parks and other public facilities. • The environment is generally clean and well-managed. • Young trees and shrubs could be seen around the area. • Decorative plants were potted at the car garage and side garden of many houses. • Proper water supply system, drainage system and waste management. • No chemical control activities had been carried out by the Department of Health or local authority as there were no reported dengue occurrences from the area up to the time that this study was performed.

Table 3.1, continued.

State	District	Study areas	Geographical description	Ecological description
Non-agricultural area : Dengue prone residential areas				
Johor	Kota Tinggi	Felda Air Tawar 2 **(Kota Tinggi DEN)**	• Coordinate: 01˚40.552'N, 104˚01.340'E • Elevation: 5 m	• A planned area of the Federal Land Development Authority (Felda) staff quarters comprising of brick- or wooden-made bungalow houses and single storey terrace houses with reported dengue cases each year. • Trees, shrubs, bushes, ornamental plants and high vegetation could be observed within the area. • Piped water supply is offered but the system to each house is self-built by the home owner. • Proper drainage system and waste management. • Chemical control strategies had been carried out by the Department of Health or local authority when there were reported dengue cases from the area.
Selangor	Shah Alam	Kg. Padang Jawa, Seksyen 17 **(Shah Alam DEN)**	• Coordinate: 03˚03.000'N, 101˚29.200'E • Elevation: 1 m	• An unplanned settlement area consisting of terraced houses, semi-detached houses, bungalows and wooden-made houses scattered along small roads with yearly reported dengue occurrences. • Matured trees, shrubs, bushes and high vegetation could be seen within the area. • Decorative plants and cash crops could be noticed around many houses. • Piped water supply is available but the system to each house is self-built by the home owner or the house developer. • Inappropriate drainage system and waste management. • Chemical control activities had been performed by the Department of Health or local authority when there were reported dengue occurrences from the area.
Federal Territory of Kuala Lumpur	Cheras	Kg. Cheras Baru **(Cheras DEN)**	• Coordinate: 03˚06.630'N, 101˚45.101'E • Elevation: 89 m	• An established residential area comprising of terraced houses, bungalows and wooden-made houses with reported dengue incidents every year. • Matured trees, shrubs, bushes and dense vegetation could be observed within the area. • Ornamental plants could be seen around many houses. • Piped water supply is available but the system varies between different roads. • Proper drainage system and waste management. • Chemical control strategies had been conducted by the Department of Health or local authority when there were reported dengue incidents from the area.

Kg. = Kampung

3.3.2 Preparation of 10% Hay Infusion Water

Hay infusion water at a concentration of 10% was prepared as described by Reiter et al. (1991) with some modifications. 41.67 g of commercially available dry grass hay (Timothi Hay Petssion; Malaysia) was steeped in 5 L of dechlorinated water for seven days in a tightly closed 10 L transparent plastic bottle which was entirely covered with aluminium foil to prevent it from any light exposure. The setting of the laboratory was maintained at 27 ± 2 ºC and 75 ± 10% relative humidity throughout the fermentation period. The mixture produced a strong foul smell. After the 7-day of fermentation period, all immersed grass hay was sieved and discarded. The prepared 10% hay infusion water was immediately poured into ovitraps which were then straightaway used for ovitrap surveillance at study areas.

3.3.3 Ovitrap Surveillance of *Aedes* Populations in Study Areas

Ovitrap surveillance was conducted once in each study area to collect field strains of *Aedes aegypti* and *Aedes albopictus* named as F0. Standardized ovitraps as defined by Lee (1992a) were deployed in this study (Plate 3.3). The ovitrap is made from a 300 ml black plastic container with an opening and a base of 6.8 cm in diameter and 9.1 cm in height. An appropriate label is attached on the outer wall of ovitrap. Each ovitrap is equipped with an oviposition paddle made from hardboard measuring 10.0 cm long x 2.5 cm width x 0.3 cm thick with two different types of surface. The oviposition paddle was positioned diagonally into each ovitrap with the rough surface of the oviposition paddle upwards as an aid for mosquito egg laying. Every ovitrap was filled with 10% hay infusion water to a level of 5.5 cm.

All ovitraps were utilized by following the guidelines of Ministry of Health Malaysia (1997). A total of 50 ovitraps per study area were placed randomly indoors and outdoors that were either partially or totally shaded to prevent direct sunlight and heavy rain

which may cause water spillage. For this study, “indoor” refers to the interior parts of the premise that are under its roof, while “outdoor” refers to the outside of the premise but limited to the immediate vicinity of the premise. All ovitraps were placed close to human dwellings and in proximity to other potential natural and artificial breeding receptacles with minimum physical and environmental disturbance (Plate 3.4). Ovitraps were left at study areas for five days before being collected and transported back to the laboratory.

Plate 3.3: An ovitrap used during ovitrap surveillance in all study areas.

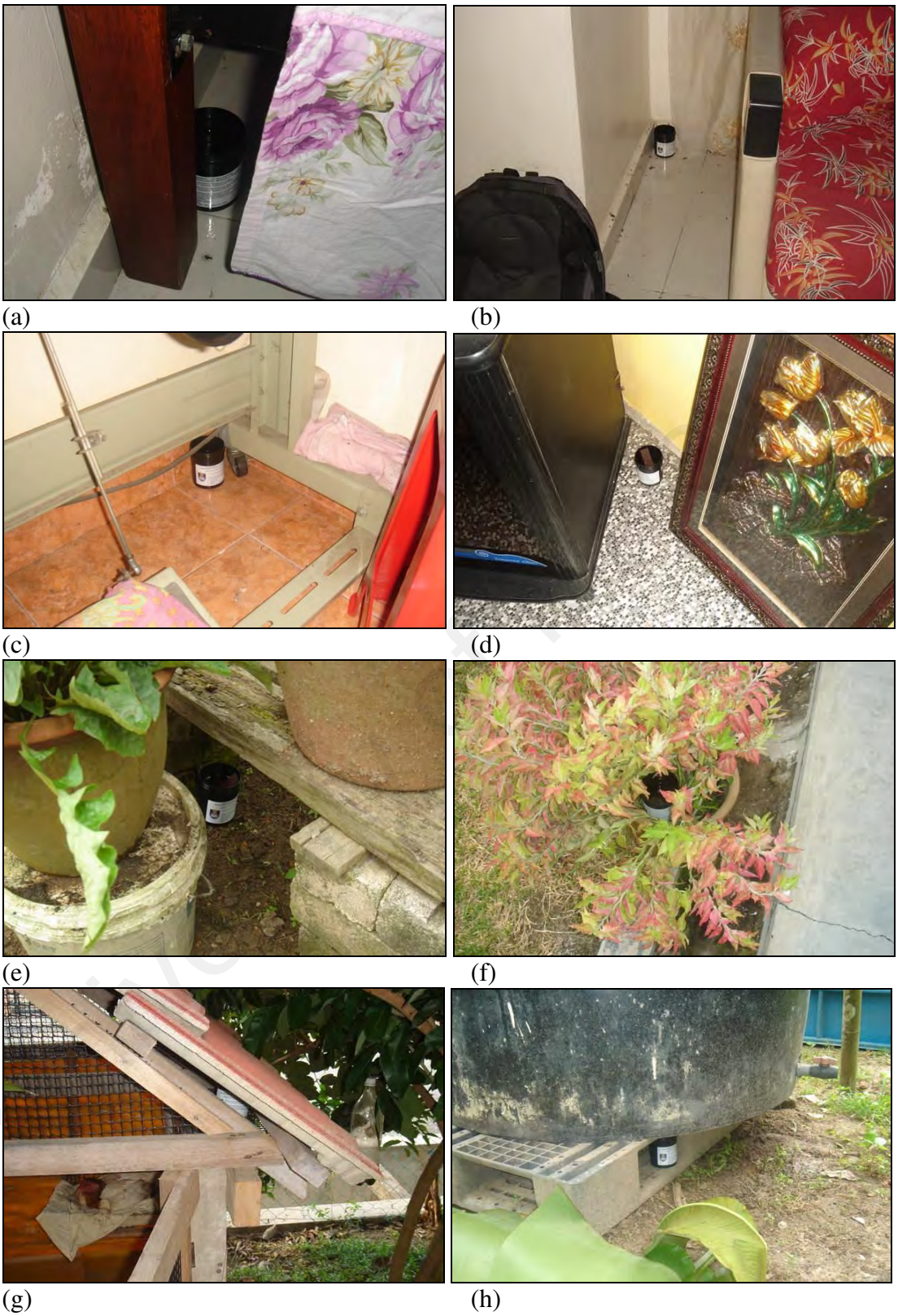

Plate 3.4: Random placement of ovitraps in all study areas : Indoors: (a) Under the bed in the bedroom (b) Behind sofa in the living room (c) Under the sewing machine (d) Behind cupboard; Outdoors: (e) Under the plant rack (f) Inside the flower pot (g) Inside the animal shelter (h) Under the outdoor water tank.

3.3.4 Identification of Larvae

Ovitraps in all study areas were collected after five days of placement and brought back to the laboratory. The contents of ovitraps including oviposition paddles were poured into plastic containers individually and topped up with dechlorinated water (Plate 3.5). A commercially available liver powder (Difco™ Liver; Becton, Dickinson & Company; France) and small pieces of partially-cooked cow liver were added into each container as larval food. All utilized containers which were modified with tiny air holes on their lids were kept covered to prevent other mosquitoes in the vicinity from ovipositing in these containers. All hatched larvae were reared before being subsequently counted and morphologically identified at fourth instar larvae using standard taxonomic keys by Division of Medical Entomology (2000a, 2000b) and Jeffery et al. (2012). Number of larvae was recorded individually for each positive ovitrap. Only *Ae. albopictus* larvae from all study areas were further colonized to adulthood in the insectarium to produce their offsprings (F1) for other studies that will be described later in this thesis.

Plate 3.5: The rearing of mosquito immatures from individual ovitraps placed at each study area.

3.3.5 Data Analysis for Ovitrap Surveillance

Data acquired from this study were analysed as follows:

(a) Distribution percentage of each larval species.

$$\frac{\text{Total number of each larval species from all study areas}}{\text{Total number of larvae from all study areas}} \times 100$$

(b) Ovitrap Index (OI):

$$\frac{\text{Number of positive ovitraps for each study area}}{\text{Total number of recovered ovitraps for each study area}} \times 100$$

(c) Mean of Ovitrap Index (OI).

(d) Normality Test for:

(i) Ovitrap Index (OI) data using Shapiro-Wilk Test.

(ii) Mean number of larvae per recovered ovitrap using Shapiro-Wilk Test.

(e) One-way ANOVA and Post Hoc Test for:

(i) Mean of Ovitrap Index (OI).

(ii) Mean number of larvae per recovered ovitrap between populations from different types of area.

(iii) Mean number of each larval species per recovered ovitrap between populations from different types of area.

(f) Mean number of each larval species per recovered ovitrap for:

(i) Each study area.

(ii) Each type of area.

(g) Percentage of positive ovitraps with single breeding for each study area:

$$\frac{\text{Number of positive ovitraps with single breeding of one larval species}}{\text{Total number of positive ovitraps}} \times 100$$

(h) Percentage of positive ovitraps with mixed breeding of different species for each study area:

$$\frac{\text{Number of positive ovitraps with mixed breeding of two larval species}}{\text{Total number of positive ovitraps}} \times 100$$

(i) Total percentage of positive ovitraps with mixed breeding for each study area.

(j) Ratio of mixed breeding between two larval species for each area.

Mean of Ovitrap Index (OI), Normality Test, One-way ANOVA, Post Hoc Test and mean number of each larval species were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at $P = 0.05$.

3.4 Susceptibility of *Aedes albopictus* Larvae against WHO Diagnostic Dosage of Larvicides

3.4.1 Larvicides

Larvicides chosen for this study included organochlorines DDT and dieldrin, as well as organophosphates malathion, fenitrothion, fenthion, temephos, chlorpyrifos and bromophos. These larvicides were in the form of 0.25 g/ 50 ml solution per bottle which were obtained from the WHO Collaborating Centre; Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang, Malaysia. All larvicides used in this study adhered to WHO diagnostic dosages for *Ae. albopictus* (World Health Organization, 1992b). Whenever the diagnostic dosage of any larvicide used in this study was not suggested by WHO for *Ae. albopictus*, the diagnostic dosage of that larvicide recommended by WHO for *Ae. aegypti* was utilized. WHO diagnostic dosages applied in this study are listed in Table 3.2.

Table 3.2: WHO diagnostic dosages (mg/L) utilized in the susceptibility study of *Aedes albopictus* larvae against WHO diagnostic dosage of larvicides.

Class of insecticides	Larvicides	WHO diagnostic dosages (mg/L)
Organochlorines	DDT	0.012*
	Dieldrin	0.050
Organophosphates	Malathion	0.125*
	Fenitrothion	0.020*
	Fenthion	0.025*
	Temephos	0.012
	Chlorpyrifos	0.012
	Bromophos	0.050*

* WHO diagnostic dosages (mg/L) for *Aedes aegypti*

Four (4) replicates of control were also prepared by the addition of 1 ml of absolute ethanol into 249 ml dechlorinated tap water per paper cup.

3.4.2 WHO Larval Bioassay using WHO Diagnostic Dosage of Larvicides

The bioassay of mosquito larvae was performed periodically according to WHO standard procedure of larvicide testing (World Health Organization, 2016a). WHO larval bioassay was carried out in the laboratory that is free from insecticidal contamination and extremes of temperature, relative humidity, wind and illumination. All bioassay testings were performed at 27 ± 2 ºC and 75 ± 10% relative humidity. Two hundred and fifty (250) ml of test solution containing an appropriate volume of the respective larvicide diluted in dechlorinated tap water to obtain the WHO diagnostic dosage was prepared in a 300 ml paper cup and left for at least half an hour. Twenty five (25) healthy late third instar larvae were then introduced into each paper cup. A total of 4 replicates were set up for each concentration of larvicide (Plate 3.6). Similar stage and number of larvae were applied for each control paper cup consisting of 1 ml of absolute ethanol in 249 ml dechlorinated tap water. Cumulative larval mortality was scored after 24 hours of exposure. Both moribund and dead larvae were counted to obtain the mortality percentage. According to World Health Organization (2016a), larvae that failed to move when they were probed with a needle in the siphon or cervical region were considered dead while larvae that were incapable to appear at the water surface or not showing any sign of diving behaviour when the water was disturbed were considered as moribund larvae. All survivors from control were collected and kept in the freezer at -70 °C before being used for the biochemical enzyme microplate assay.

Plate 3.6: WHO larval bioassay conducted in the laboratory.

3.4.3 Data Analysis for WHO Larval Bioassay using WHO Diagnostic Dosage of Larvicides

Mortality percentage was calculated based on the number of dead and moribund larvae after 24 hours post-exposure. These data were documented in the report forms. As defined by WHO (2016a), larval bioassay of the respective larvicide was discarded and repeated when more than 10% of the larvae of control population pupated during the testing. If the mortality of control population was between 5% and 20%, the mortality percentage of field strains were corrected using Abbott's formula (1925) as follows:

$$\frac{\%\ \text{Test Mortality} - \%\ \text{Control Mortality}}{100 - \%\ \text{Control Mortality}} \times 100$$

Results with control mortalities exceeded 20% were recorded but not analysed. The reliability of the data influences the accuracy of results interpretation.

Data of mortality percentage were interpreted based on guidelines by World Health Organization (2016a) as in Table 3.3.

Table 3.3: Results interpretation of mortality percentage for WHO larval and adult mosquito bioassays (World Health Organization, 2016a).

Mortality percentage	Interpretation of results
98-100%	Susceptibility is indicated.
< 98%	Resistance is suggested. Further testings are suggested for verification.
90-97% (corrected if necessary)	Probable resistance / Moderate resistance / incipient resistance / tolerance to the respective insecticide is indicated. The confirmation of resistant genes in the mosquito population should be confirmed by conducting additional bioassay testings using the same adulticide on the same mosquito population and/ or by performing molecular assays for known resistance mechanisms. Resistance is confirmed with two additional testings that constantly showed mortality below 98%.
< 90%	High resistance is indicated. Additional bioassays testings to confirm the presence of resistant genes in the mosquito population is not necessary if a minimum of 100 mosquitoes had been employed in the testing. However, further studies of resistance mechanisms and distribution are suggested.

In addition, Normality Test using Shapiro-Wilk test was conducted to confirm that the data of mortality percentage for *Ae. albopictus* larval populations against WHO diagnostic dosages of organochlorines and organophosphates were normally distributed. One-way ANOVA and Post Hoc Test were then performed to ascertain any significant difference between populations from different types of area exposed to each organochlorine and organophosphate employed. The correlation test using Pearson

Correlation Test was also carried out to determine any significant cross resistance between two larvicides of organochlorines and organophosphates based on the data of mortality percentage of *Ae. albopictus* larval populations against WHO diagnostic dosages. The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant cross resistance between two tested larvicides. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong cross resistance between two tested larvicides.

The calculation of mortality percentage, Normality Test, One-way ANOVA, Post Hoc Test and Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at $P = 0.05$.

3.5 Susceptibility of *Aedes albopictus* Larvae against Independent Diagnostic Dosage of Larvicides Established from *Aedes albopictus* Reference Strain Larvae

3.5.1 Larvicides

Larvicides used in this study included the organochlorines DDT and dieldrin; the organophosphates malathion, fenitrothion, fenthion, temephos, chlorpyrifos and bromophos; the carbamates propoxur and bendiocarb; as well as the pyrethroids permethrin, deltamethrin, lambdacyhalothrin, cyfluthrin and etofenprox. These larvicides were supplied as 0.25 g/ 50 ml solution per bottle from the WHO Collaborating Centre; Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang, Malaysia.

3.5.2 WHO Larval Bioassay for Establishment of Independent Diagnostic Dosage of Larvicides for *Aedes albopictus* Reference Strain Larvae

The WHO larval bioassay was carried out on laboratory (reference) strain by following the WHO standard procedure of larvicide testing (World Health Organization, 2016a). This testing was conducted in the laboratory without any exposure to insecticides and extremes of temperature, relative humidity, wind and illumination. The setting of the laboratory was maintained at 27 ± 2 ºC and 75 ± 10% relative humidity throughout this study. Two hundred and fifty (250) ml of test mixture consisting of an appropriate volume of the larvicide diluted in dechlorinated tap water was prepared in a 300 ml paper cup and allowed to mix together for at least half an hour. A wide range of concentrations of each larvicide was prepared and tested. A narrower range of tested concentrations of each larvicide that caused 15%, 35%, 50%, 65% and 85% mortality at 24 hours post-exposure was used to estimate lethal concentrations values (LC_{50}, LC_{95} and LC_{99}). Twenty five (25) healthy late third instar larvae were introduced into each paper cup. Four (4) replicates were employed for each concentration of each larvicide. Control paper cup comprising of 1 ml of absolute ethanol in 249 ml dechlorinated tap water per paper cup was also prepared in 4 replicates with similar stage and number of larvae.

3.5.3 WHO Larval Bioassay for Determination of Susceptibility of *Aedes albopictus* Field Strains Larvae against Independent Diagnostic Dosage of Larvicides Established from *Aedes albopictus* Reference Strain Larvae

Late third instar larvae of *Ae. albopictus* of all field strains were submitted for WHO larval bioassay which was performed in the same manners and conditions as described in 3.4.2. The susceptibility status of all *Ae. albopictus* field strains were evaluated by

exposing them to double value of lethal concentration$_{99}$ (LC_{99}) of the laboratory (reference) strain for each larvicide.

Larval mortality percentage was recorded after 24 hours of exposure by calculating both moribund and dead larvae. Larvae were probed with a needle in the siphon or cervical region and considered dead if they failed to move, whereas, larvae that were incapable to appear at the water surface or not showing any sign of diving behaviour when the water was disturbed were treated as moribund larvae. All survivors from control were collected and kept in the freezer at -70 °C before being used for the biochemical enzyme microplate assay.

3.5.4 Data Analysis for WHO Larval Bioassay for Determination of Susceptibility of *Aedes albopictus* Field Strains Larvae against Independent Diagnostic Dosage of Larvicides Established from *Aedes albopictus* Reference Strain Larvae

The mortality percentage results for all concentrations of each larvicide that caused 15%, 35%, 50%, 65% and 85% mortality among *Ae. albopictus* laboratory (reference) strain at 24 hours post-exposure were used to generate the regression line of probit analysis. Lethal concentrations values (LC_{50}, LC_{95} and LC_{99}) of the reference strain were obtained from the regression line constructed. Discriminating lethal dosages of larvicides for *Ae. albopictus* field strains larvae were values of twice the calculated lethal concentration$_{99}$ (2 x LC_{99}) of the reference strain.

Mortality percentage of each *Ae. albopictus* population upon exposures to all larvicides at independent diagnostic dosages (2xLC_{99}) was recorded in report forms by calculating the number of dead and moribund larvae at 24 hours post-exposure. According to World Health Organization (2016a), larval bioassay of the respective larvicide was discarded and repeated when more than 10% of the larvae of control

population pupated during the testing. If the mortality of control population was between 5% and 20%, the mortality percentage of field strains was corrected using Abbott's formula (1925) as follows:

$$\frac{\%\ \text{Test Mortality} - \%\ \text{Control Mortality}}{100 - \%\ \text{Control Mortality}} \times 100$$

Results with control mortalities exceeding 20% were recorded but not analysed. The reliability of the data obtained affects the accuracy of results interpretation. The susceptibility status of each *Ae. albopictus* population based on their mortality percentages was classified according to the guidelines by World Health Organization (2016a) as illustrated in Table 3.3 in 3.4.3.

Subsequently, Normality Test using Shapiro-Wilk test was carried out to validate that the data of mortality percentage for *Ae. albopictus* larval populations against independent diagnostic dosages ($2xLC_{99}$) of larvicides were normally distributed. One-way ANOVA and Post Hoc Test were then performed to determine any significant difference between populations from different types of area exposed to each larvicide. The correlation test using Pearson Correlation Test was also conducted to ascertain any significant cross resistance between two larvicides based on the data of mortality percentage of *Ae. albopictus* larval populations against independent diagnostic dosages ($2xLC_{99}$). The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant cross resistance between two tested larvicides. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong cross resistance between two tested larvicides.

The probit analysis to generate the lethal concentration regression line of each larvicide for *Ae. albopictus* reference strain, the calculation of mortality percentage, Normality Test, One-way ANOVA, Post Hoc Test and the Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at P = 0.05.

3.6 Susceptibility of *Aedes albopictus* Adults against WHO Diagnostic Dosage of Adulticides

3.6.1 Adulticides

Adulticides used in this study consisted of organochlorines: 4% DDT and 4% dieldrin; organophosphates: 5% malathion and 1% fenitrothion; carbamates: 0.1% propoxur and 0.1% bendiocarb; and pyrethroids: 0.75% permethrin, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.15% cyfluthrin and 0.5% etofenprox. These adulticides were in the form of impregnated papers which were purchased from the WHO Collaborating Centre; Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang, Malaysia. All adulticides followed diagnostic dosages designated by WHO for *Ae. aegypti* or *Ae. albopictus* (World Health Organization, 1992b; World Health Organization, 1998c; World Health Organization, 2016a) except for dieldrin (WHO recommended diagnostic dosage: 1%), malathion (0.8%), bendiocarb (no recommended diagnostic dosage by WHO), permethrin (0.25%), deltamethrin (0.03%), and lambdacyhalothrin (0.03%). This limitation was due to the unavailability of impregnated papers following diagnostic dosages suggested by WHO. Nevertheless, the exposure period of all adulticides used in this study adhered to the recommendation of WHO which was 1 hour. Impregnated papers of control were also purchased from the same source.

3.6.2 WHO Adult Mosquito Bioassay

The bioassay of adult mosquitoes was performed periodically according to WHO standard procedure of susceptibility testing (World Health Organization, 2016a). A total of 4 replicates with 25 sucrose-fed 3-5 days old adult female mosquitoes per tube were employed for this bioassay. The same number of replicates and adult female mosquitoes per tube were applied for the control.

WHO adult mosquito bioassay was conducted in the laboratory which is free from insecticidal contamination and extremes of temperature, relative humidity, wind and illumination. All bioassay testings were carried out at 27 ± 2 ºC and 75 ± 10% relative humidity. Adult mosquitoes used in this bioassay were held in the holding tubes for an hour at optimum test conditions. After an hour of the pre-testing holding period, any knocked-down, dead or damaged adult female mosquitoes were replaced with healthy ones. These adult mosquitoes were then blown gently into the exposure tubes lined with adulticide-impregnated papers or control papers, respectively. These exposure tubes were covered with black cloth and laid either vertically for organochlorines, organophosphates and carbamates; or horizontally for pyrethroids to ensure optimum contacts between all mosquitoes and impregnated papers (Plate 3.7). Cumulative knockdown counts were recorded every minute within the exposure time.

After an hour of exposure, all mosquitoes were transferred into clean paper cups which were covered with fine nylon nettings and secured with rubber bands. Sucrose solution was provided for the mosquitoes using cotton pads soaked in 10% sucrose solution placed on the nettings. All mosquitoes were held for 24 hours of recovery before the mortality was recorded again (Plate 3.8). All survivors were collected and kept in the freezer at -70 °C before being used for the biochemical enzyme microplate assay.

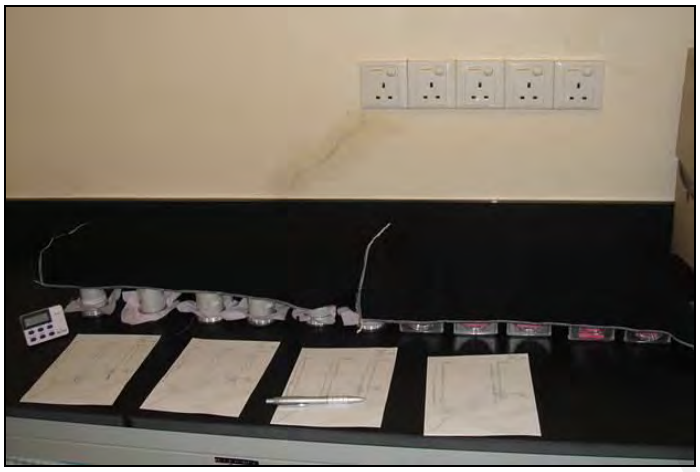

Plate 3.7: WHO adult mosquito bioassay carried out in the laboratory.

Plate 3.8: The 24-hour recovery period for adult mosquitoes subjected to WHO adult mosquito bioassay.

3.6.3 Data Analysis for WHO Adult Mosquito Bioassay

Results of knockdown percentages of *Ae. albopictus* adults throughout the one hour adulticide exposure that fell between 5% to 95% knockdown were subjected to probit analysis to obtain knockdown time$_{50}$ (KT_{50}) (Raymond, 1985). Resistance ratio (RR) was also calculated for each *Ae. albopictus* field populations using the following formula (Brown & Pal, 1971):

$$\text{Resistance Ratio (RR)} = \frac{KT_{50} \text{ of the field strain}}{KT_{50} \text{ of the reference strain}}$$

The value of RR > 10 indicated that the mosquito population is highly resistant. Moderate resistance of mosquitoes is expressed when RR is between 5 and 10 while RR < 5 showed that the mosquito population is susceptible (World Health Organization, 2016a).

Knockdown percentage of each *Ae. albopictus* adult population exposed to organochlorines, organophosphates and carbamates was calculated based on the number of knocked-down or dead adult mosquitoes at 60 minutes of the exposure time while the same parameter for pyrethroids exposures was calculated at 30 minutes of the exposure time due to rapid knockdown action of pyrethroids. On the other hand, mortality percentage of each *Ae. albopictus* adult population to all adulticides was calculated based on the number of dead or knocked-down adult mosquitoes at 24 hours post-exposure. These results were documented in the report forms. If the knockdown or mortality of control population was between 5% and 20%, the mortality percentage was corrected using Abbott's formula (1925) as described in 3.4.3. Results interpretation of knockdown and mortality percentage followed the guidelines by World Health Organization (2016a) as listed in Table 3.3 in 3.4.3 as well.

Additionally, Normality Test using Shapiro-Wilk test was conducted to confirm that the data of knockdown percentages at 60 minutes of the exposure time (organochlorines, organophosphates and carbamates) and 30 minutes of the exposure time (pyrethroids) as well as mortality percentages at 24 hours post-exposure for all *Ae. albopictus* adult populations were normally distributed. One-way ANOVA and Post Hoc Test were then performed to determine any significant difference between populations from different types of area exposed to each adulticide.

The correlation test using Pearson Correlation Test was also performed to verify any significant cross resistance between two adulticides based on the data of knockdown time$_{50}$ (KT_{50}) values of *Ae. albopictus* adult populations. The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant cross resistance between two tested adulticides. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong cross resistance between two tested adulticides.

The probit analysis to obtain knockdown time$_{50}$ of all *Ae. albopictus* adult populations exposed to each adulticide, the calculation of both knockdown and mortality percentages, Normality Test, One-way ANOVA, Post Hoc Test and Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at $P = 0.05$.

3.7 Characterization of Biochemical Enzyme Mechanisms Contributing to Insecticide Resistance in *Aedes albopictus* Larvae and Adults

Four (4) types of enzyme microassays were performed on *Ae. albopictus* larvae and adult mosquitoes collected using the enzyme-linked immunoassay (ELISA) reader which consisted of non-specific esterases (EST), mixed function oxidases (MFO), glutathione-S-transferases (GST) and insensitive acetylcholinesterase (AChE) in order to identify the causes of metabolic resistance occurred in those field strains mosquitoes (Plate 3.9).

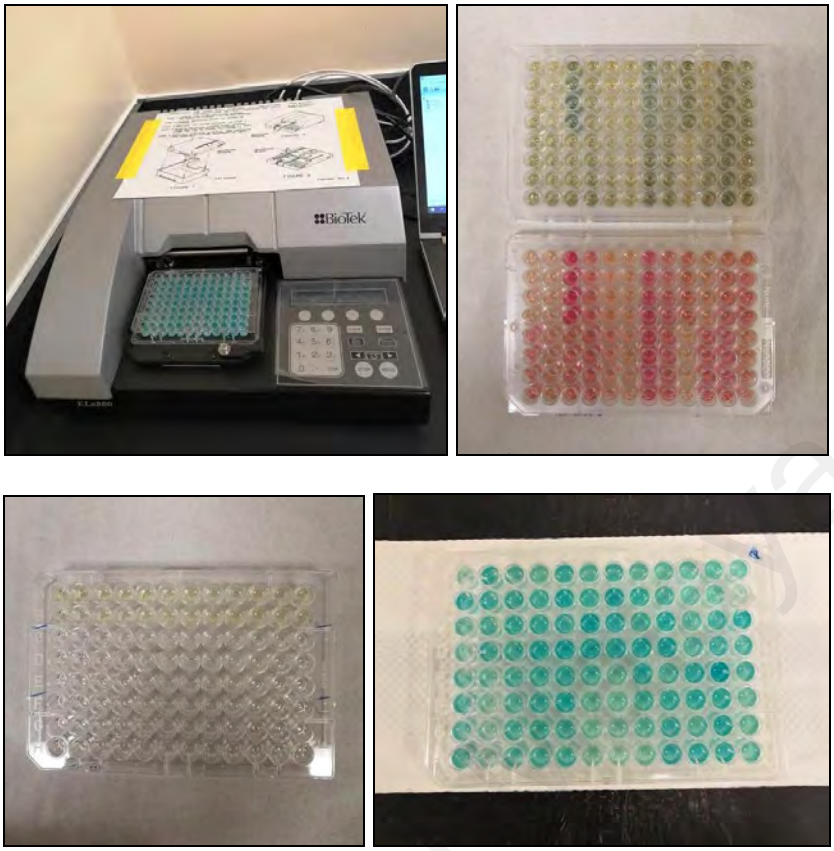

Plate 3.9: Enzyme microassays using the enzyme-linked immunoassay (ELISA) reader in the laboratory.

3.7.1 Non-specific Esterases (EST) Enzyme Microassay

The biochemical assay for non-specific esterases (EST) activities was performed according to Brogdon et al. (1988) and Lee (1990).

3.7.1.1 Preparation of Potassium Phosphate Buffer (2.0 M; pH 7.6)

Both 4.50 g sodium phosphate dibasic (Na_2HPO_4) and 1.70 g potassium phosphate monobasic (KH_2PO_4) were dissolved in 500 ml distilled water to produce 2.0 M potassium phosphate buffer. The pH was adjusted to 7.6 using pH meter.

3.7.1.2 Preparation of Substrate Solution

The stock solution was prepared first by dissolving 0.06 g α-naphthyl acetate in 10 ml acetone. The substrate solution was then prepared by adding 0.5 ml of stock solution into 50 ml potassium phosphate buffer.

3.7.1.3 Preparation of Indicator Solution/ Coupling Reagent

Both 0.875 g sodium dodecyl sulphate (SDS) and 0.075 g fast blue salt (FBS) (tetrazotized o-dianisidine) were introduced into 50 ml distilled water to produce the indicator solution or coupling reagent.

3.7.1.4 Preparation of Stopping Solution: 10% Acetic Acid

Ten (10) ml absolute acetic acid was added into 90 ml distilled water to produce 100 ml 10% acetic acid as the stopping solution.

3.7.1.5 Procedure of Non-specific Esterases (EST) Enzyme Microassay

Survived *Ae. albopictus* of all strains from bioassays kept at -70 °C were prepared. Every sample was homogenized individually in 100 µl potassium phosphate buffer in a microcentrifuge tube at 4 °C using the pestle. Another 400 µl of buffer was diluted to a total of 500 µl. The homogenate was centrifuged at 15000 rpm at 4 °C for 10 minutes. Fifty (50) µl clear homogenate were then transferred into each well of the microtiter plate using the micropipette. A total of four (4) replicate aliquots of the homogenate from every sample were obtained for this assay. Hence, four (4) wells of microtiter plate were used per sample. Fifty (50) µl substrate solution was added into each well using a multiple eight (8) channels micropipette. The microtiter plate was incubated for 1 minute at room temperature (28 °C). Fifty (50) µl indicator solution was then added into each well using a multiple eight (8) channels micropipette. The microtiter plate was

incubated again for 10 minutes at room temperature (28 °C). Change of colour reaction took place immediately where a pinkish purplish colour appeared which then turned to blue after the incubation. This is due to the hydrolysis of α-naphthyl acetate into α-naphthol which reacted with the FBS, thus producing a change in the absorbance of the solution. The reaction was stopped by the addition of 50 µl 10% acetic acid into each well using a multiple eight (8) channels micropipette. The microtiter plate was incubated for 10 minutes at room temperature (28 °C). The absorbance of the reaction which indicates the esterase activity was measured spectrophotometrically using an immunoassay (ELISA) reader at wavelength of 450 nm to determine the enzyme activity quantitatively. Esterases activity was calculated based on the absorbance standard curve for known concentration of α-naphthol and expressed as nmoles α-naphthol/min/mg protein. Similar preparation and procedure was applied to determine the β-esterase enzyme activity except that α-naphthyl acetate was replaced with β-naphthyl acetate and the absorbance standard curve for known concentration of β-naphthol was used in the calculation of β-esterases activity.

3.7.2 Mixed Function Oxidases (MFO) Enzyme Microassay

Mixed function oxidases (MFO) enzyme microassay was carried out following Brogdon et al. (1997) with some modifications as outlined by Nazni et al. (2000).

3.7.2.1 Preparation of Sodium Acetate Buffer (0.25 M; pH 5.0)

Exactly 20.51 g sodium acetate was dissolved in 1000 ml distilled water to produce 0.25 M sodium acetate buffer. The pH was adjusted to 5.0 with acetic acid using pH meter.

3.7.2.2 Preparation of Substrate Solution: 3,3'5,5'-Tetramethylbenzidine (TMBZ) Solution

The substrate solution was freshly prepared by dissolving 0.05 g 3,3'5,5'-tetramethylbenzidine (TMBZ) in 25 ml absolute methanol. 75 ml of 0.25 M sodium acetate buffer (pH 5.0) was then added into the solution.

3.7.2.3 Preparation of Indicator Solution: 3% Hydrogen Peroxide (H_2O_2) Solution

Exactly 1.935 ml 31% hydrogen peroxide was introduced into 18.065 ml distilled water to produce 20 ml 3% hydrogen peroxide.

3.7.2.4 Procedure of Mixed Function Oxidases (MFO) Enzyme Microassay

Survived *Ae. albopictus* of all strains from bioassays kept at -70 °C were prepared. Each individual sample was homogenized in 100 µl sodium acetate buffer in a microcentrifuge tube at 4 °C using the pestle. Nine hundred (900) µl of buffer was then added to a total of 1 ml. Hundred (100) µl of homogenate was transferred into each well of microtiter plate using the micropipette. A total of four (4) replicate aliquots of the homogenate from each sample were prepared for this assay. Therefore, four (4) wells of microtiter plate were used per sample. Two hundred (200) µl of substrate solution 3,3'5,5'-tetramethylbenzidine (TMBZ) solution was added into each well of the microtiter plate and left for 1 minute. Twenty five (25) µl of an indicator solution 3 % hydrogen peroxide solution was added into each well of the microtiter plate. Change of colour reaction took place immediately. The microtiter plate was incubated for 10 minutes before being read using an immunoassay (ELISA) reader at a wavelength of 630 nm. The MFO activity was then calculated using the absorbance standard curve for

known concentration of cytochrome c (Brogdon et al., 1997). The activity of MFO was expressed as nmoles cyt c/min/mg protein.

3.7.3 Glutathione-S-transferases (GST) Enzyme Microassay

The biochemical assay for glutathione-s-transferases (GST) activities was performed according to Lee & Chong (1995) and World Health Organization (1998b) with some modifications.

3.7.3.1 Preparation of Potassium Phosphate Buffer (0.5 M; pH 7.4)

Exactly 2.724 g potassium phosphate monobasic (KH_2PO_4) was dissolved in 300 ml distilled water to produce Solution A. Meanwhile, 9.47 g sodium phosphate dibasic (Na_2HPO_4) was dissolved in 1000 ml distilled water to produce Solution B. One thousand (1000) ml potassium phosphate buffer was prepared by adding 196.0 ml Solution A to 804.0 ml Solution B. The pH was adjusted to 7.4 using pH meter.

3.7.3.2 Preparation of Substrate Solution

The substrate solution was prepared by dissolving 0.03 g glutathione (GSH) in 50 ml potassium phosphate buffer (0.5 M; pH 7.4).

3.7.3.3 Preparation of Indicator Solution/ Coupling Reagent

Both 0.01 g 1-chloro-2, 4-dinitrobenzene (CDNB) and 0.5 ml acetone were introduced into 50 ml potassium phosphate buffer (0.5 M; pH 7.4) to produce the indicator solution or coupling reagent.

3.7.3.4 Procedure of Glutathione-S-transferases (GST) Enzyme Microassay

Survivors of *Ae. albopictus* of all strains from bioassays kept at -70 °C were prepared. Each individual sample was homogenized in 100 µl potassium phosphate buffer in a microcentrifuge tube at 4 °C using the pestle. Another 400 µl of buffer was diluted to the total of 500 µl. The homogenate was centrifuged at 14000 rpm at 4 °C for 10 minutes. Fifty (50) µl clear homogenate was transferred into each well of microtiter plate using a micropipette. A total of four (4) replicate aliquots of the homogenate from every sample was obtained for this assay. Therefore, four (4) wells of microtiter plate were occupied per adult mosquito. Fifty (50) µl GSH (substrate solution) was added into each well using a multiple eight (8) channels micropipette. Fifty (50) µl CDNB (indicator solution) was then added into each well using a multiple eight (8) channels micropipette. Change of colour reaction took place which yellowish colour was observed. The microtiter plate was incubated for 15 minutes at room temperature (28 °C) before it was read using an immunoassay (ELISA) reader at wavelength of 410 nm. The optical density values were obtained. GST activity was calculated by assuming that the absorbance (A) is following the Beer's law ($A = \varepsilon cl$). The extinction coefficient (ε) used was 4.39 mM^{-1} while the path length (l) which is the depth of the buffer solution in the well of the microtitre plate was 0.6 cm. GST activity was then reported as mmoles CDNB/min/mg protein.

3.7.4 Insensitive Acetylcholinesterase (AChE) Enzyme Microassay

The biochemical assay for insensitive acetylcholinesterase (AChE) activities was performed by using a modification of Ellman's method (Brogdon et al., 1988).

3.7.4.1 Preparation of Potassium Phosphate Buffer (pH 6.8)

Exactly 4.735 g sodium phosphate dibasic (Na_2HPO_4) dissolved in 500 ml distilled water was added to 4.540 g potassium phosphate monobasic (KH_2PO_4) dissolved in 500 ml distilled water to produce the potassium phosphate buffer. The pH was adjusted to 6.8 using pH meter.

3.7.4.2 Preparation of Substrate Solution: Acetylthiocholine iodide (ACTHI)

The substrate solution was prepared by dissolving 0.075 g acetylthiocholine iodide (ACTHI) and 10 ml acetone in 90 ml potassium phosphate buffer (pH 6.8). The solution was mixed in a bottle covered with aluminum foil to prevent from any exposure to light.

3.7.4.3 Preparation of Coupling Reagent: Ellman's Solution (DTNB)

Exactly 0.013 g 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) was added into 100 ml potassium phosphate buffer (pH 6.8) to produce the Ellman's solution. The reagent bottle was covered with aluminum foil to prevent from any exposure to light.

3.7.4.4 Preparation of Inhibitor

The independent diagnostic dosage of propoxur ($2xLC_{99}$) which was established from the reference strain of *Ae. albopictus* and used in the WHO larval bioassay of this study was 4.88 mg/L. On the other hand, the WHO recommended dosage of propoxur for *Ae. albopictus* adults is 0.1%, which is equivalent to 1000 mg/L. Hence, 5 mg/L and 1000 mg/L of propoxur solution were used to prepare the inhibitor as propoxur-ACTHI concentrations for samples of *Ae. albopictus* larvae and adults, respectively.

3.7.4.5 Procedure of Insensitive Acetylcholinesterase (AChE) Enzyme Microassay

Survived *Ae. albopictus* of all strains from bioassays kept at -70 °C were prepared. Every sample was homogenized in 100 µl potassium phosphate buffer in a microcentrifuge tube at 4 °C using the pestle. Another 400 µl of buffer was diluted to the total of 500 µl. The homogenate was centrifuged at 14000 rpm at 4 °C for 10 minutes. Fifty (50) µl clear homogenate was transferred into each well of microtiter plate using a micropipette. A total of eight (8) replicate aliquots of the homogenate from every sample were obtained for this assay. Hence, eight (8) wells of microtiter plate were used per sample. Fifty (50) µl aliquot of mixture of 10% acetone buffer solution of ACTHI plus 5 mg/L (for larvae samples) or 1000 mg/L (for adult mosquito samples) of propoxur were added into 4 replicates of test wells. As a positive control, 50 µl ACTHI solution without propoxur was used in 4 wells. Fifty (50) µl of aliquot of DTNB was added into each well of the microtiter plate using a multiple eight (8) channels micropipette. Change of colour reaction took place immediately in which the yellowish colour or colourless solution was observed. The microtiter plate was incubated for 30 minutes at room temperature (28 °C) before it was read using an immunoassay (ELISA) reader at wavelength of 410 nm. The optical density values were obtained. The significant difference between the absorbance of the samples with and without the addition of propoxur was determined. The mean percent of AChE activity in propoxur-inhibited fraction (%) was also calculated as below:

$$\text{Mean percent acetylcholinesterase activity in propoxur-inhibited fraction (\%)} = \frac{\text{Total mean optical density of inhibited reaction (with propoxur)}}{\text{Total mean optical density of unhibited reaction (without propoxur)}} \times 100$$

3.7.5 Protein Assay for Determination of Total Protein Content

Samples of larvae and adults of *Ae. albopictus* were slightly varied in size. In order to correct the partiality of enzyme activity findings due to size variances of these samples, the protein assay was carried out to determine the total protein content which is used as a standard correction factor in the analysis of all enzyme activities (Koou et al., 2014a).

The protein assay was performed based on the standard protocol for microtiter plates provided by the manufacturer (Bio-Rad Laboratories, Inc.), World Health Organization (1998b) and Rasli et al. (2018) with some modifications.

3.7.5.1 Procedure of Protein Assay

Ten (10) µl of the mosquito homogenate was pipetted into each well. A total of four (4) replicate aliquots of the homogenate from a single mosquito was prepared. Twenty (20) ml of diluted dye reagent was prepared by diluting 1 ml of dye reagent concentrate with 4 ml of double distilled water. Two hundred (200) µl of this diluted dye reagent was then added into each well. The homogenate solution was pipetted up and down to mix it. The microtiter plate was incubated for 10 minutes at room temperature (28 °C). The absorbance of the reaction was then measured spectrophotometrically using an immunoassay (ELISA) reader at wavelength of 595 nm. The optical density values obtained were then transformed into protein concentration. The bovine serum albumin standard curve was used as the reference of a known protein concentration in the calculation and construction of the protein concentration for *Ae. albopictus* samples of this study. Except for acetylcholinesterase (AChE) activity, all enzyme activities conducted in this study were calculated by correcting for protein content.

3.7.6 Statistical Analysis of Enzyme Activities and Their Correlation with Findings of WHO Larval and Adult Mosquito Bioassays

The level of elevated enzyme activities in *Ae. albopictus* larvae and adults from different types of area were compared with the reference strain by calculating the resistance ratio (RR) (Brown & Pal, 1971) as below:

$$\text{Resistance Ratio (RR)} = \frac{\text{Mean of elevated enzyme activity of the field strain}}{\text{Mean of elevated enzyme activity of the reference strain}}$$

The value of RR > 10 indicated that the mosquito population is highly resistant. Moderate resistance of mosquitoes is expressed when RR is between 5 and 10 while RR < 5 showed that the mosquito population is susceptible (World Health Organization, 2016a).

Normality Test using Shapiro-Wilk test was carried out to confirm that the data of all elevated enzyme activities for *Ae. albopictus* larval and adult populations were normally distributed. Any significant difference in the mean elevated enzyme activities of non-specific esterases (EST), mixed function oxidases (MFO) and glutathione-S-transferases (GST) among *Ae. albopictus* larval and adults from all types of area was determined by performing One-way ANOVA. Post Hoc Test was then conducted to determine any significant difference in the mean elevated enzyme activity of EST, MFO and GST between *Ae. albopictus* larval and adult populations from agricultural and non-agricultural areas.

Furthermore, individual samples from each enzyme microassay were also grouped into different range of elevated enzyme activities in order to compute the distribution frequency as outlined by Rasli et al. (2018) with some modifications. These elevated activities of EST, MFO and GST were then classified into low (+), moderate (++) and

high (+++) scores by referring to the heterogeneity of the elevated enzyme activity distribution.

Meanwhile, instead of revealing the level of elevated enzyme activities as shown in the EST, MFO and GST microassays, the aim of conducting the insensitive acetylcholinesterase (AChE) microassay was to determine the efficacy of propoxur in controlling *Ae. albopictus* larvae and adults in different types of area. Hence, for AChE microassay, besides the calculation of resistance ratio (RR) based on the mean percent AChE activity in propoxur-inhibited fraction (%) for each *Ae. albopictus* larval and adult population, any significant difference between the mean AChE activity with the addition of propoxur and the mean AChE activity without the addition of propoxur for each *Ae. albopictus* larval and adult populations was also determined by performing Paired samples t-test. These samples were then classified into three different heterogeneity categories based on their mean percent acetylcholinesterase activity in propoxur-inhibited fraction (%). Individual samples with more than 70 % remaining activity were indicative of homozygous resistance (RR) (+++), 30-70 % remaining activity were suggestive of heterozygous (RS) (++) while below 30 % remaining activity were interpreted as homozygous susceptible (SS) (+) (World Health Organization, 1998b; Low et al., 2013). The value of mean percent acetylcholinesterase activity in propoxur-inhibited fraction (%) for resistant samples is possible to be higher than 100%. This phenomenon is normal in resistant samples which is partly due to the optical density of propoxur in well of the microtiter plate (World Health Organization, 1998b).

Other than that, any significant increase of elevated enzyme activities of EST, MFO, GST and AChE in *Ae. albopictus* larvae and adults from different types of area as compared to the reference strain was determined by conducting Independent samples t-test.

In addition, the correlation analysis using Pearson Correlation Test was performed to determine any association between EST, MFO, GST and AChE activities at both larval and adult stages of *Ae. albopictus* using their mean elevated enzyme activities values. This correlation test was carried out to verify whether or not the elevated activity of an enzyme was influenced by the elevated activity of another enzyme within the same or different development stage of *Ae. albopictus*. Besides that, any association between the susceptibility status of *Ae. albopictus* from different types of area at both larval and adult stages as indicated by the findings of WHO bioassays, and their elevated enzyme activities was ascertained using Pearson Correlation Test as well. In this correlation test, data of percent mortality of *Ae. albopictus* larvae at 24 hours post-treatment using independent diagnostic dosages ($2xLC_{99}$) and knockdown $time_{50}$ (KT_{50}) values of *Ae. albopictus* adults were used in the comparison with all elevated enzyme activities of their respective development stage. The role of each elevated enzyme activity in the resistance development among *Ae. albopictus* populations from different types of area at either larval stage, adult stage or both stages was verified through this correlation test. The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant association between two tested parameters. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong association between two tested parameters.

Normality Test, One-way ANOVA, Post Hoc Test, Paired samples t-test, Independent samples t-test and Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at $P = 0.05$.

3.8 Synergistic Effect of Piperonyl Butoxide (PBO) in *Aedes albopictus* Adults against Organochlorines and Pyrethroids

3.8.1 Synergist

Piperonyl butoxide (PBO) (90.0% technical grade, Aldrich, St. Louis, MO, USA) was utilized as an inhibitor in this study to observe the synergistic effect in *Ae. albopictus* adults of all strains against organochlorines and pyrethroids.

3.8.2 Preparation of 4% Piperonyl Butoxide (PBO) Impregnated Papers

Piperonyl butoxide (PBO) impregnated papers were made locally at 4% as outlined by Ishak et al. (2015) and World Health Organization (2016b). PBO impregnated papers were prepared by immersing individual 14 cm x 14 cm Whatman No. 1 filter paper into the mixture solution of PBO and absolute ethanol in a tray. Immersed PBO impregnated papers were taken out from the tray using forceps and left to dry at room temperature on the styrofoam board embedded with metal pins on it to support the PBO impregnated papers (Plate 3.10). Prepared PBO impregnated papers were covered and kept individually using aluminium foil at 4 ºC before being used.

Plate 3.10: Preparation of 4% piperonyl butoxide (PBO) impregnated papers in the laboratory.

3.8.3 Adulticides

Adulticides used in this synergism study consisted of organochlorines (4% DDT and 4% dieldrin) as well as pyrethroids (0.75% permethrin, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.15% cyfluthrin and 0.5% etofenprox). Impregnated papers of these adulticides were bought from similar source as explained in 3.6.1. Hence, the exposure period of all organochlorines and pyrethroids used in this study also followed the recommendation of WHO which was 1 hour.

3.8.4 Synergism Study using Piperonyl Butoxide (PBO) Impregnated Papers

Synergist assays using piperonyl butoxide (PBO) were carried out on all strains of *Ae. albopictus* adults. In order to minimize bias, a synergism study was conducted concurrently with the susceptibility study of *Ae. albopictus* adults against WHO diagnostic dosage of adulticides. Therefore, synergist assays using PBO were conducted in the same settings and manners as explained in 3.6.2 with the use of tubes exposed to

both PBO and respective organochlorines or pyrethroids; and tubes exposed to PBO alone. For the synergist assays with PBO, a total of 4 replicates with 25 sucrose-fed 3-5 days old adult female mosquitoes per tube were utilized for the set of PBO-exposed mosquitoes prior to exposure to respective adulticide. Similar number of replicates was also applied for the set of mosquitoes exposed to PBO only. The exposure period to PBO for all tubes was 1 hour (Ishak et al., 2015). Cumulative mortality counts were scored every minute throughout the exposure time of PBO and/or adulticides used in this study.

Upon completion of synergist assays, all mosquitoes were also held in the same settings and manners as WHO adult mosquito bioassay. Surviving adult mosquitoes were collected and kept in a freezer at -70 ºC prior to use in the mixed function oxidases (MFO) microassay.

In line with the role of PBO as an inhibitor for mixed function oxidases (MFO), survived *Ae. albopictus* adults from the synergist assays were then subjected to the mixed function oxidases (MFO) microassay to observe on any changes in the level of elevated MFO activity after the pre-exposure of PBO. The conduct of mixed function oxidases microassay for samples of these synergist assays followed similar procedures as described in 3.7.2.

3.8.5 Data Analysis for Synergism Study using Piperonyl Butoxide (PBO)

The conduct of synergism study was similar to the WHO adult mosquito bioassay. Therefore, synergism study was carried out simultaneously with the WHO adult mosquito bioassay whereby data for KT_{50} of organochlorines and pyrethroids used in this synergism study was gained from that WHO adult mosquito bioassay.

Data analysis of synergism study was nearly similar to the data analysis of the WHO adult mosquito bioassay. For synergism study, another groups of *Ae. albopictus* adults

from the same batch used in the WHO adult mosquito bioassay were exposed to both PBO alone and PBO + adulticide. Hence, results of knockdown percentages of *Ae. albopictus* adults exposed to PBO alone and PBO + adulticide for one hour that fell between 5% to 95% knockdown were then subjected to probit analysis to obtain knockdown time$_{50}$ (KT_{50}) values. Instead of resistance ratio, the synergistic effect of PBO was evaluated using the following formula:

$$\text{Synergistic Ratio (SR)} = \frac{KT_{50} \text{ of the adulticide}}{KT_{50} \text{ of the PBO + adulticide}}$$

Other than that, knockdown percentage of each *Ae. albopictus* adult population at 60 minutes of PBO + organochlorine exposure, at 30 minutes of PBO + pyrethroid exposure and mortality percentage at 24 hours post-exposure of PBO + organochlorine and PBO + pyrethroid were analysed similarly as described in 3.4.3 and 3.6.3. Results of knockdown and mortality percentages were interpreted based on the guidelines by World Health Organization (2016a) as listed in Table 3.3 in 3.4.3 as well.

Normality Test using Shapiro-Wilk test was performed to verify that the data of knockdown percentages at 60 minutes of the exposure time of PBO + organochlorine and 30 minutes of the exposure time of PBO + pyrethroid as well as mortality percentages at 24 hours post-exposure for all *Ae. albopictus* adult populations were normally distributed. Subsequently, any significant difference in KT_{50} values for *Ae. albopictus* adults exposed to adulticide alone and PBO + adulticide obtained from probit analysis was determined by conducting Independent samples t-test. Additionally, One-way ANOVA and Post Hoc Test were performed to determine any significant difference between populations from different types of area exposed to PBO + adulticide.

Meanwhile, the level of elevated mixed function oxidases (MFO) activity in PBO-exposed *Ae. albopictus* adults from different types of area was compared with the non-exposed *Ae. albopictus* adults utilized in the WHO adult bioassay whereby these samples were not exposed to any synergist or insecticide. Normality Test using Shapiro-Wilk test was conducted to verify that the data of elevated MFO activity for PBO-exposed *Ae. albopictus* adults was normally distributed. Any significant difference in the mean elevated MFO activity among PBO-exposed *Ae. albopictus* adults from all types of area was determined by performing One-way ANOVA. Post Hoc Test was then conducted to determine any significant difference in the mean elevated MFO activity between *Ae. albopictus* adult populations from agricultural and non-agricultural areas. These results were compared with results of mean elevated MFO activity of non-exposed *Ae. albopictus* adult populations that were analysed as described in 3.7.6. Any significant difference in mean elevated MFO activity between non-exposed and PBO-exposed *Ae. albopictus* adult populations was determined by conducting Independent samples t-test.

Moreover, as previously in 3.7.6, individual PBO-exposed samples from MFO enzyme microassay were also grouped into different range of elevated MFO activity and classified into different scores based on their heterogeneity in the elevated MFO activity distribution. Any significant increase of elevated MFO activity in PBO-exposed *Ae. albopictus* adults from different types of area as compared to the reference strain was also determined by conducting Independent samples t-test.

The correlation test using Pearson Correlation Test was performed to determine any correlation between KT_{50} values of PBO + organochlorine and PBO + pyrethroid *Ae. albopictus* adults, and mean elevated MFO activity in PBO-exposed *Ae. albopictus* adults. This correlation test was carried out to reveal any significant association between the reduced KT_{50} values in *Ae. populations* exposed to PBO + organochlorine and PBO

+ pyrethroid, and lower elevated mean MFO activity of the same adult populations after the PBO exposure in order to verify the efficacy of PBO as a synergist. The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant association between the use of PBO and reduced mean elevated MFO activity. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong association between the use of PBO and reduced mean elevated MFO activity.

The probit analysis to obtain knockdown time$_{50}$ (KT_{50}) of all *Ae. albopictus* adult populations exposed to PBO alone, PBO + organochlorine and PBO + pyrethroid as well as the calculation of both knockdown and mortality percentages, Normality Test, Independent samples t-test, One-way ANOVA, Post Hoc Test and Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at $P = 0.05$.

CHAPTER 4: RESULTS

4.1 Ovitrap Surveillance of *Aedes* Mosquitoes in Selected Agricultural and Non-agricultural Areas in Peninsular Malaysia

A total of 20,468 larvae were collected from ovitraps placed in all study areas which comprised *Ae. albopictus* (90.35%), *Cx. quinquefasciatus* (5.63%), *Armigeres subalbatus* (2.66%), *Ae. aegypti* (1.31%) and *Uranotaenia* sp. (0.05%) (Figure 4.1). Focusing on dengue vectors, *Aedes albopictus* was obtained at the highest number in all study areas while *Ae. aegypti* was detected only in five study areas (Figure 4.2). No *Ae. aegypti* larvae were captured from any rice cultivation areas and fogging-free residential areas. Other species of mosquito larvae were also spotted in ovitraps which consisted of *Cx. quinquefasciatus* (eight study areas), *Ar. subalbatus* (five study areas) and *Uranotaenia* sp. (one study area).

Table 4.1 shows the number of productive ovitraps, the ovitrap index (OI) and the mean OI per type of study area. The OI of all study areas ranged between 64.00% and 96.00%. Cheras dengue prone residential area (Cheras DEN) demonstrated the highest OI while the lowest OI was observed in both Padang Serai fogging-free residential area (Padang Serai FF) and Temerloh FF. Among different types of study area, the highest mean OI was noted from dengue prone residential areas (84.00 ± 6.43), followed by rubber estates (83.33 ± 1.76). The lowest mean OI was seen from the fogging-free residential areas (65.33 ± 1.33). The difference between the lowest mean OI and the highest mean OI was about 1.29 times. Results of the Normality Test showed that the data of OI were normally distributed ($P > 0.05$). From the One-way ANOVA performed, significant difference ($P \leq 0.05$) was achieved for the mean OI among five

different types of study area but no significant difference was demonstrated in the Post Hoc Tukey HSD Test between the mean OI for different types of area.

Table 4.2 illustrates the mean number of larvae per recovered ovitrap of different mosquito species captured from all study areas. The Normality Test conducted for these mean number of larvae per recovered ovitrap for all study areas showed that they were normally distributed ($P > 0.05$). The mean number of *Ae. aegypti* larvae per recovered ovitrap in five study areas ranged from 0.08 to 3.42. For *Ae. albopictus*, the fogging-free residential areas showed the lowest mean number of *Ae. albopictus* larvae per recovered ovitrap (13.68 ± 0.64) whereas, the highest mean number of *Ae. albopictus* larvae per recovered ovitrap was exhibited by rubber estates (34.87 ± 7.45). The mean number of larvae per recovered ovitrap for both *Ae. aegypti* and *Ae. albopictus* were not significant ($P > 0.05$), respectively.

Meanwhile, *Cx. quinquefasciatus* was also captured from eight study areas which consisted of each type of study area. The mean number of *Cx. quinquefasciatus* larvae per recovered ovitrap ranged from 0.40 to 11.44. The dengue prone residential areas demonstrated the lowest mean number of *Cx. quinquefasciatus* larvae per recovered ovitrap (0.40 ± 0.34) while the rice cultivation areas showed the highest mean number of *Cx. quinquefasciatus* larvae per recovered ovitrap by about 12.68-fold (5.07 ± 3.22). The mean number of *Cx. quinquefasciatus* larvae per recovered ovitrap between five types of study areas was not significant ($P > 0.05$).

Another observed larval species was *Ar. subalbatus* which was captured in ovitraps placed in all three rice cultivation areas, one rubber estate and one dengue prone residential area. The highest mean number of *Ar. subalbatus* larvae per recovered ovitrap was observed in Kota Tinggi RB (8.98 ± 4.71) while the lowest mean number of *Ar. subalbatus* larvae per recovered ovitrap was from Kuala Selangor PD (0.08 ± 0.08). The mean number of *Ar. subalbatus* larvae per recovered ovitrap between rice

cultivation areas and rubber states as well as between rice cultivation areas and dengue prone residential areas were significantly different. Moreover, larvae of *Uranotaenia* sp. were captured in ovitraps placed in Kota Tinggi RB with the mean number of larvae per recovered ovitrap of 0.22 ± 0.22.

Overall, the mean number of larvae per recovered ovitrap in all types of areas were significantly different ($P \leq 0.05$) except for oil palm plantations. However, no significant difference was demonstrated in the Post Hoc Tukey HSD Test for the mean number of larvae per recovered ovitrap between different types of area. Meanwhile, only the mean number of *Ar. subalbatus* larvae per recovered ovitrap in all types of area that was significantly different ($P \leq 0.05$). Nevertheless, a significant difference ($P \leq 0.05$) was achieved through the Post Hoc Tukey HSD Test for the mean number of larvae per recovered ovitrap between *Ae. aegypti* and *Ae. albopictus*, *Ae. albopictus* and *Cx. quinquefasciatus* as well as *Ae. albopictus* and *Ar. subalbatus*.

Table 4.3 presents the distribution of various species of mosquito larvae in productive ovitraps in all study areas. Single breeding of *Ae. albopictus* larvae was demonstrated in all study areas which ranged from 64.29% to 100.00%. Contrarily, single breeding of *Cx. quinquefasciatus* larvae was only perceived in two ovitraps deployed in Klang OP. No occurrence of *Ae. aegypti*, *Ar. subalbatus* or *Uranotaenia* sp. larvae was detected in any productive ovitraps placed in all study areas.

For mixed breeding, only two different species of mosquito larvae were detected in each ovitrap positive with co-occurrence. In total, 34 ovitraps placed in five study areas were productive with mixed infestation of *Ae. aegypti* and *Ae. albopictus* larvae. Meanwhile, 32 ovitraps deployed in eight study areas showed mixed breeding of *Ae. albopictus* and *Cx. quinquefasciatus* larvae. Co-breeding of *Ae. albopictus* with *Ar. subalbatus* larvae was also displayed in ovitraps placed in all three rice cultivation areas, one rubber estate (Kota Tinggi RB; 6 ovitraps) and one dengue prone residential

area (Kota Tinggi DEN; 1 ovitrap). Other than that, one ovitrap placed in Kuala Pilah PD was positive with mixed breeding of *Cx. quinquefasciatus* and *Ar. subalbatus* larvae while another ovitrap deployed in Kota Tinggi RB was positive with co-occurrence of *Ae. albopictus* with *Uranotaenia* sp. larvae.

Generally, mixed breeding of two species of mosquito larvae was observed in eleven study areas. The highest number of positive ovitraps with mixed breeding was exhibited in Kuala Pilah PD (35.71%) and followed by Cheras DEN (33.33%). Both Temerloh RB and Temerloh FF demonstrated the lowest number of positive ovitraps with mixed breeding, each with one ovitrap, respectively.

The abundance of different species of mosquito larvae in ovitraps positive with mixed breeding is illustrated in Table 4.4. For ovitraps positive with mixed infestation of *Ae. aegypti* and *Ae. albopictus* larvae, the abundance of the latter species was higher than the former species by 5.44 to 16.29 times. For co-occurrence of *Ae. albopictus* with *Cx. quinquefasciatus* larvae, *Ae. albopictus* was also more prevalent than *Cx. quinquefasciatus* larvae by 1.96- to 33.17-fold except in ovitraps in Klang OP and Kuala Pilah PD. The domination of *Ae. albopictus* larvae continued in the mixed infestation with *Ar. subalbatus* larvae observed in ovitraps placed in all three rice cultivation areas but not in ovitraps with co-infestation collected in Kota Tinggi RB and Kota Tinggi DEN. On the other hand, co-occurrence of *Cx. quinquefasciatus* with *Ar. subalbatus* larvae detected in an ovitrap deployed in Kuala Pilah PD was dominated by *Cx. quinquefasciatus* by 4.75-fold while *Ae. albopictus* larvae in an ovitrap shared with *Uranotaenia* sp. in Kota Tinggi RB dominated by 5.64-fold.

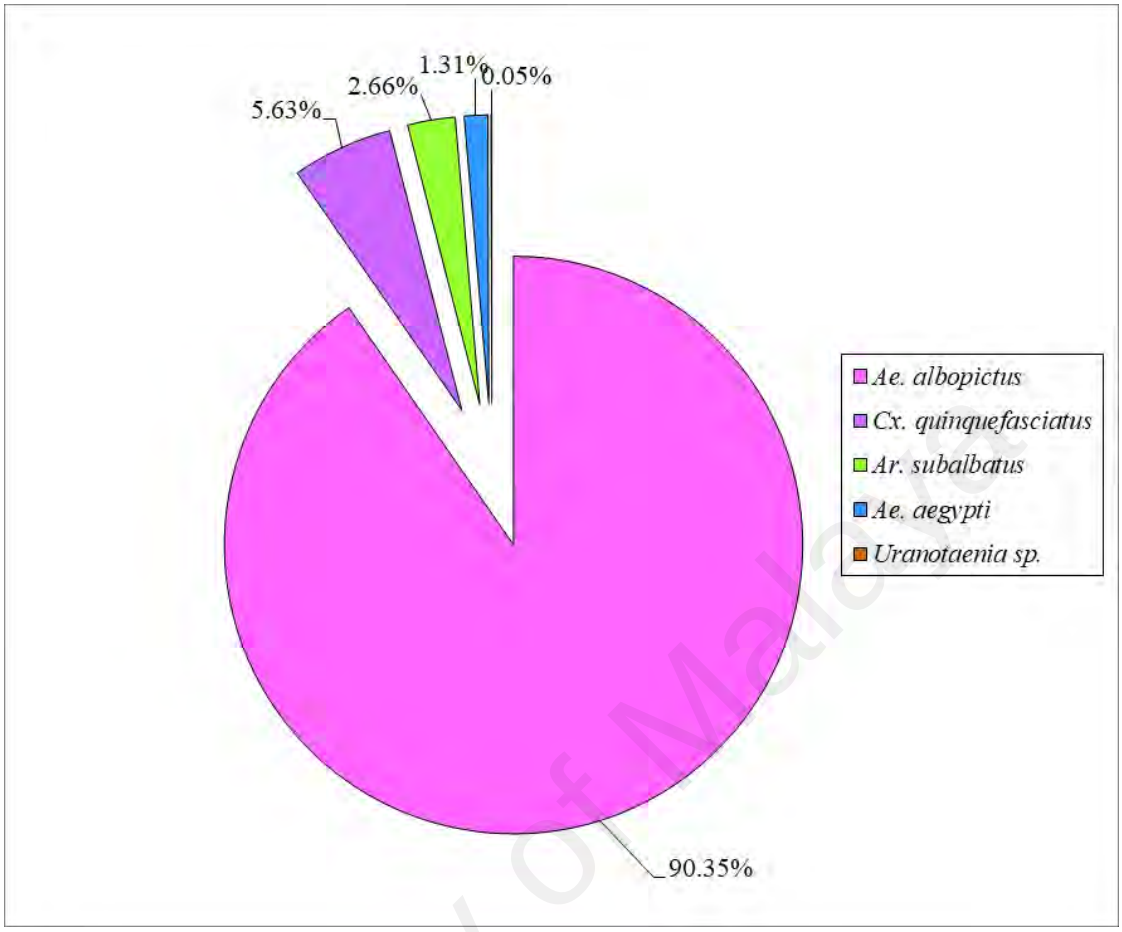

Figure 4.1: Larval prevalence of different mosquito species collected in ovitraps recovered from all study areas.

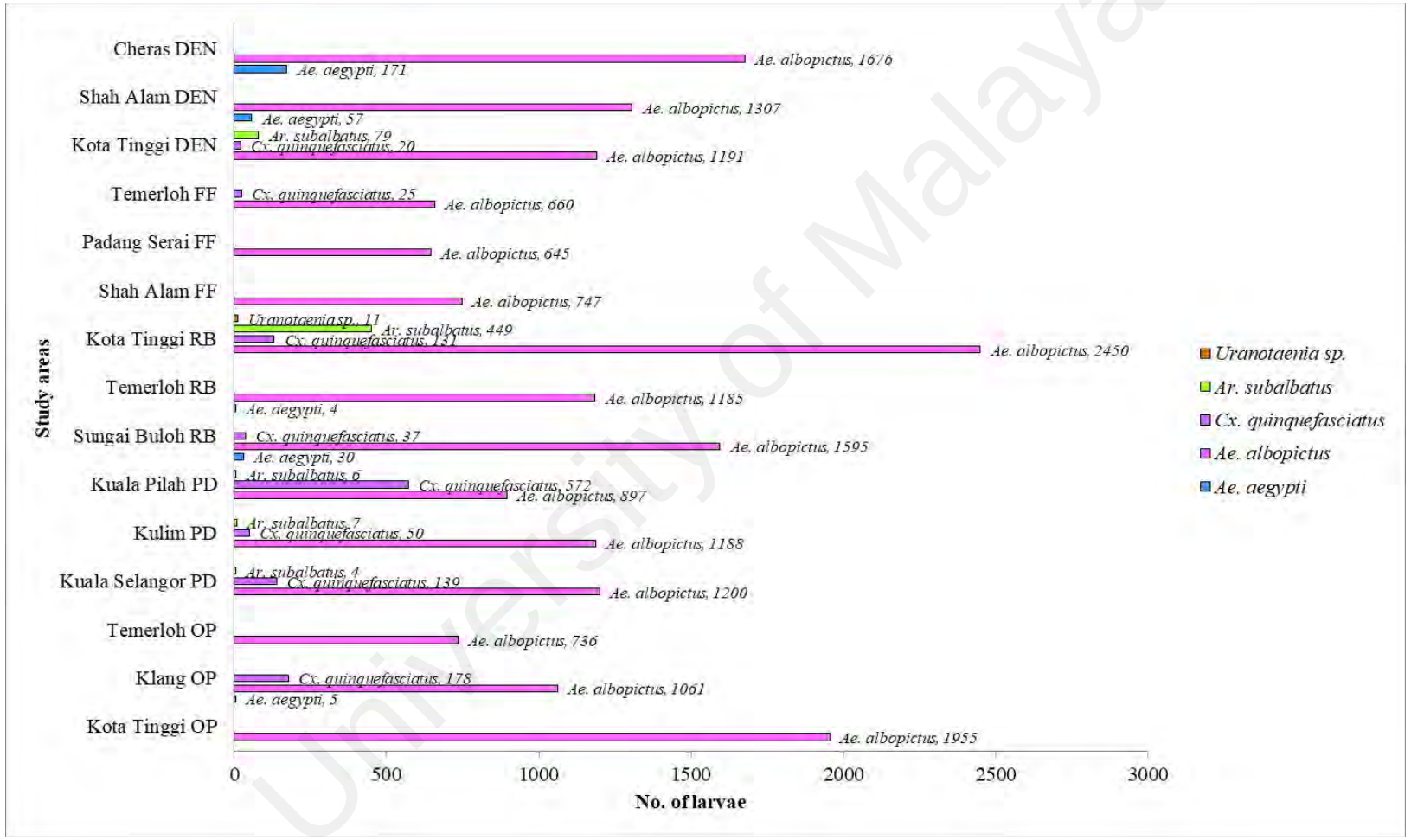

Figure 4.2: Total number of larvae collected from ovitraps recovered from each study area.

Table 4.1: Ovitrap index (OI) of all study areas and mean OI of different types of area.

Status of area	Categories of area	Types of area	Study sites	No. of placed ovitrap	No. of recovered ovitrap	No. of positive ovitrap	Ovitrap Index (OI) (%)	Mean OI ± S.E.
Fogging-free areas	Agricultural areas	Oil palm plantations	Kota Tinggi OP	50	50	40	80.00	72.00 ± 4.16
			Klang OP	50	50	35	70.00	
			Temerloh OP	50	50	33	66.00	
		Rice cultivation areas	Kuala Selangor PD	50	50	38	76.00	75.33 ± 5.21
			Kulim PD	50	50	33	66.00	
			Kuala Pilah PD	50	50	42	84.00	
		Rubber estates	Sungai Buloh RB	50	50	43	86.00	83.33 ± 1.76
			Temerloh RB	50	50	42	84.00	
			Kota Tinggi RB	50	50	40	80.00	
	Non-agricultural areas	Fogging-free residential areas	Shah Alam FF	50	50	34	68.00	65.33 ± 1.33
			Padang Serai FF	50	50	32	64.00	
			Temerloh FF	50	50	32	64.00	
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	Kota Tinggi DEN	50	50	37	74.00	84.00 ± 6.43
			Shah Alam DEN	50	50	41	82.00	
			Cheras DEN	50	50	48	96.00	
One-Way ANOVA								F = 3.419 df = 14 P = 0.050

S.E. = Standard Error

Table 4.2: Distribution of mosquito larvae in recovered ovitraps from all study areas.

Status of area	Categories of area	Types of area	Study sites	Mean number of larvae per recovered ovitrap ± S.E.										One-Way ANOVA
				Ae. aegypti		*Ae. albopictus*		*Cx. quinquefasciatus*		*Ar. subalbatus*		*Uranotaenia* sp.		
Fogging-free areas	Agricultural areas	Oil palm plantations	Kota Tinggi OP		0.10 ± 0.10	39.10 ± 6.91	25.01 ± 7.29							F = 2.04 df = 4 P = 0.329
			Klang OP	0.10 ± 0.10		21.22 ± 3.07		3.56 ± 2.48	3.56 ± 2.48					
			Temerloh OP			14.72 ± 2.24								
		Rice cultivation areas	Kuala Selangor PD			24.00 ± 2.78		2.78 ± 1.39		0.08 ± 0.08				F = 27.312 df = 8 P = 0.001
			Kulim PD			23.76 ± 4.46	21.90 ± 1.98	1.00 ± 0.90	5.07 ± 3.22	0.14 ± 0.14	0.11 ± 0.02			
			Kuala Pilah PD			17.94 ± 2.90		11.44 ± 3.87		0.12 ± 0.09				
		Rubber estates	Sungai Buloh RB	0.60 ± 0.28		31.90 ± 3.96		0.74 ± 0.74						F = 6.53 df = 8 P = 0.048
			Temerloh RB	0.08 ± 0.08	0.34 ± 0.26	23.70 ± 2.92	34.87 ± 7.45		1.68 ± 0.94		8.98 ± 4.71		0.22 ± 0.22	
			Kota Tinggi RB			49.00 ± 8.65		2.62 ± 1.43		8.98 ± 4.71		0.22 ± 0.22		
	Non-agricultural areas	Fogging-free residential areas	Shah Alam FF			14.94 ± 2.18								F = 107.38 df = 3 P = 0.009
			Padang Serai FF			12.90 ± 2.31	13.68 ± 0.64		0.50 ± 0.50					
			Temerloh FF			13.20 ± 2.06		0.50 ± 0.50						
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	Kota Tinggi DEN		2.28 ± 1.14	23.82 ± 3.27	27.83 ± 2.92	0.40 ± 0.34		1.58 ± 1.58				F = 21.86 df = 6 P = 0.015
			Shah Alam DEN	1.14 ± 0.37		26.14 ± 3.52			0.40 ± 0.34		1.58 ± 1.58			
			Cheras DEN	3.42 ± 1.21		33.52 ± 3.82								
One-Way ANOVA					F = 1.80 df = 4 P = 0.357[ab]		F = 2.49 df = 14 P = 0.110[ab]		F = 0.34 df = 7 P = 0.834[b]		F = 31828.57 df = 4 P = 0.000[b]			

S.E. = Standard Error

Significant difference (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with ***Ae. aegypti***, [b] = Significantly different with ***Ae. albopictus***, [c] = Significantly different with ***Cx. quinquefasciatus***.

Table 4.3: Distribution of single and mixed breeding of mosquito larvae in positive ovitraps recovered from study areas.

Status of area	Categories of area	Types of area	Study localities	No. of placed ovitrap	No. of recovered ovitrap	No. of positive ovitrap	No. of positive ovitrap with single species (Percentage, %)		No. of positive ovitrap with mixed infestation of 2 species (Percentage, %)					Total no. of positive ovitrap with mixed infestation of 2 species (Percentage, %)
							Ae. albopictus	*Cx. quinquefasciatus*	*Ae. aegypti* + *Ae. albopictus*	*Ae. albopictus* + *Cx. quinquefasciatus*	*Ae. albopictus* + *Ar. subalbatus*	*Cx. quinquefasciatus* + *Ar. subalbatus*	*Ae. albopictus* + *Uranotaenia* sp.	
Fogging-free areas	Agricultural areas	Oil palm plantations	Kota Tinggi OP	50	50	40	40 (100.00)							
			Klang OP	50	50	35	31 (88.56)	2 (5.72)	1 (2.86)	1 (2.86)				2 (5.72)
			Temerloh OP	50	50	33	33 (100.00)							
		Rice cultivation areas	Kuala Selangor PD	50	50	38	31 (81.58)			6 (15.79)	1 (2.63)			7 (18.42)
			Kulim PD	50	50	33	30 (90.91)			2 (6.06)	1 (3.03)			3 (9.09)
			Kuala Pilah PD	50	50	42	27 (64.29)			13 (30.95)	1 (2.38)	1 (2.38)		15 (35.71)
		Rubber estates	Sungai Buloh RB	50	50	43	36 (83.72)		6 (13.95)	1 (2.33)				7 (16.28)
			Temerloh RB	50	50	42	41 (97.62)		1 (2.38)					1 (2.38)
			Kota Tinggi RB	50	50	40	28 (70.00)			5 (12.50)	6 (15.00)		1 (2.50)	12 (30.00)
	Non-agricultural areas	Fogging-free residential areas	Shah Alam FF	50	50	34	34 (100.00)							
			Padang Serai FF	50	50	32	32 (100.00)							
			Temerloh FF	50	50	32	31 (96.88)			1 (3.12)				1 (3.12)
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	Kota Tinggi DEN	50	50	37	33 (89.19)			3 (8.11)	1 (2.70)			4 (10.81)
			Shah Alam DEN	50	50	41	31 (75.61)		10 (24.39)					10 (24.39)
			Cheras DEN	50	50	48	32 (66.67)		16 (33.33)					16 (33.33)

Table 4.4: Abundance of different species of mosquito larvae in ovitraps positive with mixed breeding.

Status of area	Categories of area	Types of area	Study localities	Ratio of mixed infestation				
				AE:AL	AL:CQ	AL:AR	CQ:AR	AL:UR
Fogging-free areas	Agricultural areas	Oil palm plantations	Klang OP	1.00 : 15.60	1.00 : 2.02			
		Rice cultivation areas	Kuala Selangor PD		1.96 : 1.00	13.00 : 1.00		
			Kulim PD		6.46 : 1.00	1.57 : 1.00		
			Kuala Pilah PD		1.00 : 4.09	21.50 : 1.00	4.75 : 1.00	
		Rubber estates	Sungai Buloh RB	1.00 : 16.29	3.32 : 1.00			
			Temerloh RB	1.00 : 10.75				
			Kota Tinggi RB		7.02 : 1.00	2.06 : 6.99		5.64 : 1.00
	Non-agricultural areas	Fogging-free residential areas	Temerloh FF		2.64 : 1.00			
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	Kota Tinggi DEN		33.17 : 1.00	1.00 : 8.78		
			Shah Alam DEN	1.00 : 5.44				
			Cheras DEN	1.00 : 8.26				

AE = *Ae. aegypti*
AL = *Ae. albopictus*
CQ = *Cx. quinquefasciatus*
AR = *Ar. subalbatus*
UR = *Uranotaenia* sp.

4.2 Susceptibility of *Aedes albopictus* Larvae against WHO Diagnostic Dosage of Larvicides

The susceptibility study of *Ae. albopictus* larvae from different types of area against eight larvicides of organochlorines (DDT; Dieldrin) and organophosphates (Fenitrothion; Fenthion; Temephos; Chlorpyrifos; Bromophos) were carried out according to WHO recommended dosages (World Health Organization, 2016a). At 24 hours post-treatment, *Ae. albopictus* larvae from all types of area including the reference strain were highly resistant to DDT, temephos, chlorpyrifos and bromophos (Table 4.5). Varied results were obtained upon the exposure to dieldrin in which *Ae. albopictus* larvae of the reference strain, oil palm plantations and fogging-free residential areas were susceptible to dieldrin while *Ae. albopictus* larvae from paddy cultivation areas and rubber estates developed tolerance to dieldrin. At the same time, *Ae. albopictus* larvae from dengue prone residential areas were resistant to dieldrin.

Moreover, a similar trend of susceptibility was observed in the selection of malathion and fenitrothion. *Aedes albopictus* larvae of both the reference strain and the oil palm plantations showed incipient resistance against malathion, whereas *Ae. albopictus* larvae of both the reference strain and fogging-free residential areas developed moderate resistance against fenitrothion. The rest of the field strains were resistant to malathion and fenitrothion, respectively. Meanwhile, only *Ae. albopictus* larvae from fogging-free residential areas were tolerance to fenthion while *Ae. albopictus* larvae from dengue prone residential areas developed high resistance against the same larvicide.

Results from the Normality Test verified that data of mortality percentage of *Ae. albopictus* larval populations from different types of area against WHO diagnostic dosages were normally distributed ($P > 0.05$). One-way ANOVA demonstrated significant differences in the susceptibility status of *Ae. albopictus* larvae from different agricultural and non-agricultural areas only in the exposure of fenthion, temephos and

bromophos ($P \leq 0.05$). Significant differences were also recorded in the mortality percentage against WHO diagnostic dosages between certain *Ae. albopictus* larval populations exposed to dieldrin, malathion, fenitrothion, fenthion and temephos ($P \leq 0.05$) through the Post Hoc Tukey HSD Test.

From the Pearson Correlation Test performed, cross resistance between intraclass larvicides was found between malathion and fenthion ($r = 0.628$, $P = 0.009$) as well as temephos and chlorpyrifos ($r = 0.622$, $P = 0.010$) (Table 4.6). Additionally, cross resistance between organochlorines and organophosphates was shown involving dieldrin with malathion ($r = 0.527$, $P = 0.036$) and fenthion ($r = 0.590$, $P = 0.016$).

Table 4.5: Percent mortality of *Aedes albopictus* larvae from different types of area against WHO diagnostic dosage (mg/L) of organochlorines and organophosphates for larval bioassay at 24 hours post-treatment.

Types of area	Insecticides / Study areas	Organochlorines		Organophosphates					
		DDT 0.012 mg/L*	**Dieldrin 0.050 mg/L**	**Malathion 0.125 mg/L***	**Fenitrothion 0.020 mg/L***	**Fenthion 0.025 mg/L***	**Temephos 0.012 mg/L**	**Chlorpyrifos 0.012 mg/L**	**Bromophos 0.050 mg/L***
Reference	Laboratory	$^{R}0.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{M}90.00 \pm 3.46$	$^{M}92.00 \pm 2.31$	$^{S}100.00 \pm 0.00$	$^{R}1.00 \pm 1.00$	$^{R}0.00 \pm 0.00$	$^{R}3.00 \pm 1.91$
Oil palm plantations	Kota Tinggi OP	$^{R}0.33 \pm 0.33$	$^{S}98.33 \pm 1.67^{a}$	$^{M}95.33 \pm 1.76^{a}$	$^{R}73.00 \pm 7.55$	$^{S}99.33 \pm 0.33^{a}$	$^{R}1.33 \pm 0.67^{a}$	$^{R}0.00 \pm 0.00$	$^{R}0.00 \pm 0.00$
	Klang OP								
	Temerloh OP								
Paddy cultivation areas	Kuala Selangor PD	$^{R}0.00 \pm 0.00$	$^{M}92.33 \pm 4.26$	$^{R}50.33 \pm 21.87$	$^{R}74.33 \pm 9.74^{b}$	$^{S}98.67 \pm 0.88^{b}$	$^{R}0.33 \pm 0.33^{b}$	$^{R}0.00 \pm 0.00$	$^{R}0.00 \pm 0.00$
	Kulim PD								
	Kuala Pilah PD								
Rubber estates	Sungai Buloh RB	$^{R}0.00 \pm 0.00$	$^{M}97.00 \pm 2.52$	$^{R}79.67 \pm 8.65^{c}$	$^{R}83.00 \pm 11.59$	$^{S}100.00 \pm 0.00^{c}$	$^{R}11.00 \pm 2.52^{abc}$	$^{R}0.33 \pm 0.33$	$^{R}0.33 \pm 0.33$
	Temerloh RB								
	Kota Tinggi RB								
Fogging-free residential areas	Shah Alam FF	$^{R}0.00 \pm 0.00$	$^{S}98.33 \pm 1.20^{d}$	$^{R}79.67 \pm 6.69^{d}$	$^{M}92.67 \pm 3.48^{b}$	$^{M}97.33 \pm 2.19^{d}$	$^{R}2.67 \pm 2.67$	$^{R}0.00 \pm 0.00$	$^{R}0.00 \pm 0.00$
	Padang Serai FF								
	Temerloh FF								
Dengue prone residential areas	Kota Tinggi DEN	$^{R}0.00 \pm 0.00$	$^{R}89.00 \pm 1.53^{ad}$	$^{R}33.67 \pm 15.19^{acd}$	$^{R}77.00 \pm 9.71$	$^{R}88.33 \pm 0.88^{abcd}$	$^{R}0.00 \pm 0.00^{c}$	$^{R}0.00 \pm 0.00$	$^{R}0.00 \pm 0.00$
	Shah Alam DEN								
	Cheras DEN								
One way ANOVA		F = 0.812 df = 15 P = 0.567	F = 2.489 df = 15 P = 0.103	F = 3.205 df = 15 P = 0.055	F = 0.776 df = 15 P = 0.589	F = 14.807 df = 15 P = 0.000	F = 6.022 df = 15 P = 0.008	F = 0.812 df = 15 P = 0.567	F = 25.000 df = 15 P = 0.000

* WHO diagnostic dosages (mg/L) for *Aedes aegypti*

Percent mortality after 24 h (%) = Mean of mortality for larvae + Standard Error (S.E.)

S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).

Percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b] = Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.

Table 4.6: Cross resistance between larvicides based on the correlation of percent mortality of *Aedes albopictus* larvae from different types of area between organochlorines and organophosphates utilized in WHO larval bioassay using WHO diagnostic dosages (mg/L) at 24 hours post-treatment.

Insecticides		Organochlorines		Organophosphates				
		DDT 0.012 mg/L	Dieldrin 0.050 mg/L	Malathion 0.125 mg/L	Fenitrothion 0.020 mg/L	Fenthion 0.025 mg/L	Temephos 0.012 mg/L	Chlorpyrifos 0.012 mg/L
Organochlorines	Dieldrin 0.050 mg/L	r = -0.016 P = 0.954						
Organophosphates	Malathion 0.125 mg/L	r = 0.207 P = 0.441	r = 0.527 P = 0.036					
	Fenitrothion 0.020 mg/L	r = 0.131 P = 0.629	r = 0.258 P = 0.335	r = 0.369 P = 0.160				
	Fenthion 0.025 mg/L	r = 0.118 P = 0.663	r = 0.590 P = 0.016	r = 0.628 P = 0.009	r = 0.176 P = 0.514			
	Temephos 0.012 mg/L	r = -0.165 P = 0.541	r = 0.126 P = 0.642	r = 0.296 P = 0.265	r = 0.251 P = 0.348	r = 0.396 P = 0.129		
	Chlorpyrifos 0.012 mg/L	r = -0.067 P = 0.806	r = -0.167 P = 0.535	r = 0.207 P = 0.441	r = 0.294 P = 0.270	r = 0.175 P = 0.516	r = 0.622 P = 0.010	
	Bromophos 0.050 mg/L	r = -0.086 P = 0.751	r = 0.175 P = 0.516	r = 0.250 P = 0.350	r = 0.292 P = 0.273	r = 0.227 P = 0.399	r = 0.095 P = 0.725	r = 0.258 P = 0.334

Cross resistance between two larvicides (Pearson Correlation Test) based on the correlation of percent mortality at 24 hours post-treatment for two tested larvicides: r > 0.4 = Correlated (Two tested larvicides showed cross resistance between one another); r > 0.8 = Highly correlated (Two tested larvicides showed strong cross resistance between one another).

$P \leq 0.05$ = Significant

4.3 Susceptibility of *Aedes albopictus* Field Strains Larvae against Independent Diagnostic Dosage of Larvicides Established from *Aedes albopictus* Reference Strain Larvae

Since only recommended diagnostic dosages of organochlorines and organophosphates as larvicides were listed by World Health Organization, an attempt has been performed to determine the diagnostic dosages of larvicides covering all main insecticide classes namely organochlorines (DDT; Dieldrin), organophosphates (Malathion; Fenitrothion; Fenthion; Temephos; Chlorpyrifos; Bromphos), carbamates (Propoxur; Bendiocarb) and pyrethroids (Permethrin; Deltamethrin; Lambdacyhalothrin; Cyfluthrin; Etofenprox). For this purpose, an establishment of independent diagnostic dosages of these larvicides needs to be conducted on *Ae. albopictus* reference strain larvae so that the double LC_{99} values of these larvicides could be used as the discriminated dosages to determine the susceptibility status of *Ae. albopictus* larvae from field strains against these larvicides as defined by WHO.

Hence, larvae of *Ae. albopictus* reference strain were exposed to wide range of concentrations for each larvicide which caused mortality between 5% to 95% at 24 hours post-treatment. The LC_{50} and LC_{99} values were generated from the regression lines of probit analysis constructed from the results of mortality percentages of *Ae. albopictus* reference strain at 24 hours post-exposure to each larvicide. These regression lines generated are listed in Table 4.7. All LC_{99} values obtained were then been doubled up in which these values were then used for all strains of *Ae. albopictus* larvae. Diverse range of independent diagnostic dosage ($2xLC_{99}$) values were obtained for these larvicides. In comparison between the $2xLC_{99}$ values calculated with the WHO recommended dosages for organochlorines and organophosphates, the $2xLC_{99}$ values generated were much higher than the WHO recommended dosages except for the fenthion.

Table 4.7: Lethal concentration values at 50% (LC_{50}) and 99% (LC_{99}) for *Aedes albopictus* reference strain and independent diagnostic dosage ($2xLC_{99}$) values calculated as compared to WHO diagnostic dosages.

Classes of Insecticides	Insecticides	LC_{50} (mg/L) 95% C.L.	LC_{99} (mg/L) 95% C.L.	Regression Line	Independent diagnostic dosage, $2xLC_{99}$ (mg/L)	WHO diagnostic dosage (mg/L)
Organochlorines	DDT	0.2160 (0.2090-0.2240)	0.4190 (0.3760-0.4910)	Y = 8.066x+5.370	0.8384	0.012
	Dieldrin	0.0820 (0.0790-0.0850)	0.1730 (0.1530-0.2070)	Y = 7.204x+7.816	0.3460	0.050
Organophosphates	Malathion	0.1610 (0.1390-0.1850)	2.5170 (1.5970-4.8550)	Y = 1.947x+1.546	5.0340	0.125
	Fenitrothion	0.0180 (0.0180-0.0190)	0.0270 (0.0250-0.0290)	Y = 13.786x+23.973	0.0540	0.020
	Fenthion	0.0050 (0.0050-0.0060)	0.0090 (0.0090-0.0110)	Y = 9.768x+22.141	0.0180	0.025
	Temephos	0.0180 (0.0180-0.0190)	0.0330 (0.0300-0.0380)	Y = 9.031x+15.721	0.0660	0.012
	Chlorpyrifos	0.0040 (0.0040-0.0050)	0.0080 (0.0070-0.0090)	Y = 8.925x+20.987	0.0160	0.012
	Bromophos	0.0510 (0.0490-0.0530)	0.1170 (0.1020-0.1420)	Y = 6.475x+8.370	0.2340	0.050
Carbamates	Propoxur	1.2590 (1.0780-1.4600)	2.4400 (1.8530-8.6600)	Y = 8.097x-0.811	4.8800	-
	Bendiocarb	0.7580 (0.6410-0.9070)	2.0380 (1.3980-7.2940)	Y = 5.413x+0.652	4.0760	-
Pyrethroids	Permethrin	0.0200 (0.0200-0.0210)	0.0290 (0.0280-0.0320)	Y = 14.461x+24.487	0.0580	-
	Deltamethrin	0.0090 (0.0080-0.0090)	0.0230 (0.0200-0.0290)	Y = 5.635x+11.570	0.0460	-
	Lambdacyhalothrin	0.0100 (0.0080-0.0130)	0.0440 (0.0250-0.2440)	Y = 3.675x+7.325	0.0880	-
	Cyfluthrin	0.0120 (0.0110-0.0120)	0.0370 (0.0310-0.0490)	Y = 4.595x+8.899	0.0740	-
	Etofenprox	0.0290 (0.0270-0.0300)	0.0760 (0.0650-0.0960)	Y = 5.484x+8.467	0.1520	-

C.L. = Confidence Limit

Regression Line generated from probit analysis using the mortality percentages of *Ae. albopictus* reference strain at 24 hours post-exposure.

The independent diagnostic dosage of all classes of larvicides established from the calculation of 2xLC_{99} values of the reference strain of *Ae. albopictus* were then applied in the WHO larval bioassays involving all *Ae. albopictus* larval populations. *Aedes albopictus* larvae from all different types of area were found to be susceptible against both DDT and dieldrin except for *Ae. albopictus* larvae from dengue prone residential areas which developed incipient resistance against DDT (Table 4.8).

For organophosphates, *Ae. albopictus* larvae from all types of area were susceptible to both malathion and bromophos. The susceptibility against fenitrothion was also displayed in *Ae. albopictus* larvae from most types of area except for *Ae. albopictus* larvae from rubber estates and dengue prone residential areas that were moderately and highly resistant to fenitrothion, respectively. As for fenthion, *Ae. albopictus* larvae from oil palm plantations and rubber estates were susceptible against this larvicide, but incipient resistance was detected in *Ae. albopictus* larvae from paddy cultivation areas and fogging-free residential areas while high resistance was demonstrated in *Ae. albopictus* larvae of dengue prone residential areas. Subsequently, incipient resistance against temephos was exhibited in *Ae. albopictus* larvae from oil palm plantations, paddy cultivation areas and rubber estates, whereas *Ae. albopictus* larvae from both fogging-free residential areas and dengue prone residential areas were highly resistant to temephos. Furthermore, only *Ae. albopictus* larvae from oil palm plantations were moderately resistant to chlorpyrifos while the rest of the populations developed high resistance against the same larvicide.

In addition, mixed level of resistance was observed in *Ae. albopictus* larvae from different types of area against propoxur (Table 4.9). *Aedes albopictus* larvae from both oil palm plantations and dengue prone residential areas were the least susceptible and highly resistant against propoxur, respectively, while the rest of *Ae. albopictus* populations were moderately resistant to the same larvicide. In contrast, only *Ae.*

albopictus larvae from oil palm plantations were susceptible to bendiocarb while other populations of *Ae. albopictus* larvae were highly resistant to bendiocarb. As for pyrethroids, incipient resistance was detected only in *Ae. albopictus* larvae from rubber estates and dengue prone residential areas against permethrin while susceptible status was achieved for the rest of the populations against all pyrethroids tested.

Results obtained from the Normality Test validated that data of mortality percentage of *Ae. albopictus* larval populations from different types of area against independent diagnostic dosages were normally distributed ($P > 0.05$). In terms of differences in the mortality percentages at 24 hours post-treatment of each larvicide between all *Ae. albopictus* larval field populations, One-way ANOVA revealed that significant differences were demonstrated in the selection of malathion, fenitrothion, fenthion, bromophos, propoxur, bendiocarb, permethrin, lambdacyhalothrin, cyfluthrin and etofenprox ($P \leq 0.05$). However, the Post Hoc Tukey HSD Test showed significant differences in the susceptibility status of *Ae. albopictus* larvae collected from agricultural and non-agricultural areas only for DDT, fenitrothion, fenthion, temephos, chlorpyrifos, carbamates, bendiocarb and permethrin exposures ($P \leq 0.05$).

The correlation analysis using the Pearson Correlation Test was performed to determine any cross resistance between two tested larvicides using the percent mortality of *Ae. albopictus* larvae at independent diagnostic dosages ($2xLC_{99}$). Cross resistance between intraclass larvicides was demonstrated in organochlorines, organophosphates and carbamates (Table 4.10). Cross resistance was detected between DDT and dieldrin for organochlorines ($r = 0.514$, $P = 0.042$). Cross resistance within organophosphates was also exhibited among fenitrothion with fenthion ($r = 0.756$, $P = 0.001$) and temephos ($r = 0.646$, $P = 0.007$); fenthion with temephos ($r = 0.770$, $P = 0.000$) and chlorpyrifos ($r = 0.589$, $P = 0.016$); as well as temephos with chlorpyrifos ($r = 0.589$, $P = 0.016$). In carbamates, cross resistance was also displayed between propoxur and

bendiocarb (r = 0.789, P = 0.000). Cross resistance among larvicides of pyrethroids was either not achieved or not able to be determined due to complete mortalities observed at 24 hours post-treatment.

Cross resistance between interclass larvicides was also exhibited among DDT with permethrin (r = 0.615, P = 0.011) and deltamethrin (r = 0.641, P = 0.007) as well as dieldrin with deltamethrin (r = 0.554, P = 0.026). Cross resistance was also displayed among fenitrothion with propoxur (r = 0.720, P = 0.002), bendiocarb (r = 0.654, P = 0.006) and permethrin (r = 0.818, P = 0.000) as well as fenthion with propoxur (r = 0.928, P = 0.000), bendiocarb (r = 0.719, P = 0.002) and permethrin (r = 0.713, P = 0.002). Moreover, temephos was cross resistant with propoxur (r = 0.835, P = 0.000), bendiocarb (r = 0.723, P = 0.002) and permethrin (r = 0.609, P = 0.012). Meanwhile, chlorpyrifos was cross resistant with propoxur (r = 0.649, P = 0.007) and bendiocarb (r = 0.661, P = 0.005). Cross resistance was also demonstrated between propoxur and permethrin (r = 0.667, P = 0.005) as well as between bendiocarb and permethrin (r = 0.504, P = 0.047).

Table 4.8: Percent mortality of *Aedes albopictus* larvae from different types of area against independent diagnostic dosage of larvicides ($2xLC_{99}$) for organochlorines and organophosphates larval bioassay at 24 hours post-treatment.

Types of area	Insecticides / Study areas	Organochlorines		Organophosphates					
		DDT 0.8384 mg/L	**Dieldrin 0.3460 mg/L**	**Malathion 5.0340 mg/L**	**Fenitrothion 0.0540 mg/L**	**Fenthion 0.0180 mg/L**	**Temephos 0.0660 mg/L**	**Chlorpyrifos 0.0160 mg/L**	**Bromophos 0.2340 mg/L**
Reference	Laboratory	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
Oil palm plantations	Kota Tinggi OP	$^{S}98.67 \pm 0.88$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}98.00 \pm 1.53^{a}$	$^{S}98.67 \pm 0.67^{a}$	$^{M}96.33 \pm 3.67$	$^{M}95.67 \pm 4.33^{a}$	$^{S}100.00 \pm 0.00$
	Klang OP								
	Temerloh OP								
Paddy cultivation areas	Kuala Selangor PD	$^{S}98.33 \pm 0.33^{b}$	$^{S}99.33 \pm 0.67$	$^{S}100.00 \pm 0.00$	$^{S}98.67 \pm 0.88^{b}$	$^{M}94.33 \pm 3.48^{b}$	$^{M}97.67 \pm 1.86^{b}$	$^{R}74.67 \pm 10.74$	$^{S}100.00 \pm 0.00$
	Kulim PD								
	Kuala Pilah PD								
Rubber estates	Sungai Buloh RB	$^{S}98.67 \pm 0.88$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{M}96.67 \pm 0.88^{c}$	$^{S}99.00 \pm 0.00^{c}$	$^{M}95.00 \pm 2.08^{c}$	$^{R}84.00 \pm 8.08$	$^{S}100.00 \pm 0.00$
	Temerloh RB								
	Kota Tinggi RB								
Fogging-free residential areas	Shah Alam FF	$^{S}100.00 \pm 0.00^{b}$	$^{S}99.00 \pm 1.00$	$^{S}100.00 \pm 0.00$	$^{S}99.33 \pm 0.33^{cd}$	$^{M}94.00 \pm 3.06^{cd}$	$^{R}89.33 \pm 5.49$	$^{R}83.33 \pm 4.37^{d}$	$^{S}100.00 \pm 0.00$
	Padang Serai FF								
	Temerloh FF								
Dengue prone residential areas	Kota Tinggi DEN	$^{M}97.33 \pm 1.76$	$^{S}98.67 \pm 1.33$	$^{S}100.00 \pm 0.00$	$^{R}88.67 \pm 2.40^{abcd}$	$^{R}83.33 \pm 1.76^{abcd}$	$^{R}84.00 \pm 2.52^{bc}$	$^{R}63.00 \pm 11.00^{ad}$	$^{S}100.00 \pm 0.00$
	Shah Alam DEN								
	Cheras DEN								
One way ANOVA		F = 0.891 df = 15 P = 0.522	F = 0.476 df = 15 P = 0.786	F = 0.000 df = 15 P = 0.000	F = 8.227 df = 15 P = 0.003	F = 6.893 df = 15 P = 0.005	F = 2.560 df = 15 P = 0.097	F = 2.100 df = 15 P = 0.149	F = 0.000 df = 15 P = 0.000

Percent mortality after 24 h (%) = Mean of mortality for larvae + Standard Error (S.E.)

S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).

Percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b] = Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.

Table 4.9: Percent mortality of *Aedes albopictus* larvae from different types of area against independent diagnostic dosage of larvicides ($2xLC_{99}$) for carbamates and pyrethroids larval bioassay at 24 hours post-treatment.

Types of area	Insecticides / Study areas	Carbamates		Pyrethroids				
		Propoxur 4.8800 mg/L	**Bendiocarb 4.0760 mg/L**	**Permethrin 0.0580 mg/L**	**Deltamethrin 0.0460 mg/L**	**Lambdacyhalothrin 0.0880 mg/L**	**Cyfluthrin 0.0740 mg/L**	**Etofenprox 0.1520 mg/L**
Reference	Laboratory	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
Oil palm plantations	Kota Tinggi OP	$^{S}99.67 \pm 0.33^{a}$	$^{S}99.33 \pm 0.67^{a}$	$^{S}99.67 \pm 0.33^{a}$	$^{S}99.33 \pm 0.67$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
	Klang OP							
	Temerloh OP							
Paddy cultivation areas	Kuala Selangor PD	$^{M}92.67 \pm 5.33^{a}$	$^{R}84.67 \pm 2.85^{a}$	$^{S}100.00 \pm 0.00^{b}$	$^{S}99.67 \pm 0.33$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
	Kulim PD							
	Kuala Pilah PD							
Rubber estates	Sungai Buloh RB	$^{M}95.67 \pm 2.33^{c}$	$^{R}89.67 \pm 7.84$	$^{M}97.00 \pm 1.73$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
	Temerloh RB							
	Kota Tinggi RB							
Fogging-free residential areas	Shah Alam FF	$^{M}92.33 \pm 5.36$	$^{R}83.00 \pm 8.19$	$^{S}99.33 \pm 0.67^{d}$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
	Padang Serai FF							
	Temerloh FF							
Dengue prone residential areas	Kota Tinggi DEN	$^{R}79.67 \pm 2.73^{ac}$	$^{R}65.67 \pm 7.06^{a}$	$^{M}91.00 \pm 2.52^{abd}$	$^{S}98.33 \pm 1.20$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$	$^{S}100.00 \pm 0.00$
	Shah Alam DEN							
	Cheras DEN							
One way ANOVA		F = 3.487 df = 15 P = 0.044	F = 3.636 df = 15 P = 0.039	F = 5.953 df = 15 P = 0.008	F = 1.000 df = 15 P = 0.465	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent mortality after 24 h (%) = Mean of mortality for larvae + Standard Error (S.E.)

S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).

Percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b] = Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.

Table 4.10: Correlation of percent mortality of *Aedes albopictus* larvae at 24 hours post-treatment of independent diagnostic dosage of larvicides ($2xLC_{99}$).

Insecticides		OC		OP						CARB		PY			
		DDT 0.8384 mg/L	Dieldrin 0.3460 mg/L	Malathion 5.0340 mg/L	Fenitrothion 0.0540 mg/L	Fenthion 0.0180 mg/L	Temephos 0.0660 mg/L	Chlorpyrifos 0.0160 mg/L	Bromophos 0.2340 mg/L	Propoxur 4.8800 mg/L	Bendiocarb 4.0760 mg/L	Permethrin 0.0580 mg/L	Deltamethrin 0.0460 mg/L	Lambdacyhalothrin 0.0880 mg/L	Cyfluthrin 0.0740 mg/L
OC	Dieldrin 0.3460 mg/L	r = 0.514 P = 0.042													
OP	Malathion 5.0340 mg/L	N.D.	N.D.												
	Fenitrothion 0.0540 mg/L	r = 0.291 P = 0.273	r = 0.006 P = 0.983	N.D.											
	Fenthion 0.0180 mg/L	r = 0.368 P = 0.160	r = 0.271 P = 0.310	N.D.	r = 0.756 P = 0.001										
	Temephos 0.0660 mg/L	r = 0.065 P = 0.812	r = 0.112 P = 0.679	N.D.	r = 0.646 P = 0.007	r = 0.770 P = 0.000									
	Chlorpyrifos 0.0160 mg/L	r = 0.192 P = 0.476	r = 0.273 P = 0.306	N.D.	r = 0.437 P = 0.091	r = 0.589 P = 0.016	r = 0.589 P = 0.016								
	Bromophos 0.2340 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.							
CARB	Propoxur 4.8800 mg/L	r = 0.240 P = 0.371	r = 0.069 P = 0.799	N.D.	r = 0.720 P = 0.002	r = 0.928 P = 0.000	r = 0.835 P = 0.000	r = 0.649 P = 0.007	N.D.						
	Bendiocarb 4.0760 mg/L	r = -0.002 P = 0.993	r = 0.156 P = 0.564	N.D.	r = 0.654 P = 0.006	r = 0.719 P = 0.002	r = 0.723 P = 0.002	r = 0.661 P = 0.005	N.D.	r = 0.789 P = 0.000					
PY	Permethrin 0.0580 mg/L	r = 0.615 P = 0.011	r = 0.294 P = 0.270	N.D.	r = 0.818 P = 0.000	r = 0.713 P = 0.002	r = 0.609 P = 0.012	r = 0.395 P = 0.129	N.D.	r = 0.667 P = 0.005	r = 0.504 P = 0.047				
	Deltamethrin 0.0460 mg/L	r = 0.641 P = 0.007	r = 0.554 P = 0.026	N.D.	r = 0.367 P = 0.162	r = 0.438 P = 0.090	r = 0.281 P = 0.292	r = 0.230 P = 0.392	N.D.	r = 0.291 P = 0.274	r = 0.124 P = 0.648	r = 0.468 P = 0.068			
	Lambdacyhalothrin 0.0880 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	Cyfluthrin 0.0740 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
	Etofenprox 0.1520 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Cross resistance between two larvicides (Pearson Correlation Test) based on the correlation of percent mortality at 24 hours post-treatment for two tested larvicides: r > 0.4 = Correlated (Two tested larvicides showed cross resistance between one another); r > 0.8 = Highly correlated (Two tested larvicides showed strong cross resistance between one another).

N.D. = Not Determined due to 100% mortality achieved for either one of the insecticide tested for correlation.

OC = Organochlorines; OP = Organophosphates; CARB = Carbamates; PY = Pyrethroids.

$P \leq 0.05$ = Significant

4.4 Susceptibility of *Aedes albopictus* Adults against WHO Diagnostic Dosage of Adulticides

The susceptibility status of *Ae. albopictus* adults from different types of area against selected organochlorines, organophosphates and carbamates is illustrated in Table 4.11. The 50% knockdown time (KT_{50}) recorded for *Ae. albopictus* adults exposed to DDT 4% ranged from 52.73 to 74.40 minutes. *Ae. albopictus* adults from rubber estates were the most susceptible to DDT 4% (KT_{50} = 52.73 ± 5.49 min) while *Ae. albopictus* adults collected from fogging-free residential areas were the least susceptible to DDT 4% (KT_{50} = 74.40 ± 4.10 min). The resistance ratios of *Ae. albopictus* adults from different types of area that were exposed to DDT 4% were less than 1.50 fold. For exposure to malathion 5%, the KT_{50} values demonstrated by different types of area were between 39.62 minutes and 68.58 minutes. The resistance ratios of these adult mosquitoes against malathion 5% were between 1.27 fold and 1.73 fold. On the other hand, less than 70 minutes of KT_{50} values were presented in *Ae. albopictus* adults of all types of area upon exposure to propoxur 0.1%. *Aedes albopictus* adults from oil palm plantations were the most susceptible to propoxur 0.1% (KT_{50} = 34.14 ± 1.92) while *Ae. albopictus* adults from dengue prone residential areas were the least susceptible (KT_{50} = 66.03 ± 10.97) against the same insecticide. The resistance ratios of *Ae. albopictus* adults from different types of area exposed to propoxur 0.1% ranged from 0.98 to 1.90 fold. As for the selection pressure of bendiocarb 0.1%, the KT_{50} values obtained were as low as 35.76 minutes in reference strain up to 93.38 ± 17.91 minutes in *Ae. albopictus* adults of dengue prone residential areas. The KT_{50} values for selection pressure of dieldrin 4% and fenitrothion 1% were not available due to no knockdown being observed in *Ae. albopictus* adults from all types of area throughout the exposure time.

Meanwhile, the susceptibility status of *Ae. albopictus* adults from different types of area against pyrethroids which include permethrin 0.75%, deltamethrin 0.05%,

lambdacyhalothrin 0.05%, cyfluthrin 0.15% and etofenprox 0.5% were displayed in Table 4.12. The KT_{50} values of *Ae. albopictus* adults from different types of area that were exposed to permethrin 0.75% ranged from 21.40 to 26.20 minutes with their resistance ratios between 0.82 fold to 1.00 fold. For the selection pressure of deltamethrin 0.05%, the lowest KT_{50} value was demonstrated by *Ae. albopictus* adults of rubber estates (KT_{50} = 21.38 ± 1.30 min) while the highest KT_{50} value was recorded from *Ae. albopictus* adults originating from fogging-free residential areas (KT_{50} = 30.22 ± 1.37). The resistance ratios of *Ae. albopictus* adults exposed to deltamethrin 0.05% were between 0.82 fold and 1.16 fold. Following the exposure of lambdacyhalothrin 0.05%, *Ae. albopictus* adults from oil palm plantations took the least time to achieve 50% knockdown (KT_{50} = 27.00 ± 0.56 min) while *Ae. albopictus* adults collected from fogging-free residential areas required the longest time to reach 50% knockdown (KT_{50} = 36.56 ± 1.28 min). The resistance ratios calculated for *Ae. albopictus* adults from different types of area upon exposure to lambdacyhalothrin 0.05% ranged from 0.88 to 1.22 fold. Furthermore, as low as 19.59 ± 0.78 minutes of KT_{50} value to as high as 26.41 ± 2.62 minutes of KT_{50} value with resistance ratios that ranged between 0.85 and 1.15 were demonstrated in *Ae. albopictus* adults from different types of area upon the selection of cyfluthrin 0.15%. As for the selection pressure of etofenprox 0.5%, the lowest KT_{50} value was observed in reference strain (KT_{50} = 25.53 min) while the longest time to obtain 50% knockdown was 36.46 ± 2.99 minutes which was in *Ae. albopictus* adults from dengue prone residential areas. The resistance ratios obtained in *Ae. albopictus* adults from different types of area upon the exposure to etofenprox 0.5% ranged from 1.03 to 1.43 fold.

In addition, the percent knockdown of *Ae. albopictus* adults from all types of area was also calculated at 60 minutes of the exposure time for organochlorines, organophosphates and carbamates as well as at 30 minutes of the exposure period for

pyrethroids. The percent knockdown of *Ae. albopictus* adults exposed to organochlorines, organophosphates and carbamates at 60 minutes of the exposure time is shown in Table 4.13. At an hour of DDT 4% exposure time, between 27.67% and 63.67% of knocked down *Ae. albopictus* adults from different types of area was exhibited. Nevertheless, the range of the percent knockdown for the same mosquito populations at 60 minutes of malathion 5% exposure time was much higher starting from 37.33% up to 85.00%. As for carbamates, after 60 minutes of the selection with propoxur 0.1% and bendiocarb 0.1%, the percent knockdown of *Ae. albopictus* adults from various types of area ranged from 41.67% to 97.00% and from 27.00% to 96.00%, respectively. No knockdown of *Ae. albopictus* adults was observed from any populations tested upon 60 minutes exposure of dieldrin 4%. Similar scenario was demonstrated in the exposure to fenitrothion 1% except that only the reference strain displayed 1.00% knockdown of the mosquitoes at 60 minutes of exposure time.

The cumulative mortality percentage at 24 hours after the selection pressure of insecticides were calculated in order to determine the susceptibility status of *Ae. albopictus* adults from different types of area against insecticides tested. The reference strain remained as susceptible against all organochlorines, organophosphates, carbamates and pyrethroids tested at 24 hours post-treatment (Table 4.13). For organochlorines, the percent mortality of *Ae. albopictus* adults from different types of area at 24 hours post-treatment using DDT 4% and dieldrin 4% ranged from 73.33% to 100.00% and from 95.33% to 100.00%, respectively. Only *Ae. albopictus* adults from rubber estates showed moderate resistance to DDT 4% while *Ae. albopictus* adults from other types of area were resistant to DDT 4%. No resistance to dieldrin 4% was detected in any populations of *Ae. albopictus* adults. However, *Ae. albopictus* adults from paddy cultivation areas, rubber estates and dengue prone residential areas had developed incipient resistance against dieldrin 4%.

Furthermore, between 82.00% and 100.00% as well as between 62.00% and 100.00% of mortality was demonstrated in *Ae. albopictus* adults from various types of area exposed to malathion 5% and fenitrothion 1%, respectively, at 24 hours post-treatment. Only *Ae. albopictus* adults from dengue prone residential areas were resistant to malathion 5% while the rest fell under the moderate resistance category. Nevertheless, *Ae. albopictus* adults from all types of area were resistant to fenitrothion 1%. As for carbamates, the percent mortality of *Ae. albopictus* adults from various types of area at 24 hours after the exposure to propoxur 0.1% and bendiocarb 0.1% ranged from 48.33% to 100.00% and from 32.67% to 100.00%, respectively. *Aedes albopictus* adults from fogging-free residential areas were moderately resistant to propoxur 0.1% while *Ae. albopictus* adults from paddy cultivation areas, rubber estates and dengue prone residential areas were classified as resistant to propoxur 0.1%. *Aedes albopictus* adults from different types of area also developed resistance against bendiocarb 0.1% except for *Ae. albopictus* adults from oil palm plantations that were classified as moderately resistant to bendiocarb 0.1%.

The percent knockdown of *Ae. albopictus* adults at 30 minutes of the pyrethroids exposure time is displayed in Table 4.14. At 30 minutes of the selection to permethrin 0.75%, 68.33% to 84.33% of *Ae. albopictus* adults from various types of area were knocked down. The knockdown of *Ae. albopictus* adults was also exhibited at 30 minutes of the exposure to deltamethrin 0.05% starting from 50.33% to 88.33%. A much lower range of percent knockdown among *Ae. albopictus* adults was recorded at 30 minutes of exposure to lambdacyhalothrin 0.05% which covered from 27.00% to 69.00%. In contrast, higher percentages of knockdown were presented in *Ae. albopictus* adults from different types of area at 30 minutes of selection to cyfluthrin 0.15% which ranged from 67.67% to 92.33%. On the other hand, at 30 minutes of exposure time to etofenprox 0.5%, between 30.67% and 70.00% of *Ae. albopictus* adults from various

types of area were knocked down. Contrarily, complete mortalities were achieved for all populations of *Ae. albopictus* adults exposed to all pyrethroids at 24 hours post-treatment (Table 4.14).

Results acquired from Normality Test confirmed that data of both knockdown and mortality percentages of *Ae. albopictus* adult populations from different types of area exposed to eleven adulticides were normally distributed ($P > 0.05$). One-way ANOVA showed significant difference ($P \leq 0.05$) only for the knockdown percentage of all *Ae. albopictus* adult field populations at 30 minutes of the exposure time for deltamethrin as well as the mortality percentage at 24 hours post-treatment for fenitrothion, propoxur and all pyrethroids. However, in comparison between *Ae. albopictus* adults from agricultural areas and non-agricultural areas with or without the history of insecticide exposure, significant differences were displayed through Post Hoc Tukey HSD Test in the knockdown percentage of malathion, propoxur and bendiocarb exposures as well as the mortality percentage of DDT, fenitrothion, propoxur and bendiocarb exposures.

Any correlation of KT_{50} values of *Ae. albopictus* adults from different types of area between two different adulticides were also analysed using Pearson Correlation Test in order to ascertain any cross resistance presented between these insecticides as portrayed in Table 4.15. Interclass cross resistance between organophosphate and carbamate particularly malathion 5% with bendiocarb 0.1% ($r = 0.497$, $P = 0.050$) was discovered. The interclass cross resistance between carbamates which include both propoxur 0.1% and bendiocarb 0.1% with all pyrethroids used in the study were also detected. Furthermore, it is also noteworthy that the intraclass cross resistance among adulticides namely between propoxur 0.1% and bendiocarb 0.1% as well as among all pyrethroids was presented in *Ae. albopictus* adults from various types of area tested in the study.

Table 4.11: Knockdown time values at 50% (KT_{50}) and resistance ratio (RR) of *Aedes albopictus* adults from different types of area exposed to organochlorines, organophosphates and carbamates.

		Organochlorines				**Organophosphates**				**Carbamates**			
		DDT 4%		**Dieldrin 4%**		**Malathion 5%**		**Fenitrothion 1%**		**Propoxur 0.1%**		**Bendiocarb 0.1%**	
Types of area	**Study areas**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**
Reference	Laboratory	55.29 (54.11-56.68)	-	N.D.	N.D.	39.62 (38.87-40.38)	-	N.D.	N.D.	34.65 (34.10-35.19)	-	35.76 (35.28-36.24)	-
Oil palm plantations	Kota Tinggi OP	66.93 ± 4.44 (61.28-75.68)	1.21 ± 0.08	N.D.	N.D.	54.36 ± 3.48 (48.89-60.82	1.37 ± 0.09	N.D.	N.D.	34.14 ± 1.92 (31.34-37.83)	0.98 ± 0.06	35.90 ± 2.15 (31.71-38.81)	1.01 ± 0.06
	Klang OP												
	Temerloh OP												
Paddy cultivation areas	Kuala Selangor PD	67.50 ± 6.48 (55.65-77.97)	1.22 ± 0.12	N.D.	N.D.	68.58 ± 8.23 (52.16-77.66)	1.73 ± 0.21	N.D.	N.D.	43.11 ± 8.36 (26.61-53.69)	1.25 ± 0.24	55.49 ± 12.78 (30.17-71.20)	1.55 ± 0.36
	Kulim PD												
	Kuala Pilah PD												
Rubber estates	Sungai Buloh RB	52.73 ± 5.49 (42.97-61.95)	0.95 ± 0.10	N.D.	N.D.	50.48 ± 4.87 (41.57-58.35)	1.27 ± 0.12	N.D.	N.D.	42.42 ± 7.87 (31.13-57.56)	1.23 ± 0.23	54.54 ± 15.62 (34.95-85.41)	1.53 ± 0.44
	Temerloh RB												
	Kota Tinggi RB												
Fogging-free residential areas	Shah Alam FF	74.40 ± 4.10 (70.03-82.59)	1.35 ± 0.07	N.D.	N.D.	52.53 ± 0.54 (51.84-53.60)	1.33 ± 0.01	N.D.	N.D.	51.51 ± 2.22 (49.26-55.96)	1.49 ± 0.07	58.52 ± 6.01 (51.00-70.40)	1.64 ± 0.17
	Padang Serai FF												
	Temerloh FF												
Dengue prone residential areas	Kota Tinggi DEN	68.24 ± 4.03 (62.06-75.81)	1.23 ± 0.07	N.D.	N.D.	61.71 ± 0.83 (60.67-63.35)	1.56 ± 0.02	N.D.	N.D.	66.03 ± 10.97 (44.10-77.43)	1.90 ± 0.32	93.38 ± 17.91 (60.14-121.55)	2.61 ± 0.50
	Shah Alam DEN												
	Cheras DEN												

C.L. = Confidence Limit
Resistance Ratio (RR) = KT_{50} of the field population / KT_{50} of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
N.D. = Not Determined due to no knockdown.

Table 4.12: Knockdown time values at 50% (KT_{50}) and resistance ratio (RR) of *Aedes albopictus* adults from different types of area exposed to five pyrethroids.

		Permethrin 0.75%		**Deltamethrin 0.05%**		**Lambdacyhalothrin 0.05%**		**Cyfluthrin 0.15%**		**Etofenprox 0.5%**	
Types of area	**Study areas**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**	**KT_{50} (min) 95% C.L.**	**Resistance Ratio (RR)**
Reference	Laboratory	26.20 (25.54-26.81)	-	26.06 (25.58-26.52)	-	29.94 (29.54-30.33)	-	23.08 (22.69-23.48)	-	25.53 (25.04-26.01)	-
Oil palm plantations	Kota Tinggi OP	24.40 ± 0.98 (23.11-26.33)	0.93 ± 0.04	24.11 ± 1.17 (21.79-25.54)	0.93 ± 0.04	27.00 ± 0.56 (26.10-28.04)	0.90 ± 0.02	20.78 ± 0.49 (20.24-21.76)	0.90 ± 0.02	30.94 ± 0.94 (29.76-32.79)	1.21 ± 0.03
	Klang OP										
	Temerloh OP										
Paddy cultivation areas	Kuala Selangor PD	24.29 ± 3.69 (18.56-31.19)	0.93 ± 0.14	23.85 ± 3.94 (17.53-31.09)	0.91 ± 0.15	28.97 ± 4.50 (20.76-36.28)	0.97 ± 0.15	21.88 ± 3.32 (17.63-28.43)	0.95 ± 0.14	26.31 ± 1.69 (23.56-29.38)	1.03 ± 0.07
	Kulim PD										
	Kuala Pilah PD										
Rubber estates	Sungai Buloh RB	21.40 ± 1.52 (18.94-24.17)	0.82 ± 0.06	21.38 ± 1.30 (19.03-23.53	0.82 ± 0.05	26.42 ± 0.89 (25.32-28.19)	0.88 ± 0.03	19.59 ± 0.78 (18.06-20.58)	0.85 ± 0.03	29.92 ± 4.29 (21.75-36.26)	1.17 ± 0.17
	Temerloh RB										
	Kota Tinggi RB										
Fogging-free residential areas	Shah Alam FF	25.93 ± 0.65 (24.63-26.66)	0.99 ± 0.03	30.22 ± 1.37 (27.65-32.33)	1.16 ± 0.05	36.56 ± 1.28 (34.27-38.68)	1.22 ± 0.04	26.41 ± 2.62 (21.38-30.19)	1.15 ± 0.11	31.81 ± 1.69 (28.61-34.34)	1.25 ± 0.07
	Padang Serai FF										
	Temerloh FF										
Dengue prone residential areas	Kota Tinggi DEN	26.11 ± 3.10 (20.11-30.47)	1.00 ± 0.12	28.15 ± 1.95 (24.26-30.35)	1.08 ± 0.07	34.17 ± 2.28 (29.62-36.76)	1.14 ± 0.08	25.33 ± 1.54 (22.36-27.54)	1.10 ± 0.07	36.46 ± 2.99 (30.92-41.16)	1.43 ± 0.12
	Shah Alam DEN										
	Cheras DEN										

C.L. = Confidence Limit
Resistance Ratio (RR) = KT_{50} of the field population / KT_{50} of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
N.D. = Not Determined due to no knockdown.

Table 4.13: Percent knockdown at 60 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against organochlorines, organophosphates and carbamates.

Types of area	Insecticides / Study areas	Percent knockdown at 60 minutes of the exposure time (%)						Percent mortality after 24 h (%)					
		Organochlorines		Organophosphates		Carbamates		Organochlorines		Organophosphates		Carbamates	
		DDT 4%	Dieldrin 4%	Malathion 5%	Fenitrothion 1%	Propoxur 0.1%	Bendiocarb 0.1%	DDT 4%	Dieldrin 4%	Malathion 5%	Fenitrothion 1%	Propoxur 0.1%	Bendiocarb 0.1%
Reference	Laboratory	^{R}58.00 ± 8.87	N.D.	^{R}85.00 ± 1.91	^{R}1.00 ± 1.00	^{M}97.00 ± 3.00	^{M}96.00 ± 2.83	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP Klang OP Temerloh OP	^{R}33.33 ± 7.75^{a}	N.D.	^{R}65.00 ± 9.61	N.D.	^{M}96.67 ± 2.03^{a}	^{M}93.33 ± 2.85^{a}	^{R}75.33 ± 12.81	^{S}100.00 ± 0.00	^{M}97.00 ± 3.00	^{R}85.67 ± 1.76^{a}	^{S}98.33 ± 0.88^{a}	^{M}94.33 ± 2.85^{a}
Paddy cultivation areas	Kuala Selangor PD Kulim PD Kuala Pilah PD	^{R}45.00 ± 11.27	N.D.	^{R}37.33 ± 13.86	N.D.	^{R}78.33 ± 10.93	^{R}57.33 ± 19.85	^{R}82.00 ± 4.04^{b}	^{M}96.33 ± 3.67	^{M}90.33 ± 2.40	^{R}84.00 ± 1.15^{b}	^{R}88.67 ± 5.78^{b}	^{R}66.00 ± 15.95
Rubber estates	Sungai Buloh RB Temerloh RB Kota Tinggi RB	^{R}63.67 ± 10.11^{ac}	N.D.	^{R}70.00 ± 10.44^{c}	N.D.	^{R}80.67 ± 13.38	^{R}69.67 ± 20.93	^{M}93.33 ± 4.81	^{M}97.67 ± 2.33	^{M}93.67 ± 4.37	^{R}80.33 ± 1.76^{c}	^{R}89.67 ± 4.48^{ac}	^{R}74.67 ± 17.03
Fogging-free residential areas	Shah Alam FF Padang Serai FF Temerloh FF	^{R}27.67 ± 2.85^{c}	N.D.	^{R}66.67 ± 1.86^{d}	N.D.	^{R}72.33 ± 6.12^{a}	^{R}52.67 ± 11.35^{a}	^{R}73.33 ± 0.88^{b}	^{S}100.00 ± 0.00	^{M}94.67 ± 0.33^{d}	^{R}74.33 ± 0.88^{abcd}	^{M}91.33 ± 0.33^{ad}	^{R}68.33 ± 0.88^{ad}
Dengue prone residential areas	Kota Tinggi DEN Shah Alam DEN Cheras DEN	^{R}32.33 ± 6.84	N.D.	^{R}43.00 ± 1.73^{cd}	N.D.	^{R}41.67 ± 15.67^{a}	^{R}27.00 ± 10.15^{a}	^{R}82.00 ± 9.07	^{M}95.33 ± 4.67	^{R}82.00 ± 5.51^{d}	^{R}62.00 ± 1.15^{abcd}	^{R}48.33 ± 13.98^{abcd}	^{R}32.67 ± 14.71^{ad}
One way ANOVA		F = 2.721 df = 15 P = 0.084	N.D.	F = 2.908 df = 15 P = 0.071	N.D.	F = 3.059 df = 15 P = 0.062	F = 2.574 df = 15 P = 0.095	F = 1.239 df = 15 P = 0.360	F = 0.475 df = 15 P = 0.787	F = 2.471 df = 15 P = 0.105	F = 54.670 df = 15 P = 0.000	F = 6.683 df = 15 P = 0.006	F = 3.018 df = 15 P = 0.065

Percent knockdown at 60 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.
N.D. = Not Determined due to no knockdown.

Table 4.14: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against pyrethroids.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure times (%)					Percent mortality after 24 h (%)				
		Permethrin 0.75%	Deltamethrin 0.05%	Lambdacyhalothrin 0.05%	Cyfluthrin 0.15%	Etofenprox 0.5%	Permethrin 0.75%	Deltamethrin 0.05%	Lambdacyhalothrin 0.05%	Cyfluthrin 0.15%	Etofenprox 0.5%
Reference	Laboratory	^{R}76.00 ± 6.73	^{R}71.00 ± 15.95	^{R}49.00 ± 17.92	^{M}90.00 ± 7.57	^{R}70.00 ± 4.16	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP Klang OP Temerloh OP	^{R}81.67 ± 0.33^{a}	^{R}85.00 ± 2.31^{a}	^{R}66.33 ± 3.38^{a}	^{M}90.00 ± 3.61^{a}	^{R}48.00 ± 7.37	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Paddy cultivation areas	Kuala Selangor PD Kulim PD Kuala Pilah PD	^{R}75.67 ± 17.25	^{R}81.00 ± 13.05	^{R}57.33 ± 19.20	^{R}82.33 ± 14.31	^{R}64.33 ± 8.21^{b}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Rubber estates	Sungai Buloh RB Temerloh RB Kota Tinggi RB	^{R}84.33 ± 4.70	^{R}88.33 ± 5.17^{c}	^{R}69.00 ± 5.51^{c}	^{M}92.33 ± 1.86^{c}	^{R}53.67 ± 14.84	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Fogging-free residential areas	Shah Alam FF Padang Serai FF Temerloh FF	^{R}68.33 ± 4.10^{a}	^{R}50.33 ± 12.42^{ac}	^{R}27.00 ± 5.13^{ac}	^{R}69.00 ± 8.19^{c}	^{R}41.67 ± 3.76	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Dengue prone residential areas	Kota Tinggi DEN Shah Alam DEN Cheras DEN	^{R}70.00 ± 10.02	^{R}58.67 ± 5.17^{ac}	^{R}34.00 ± 7.77^{ac}	^{R}67.67 ± 4.18^{ac}	^{R}30.67 ± 7.13^{b}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
One way ANOVA		F = 0.450 df = 15 P = 0.804	F = 4.142 df = 15 P = 0.027	F = 2.946 df = 15 P = 0.069	F = 1.832 df = 15 P = 0.194	F = 1.954 df = 15 P = 0.172	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.15: Correlation of KT_{50} values of *Aedes albopictus* between insecticides used in WHO bioassay for adult mosquitoes.

Insecticides		OC		OP		CARB		PY			
		DDT 4%	Dieldrin 4%	Malathion 5%	Fenitrothion 1%	Propoxur 0.1%	Bendiocarb 0.1%	Permethrin 0.75%	Deltamethrin 0.05%	Lambdacyhalothrin 0.05%	Cyfluthrin 0.15%
OC	Dieldrin 4%	N.D.									
OP	Malathion 5%	r = 0.074 P = 0.785	N.D.								
	Fenitrothion 1%	N.D.	N.D.	N.D.							
CARB	Propoxur 0.1%	r = 0.031 P = 0.910	N.D.	r = 0.446 P = 0.083	N.D.						
	Bendiocarb 0.1%	r = -0.012 P = 0.966	N.D.	r = 0.497 P = 0.050	N.D.	r = 0.963 P = 0.000					
PY	Permethrin 0.75%	r = 0.170 P = 0.530	N.D.	r = 0.320 P = 0.227	N.D.	r = 0.607 P = 0.013	r = 0.528 P = 0.035				
	Deltamethrin 0.05%	r = 0.319 P = 0.229	N.D.	r = 0.220 P = 0.412	N.D.	r = 0.633 P = 0.009	r = 0.535 P = 0.033	r = 0.847 P = 0.000			
	Lambdacyhalothrin 0.05%	r = 0.225 P = 0.403	N.D.	r = 0.306 P = 0.250	N.D.	r = 0.747 P = 0.001	r = 0.646 P = 0.007	r = 0.783 P = 0.000	r = 0.959 P = 0.000		
	Cyfluthrin 0.15%	r = 0.215 P = 0.423	N.D.	r = 0.219 P = 0.415	N.D.	r = 0.607 P = 0.013	r = 0.543 P = 0.030	r = 0.742 P = 0.001	r = 0.937 P = 0.000	r = 0.918 P = 0.000	
	Etofenprox 0.5%	r = 0.184 P = 0.496	N.D.	r = 0.141 P = 0.602	N.D.	r = 0.706 P = 0.002	r = 0.682 P = 0.004	r = 0.585 P = 0.017	r = 0.620 P = 0.010	r = 0.570 P = 0.021	r = 0.584 P = 0.017

Cross resistance between two adulticides (Pearson Correlation Test) based on the correlation of KT_{50} values for two tested adulticides: r > 0.4 = Correlated (Two tested adulticides showed cross resistance between one another); r > 0.8 = Highly correlated (Two tested adulticides showed strong cross resistance between one another).
N.D. = Not Determined due to no knockdown throughout the exposure period.
OC = Organochlorines; OP = Organophosphates; CARB = Carbamates; PY = Pyrethroids.
$P \leq 0.05$ = Significant

4.5 Characterization of Biochemical Enzyme Mechanisms Contributing to Insecticide Resistance in *Aedes albopictus* Larvae and Adults

Enzyme microassays of non-specific esterases (EST), mixed function oxidases (MFO), glutathione-S-transferases (GST) and insensitive acetylcholinesterase were conducted in order to reveal the underlying mechanisms of the metabolic resistance detected in *Ae. albopictus* larvae and adults derived from diverse types of area selected for this study.

4.5.1 Non-specific Esterases (EST) Enzyme Microassay

Results of Normality Test validated that data of mean elevated non-specific esterases (EST) activities was normally distributed ($P > 0.05$). One-way ANOVA showed that there was no significant difference for both elevated α-EST and β-EST activities among all *Ae. albopictus* larval populations. In comparison with the reference strain, a significant increase of α-EST activity was demonstrated only in *Ae. albopictus* larval populations from non-agricultural areas covering both fogging-free residential areas and dengue prone residential areas ($P \leq 0.05$) (Table 4.16). Concurrently, there was no significant rise of β-EST activity in any *Ae. albopictus* field populations of larvae as compared to the reference strain. No significant difference was demonstrated via Post Hoc Test for elevated α-EST and β-EST activities between *Ae. albopictus* larval populations from different types of area. Resistance ratio (RR) calculated for each field strain of *Ae. albopictus* larvae that was based on their α-EST and β-EST activities were all below 1.50 fold.

On the contrary, significant difference was displayed through One-way ANOVA for elevated β-EST activity among all *Ae. albopictus* adult populations ($P \leq 0.05$). Post Hoc Test showed significant difference for elevated α-EST and β-EST activities between certain *Ae. albopictus* adult populations from agricultural areas and non-agricultural

areas. Significant elevated α-EST activity was exhibited in *Ae. albopictus* adults from all types of area as compared to elevated α-EST activity of the reference strain (Table 4.17). Similar scenario was observed for elevated β-EST activity in adults of *Ae. albopictus* except for the population from the oil palm plantations. Resistance ratios recorded for each field population of *Ae. albopictus* adults corresponding to their α-EST and β-EST activities fell in the range of 1.00 until 3.00 fold.

For both α-EST and β-EST activities, *Ae. albopictus* larvae and adults from all types of area were categorized based on either low [≤ 30 nmoles α-naphthol(or β-naphthol)/min/mg protein], moderate (31-70 nmoles α-naphthol(or β-naphthol)/min/mg protein) or high (≥ 71 nmoles α-naphthol(or β-naphthol)/min/mg protein) activity. *Ae. albopictus* larvae from all types of area possessed either low or moderate activity of α-EST but all of them showed low activity of β-EST (Table 4.18, Figure 4.3 and Figure 4.4). At adult stage, almost all *Ae. albopictus* samples demonstrated low activity of α-EST and β-EST (Table 4.19, Figure 4.5 and Figure 4.6).

Table 4.16: Mean (± S.E.) values of non-specific α-esterases (α-EST) and β-esterases (β-EST) activities of *Aedes albopictus* larvae from different types of area at absorbance 450 nm.

Types of area	Study areas	Mean ± S.E. (nmoles α-naphthol/min/mg protein)	Resistance Ratio (RR)	Mean ± S.E. (nmoles β-naphthol/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	18.8639 ± 1.10	-	11.2378 ± 0.91	-
Oil palm plantations	Kota Tinggi OP	14.8198 ± 5.14	0.79	8.9400 ± 1.76	0.80
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	19.7218 ± 5.63	1.05	12.3058 ± 1.44	1.10
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	20.7581 ± 2.50	1.10	10.5679 ± 2.34	0.94
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	*24.6858 ± 7.23	1.31	11.8815 ± 1.96	1.06
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	*22.2183 ± 4.51	1.18	10.4662 ± 1.03	0.93
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 0.396 df = 15 P = 0.841		F = 0.457 df = 15 P = 0.799	

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean non-specific esterases of the field population / Mean non-specific esterases of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean non-specific esterases followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean non-specific esterases of the field population was significantly different with mean non-specific esterases of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.17: Mean (± S.E.) values of non-specific α-esterases (α-EST) and β-esterases (β-EST) activities of *Aedes albopictus* adults from different types of area at absorbance 450 nm.

Types of area	Study areas	Mean ± S.E. (nmoles α-naphthol/min/mg protein)	Resistance Ratio (RR)	Mean ± S.E. (nmoles β-naphthol/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	4.5825 ± 0.26	-	3.7149 ± 0.17	-
Oil palm plantations	Kota Tinggi OP	*5.8624 ± 1.47[a]	1.28	3.8500 ± 0.61[a]	1.04
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	*12.8889 ± 3.58	2.81	*6.2815 ± 0.34[ab]	1.69
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	*13.7865 ± 2.18[a]	3.01	*5.8590 ± 1.45	1.58
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	*13.9118 ± 3.01[a]	3.04	*8.8438 ± 0.60[ab]	2.38
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	*8.7189 ± 1.75	1.90	*7.4739 ± 1.06[a]	2.01
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 2.023 df = 15 P = 0.161		F = 4.001 df = 15 P = 0.030	

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean non-specific esterases of the field population / Mean non-specific esterases of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean non-specific esterases followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean non-specific esterases of the field population was significantly different with mean non-specific esterases of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.18: The distribution frequency of elevated enzyme activities in *Aedes albopictus* larvae from different types of area.

Types of area	Study areas	Frequency (%) population														
		α-esterases			β-esterases			Mixed function oxidases			Glutathione-S-transferases			Acetylcholinesterase		
		Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)
Reference	Laboratory	100.00	0.00	0.00	100.00	0.00	0.00	62.50	37.50	0.00	100.00	0.00	0.00	0.00	0.00	100.00
Oil palm plantations	Kota Tinggi OP	97.22	2.78	0.00	100.00	0.00	0.00	54.17	45.83	0.00	100.00	0.00	0.00	0.00	1.39	98.61
	Klang OP															
	Temerloh OP															
Paddy cultivation areas	Kuala Selangor PD	81.94	18.06	0.00	100.00	0.00	0.00	73.61	26.39	0.00	65.28	34.72	0.00	1.39	2.78	95.83
	Kulim PD															
	Kuala Pilah PD															
Rubber estates	Sungai Buloh RB	95.83	4.17	0.00	100.00	0.00	0.00	75.00	25.00	0.00	61.11	38.89	0.00	1.39	9.72	88.89
	Temerloh RB															
	Kota Tinggi RB															
Fogging-free residential areas	Shah Alam FF	70.83	29.17	0.00	100.00	0.00	0.00	84.72	15.28	0.00	76.39	23.61	0.00	0.00	9.72	90.28
	Padang Serai FF															
	Temerloh FF															
Dengue prone residential areas	Kota Tinggi DEN	80.56	19.44	0.00	100.00	0.00	0.00	68.06	31.94	0.00	91.67	8.33	0.00	1.39	4.17	94.44
	Shah Alam DEN															
	Cheras DEN															

α-EST = Low (+) = ≤ 30, Moderate (++) = 31-70, High (+++) = ≥ 71
β-EST = Low (+) = ≤ 30, Moderate (++) = 31-70, High (+++) = ≥ 71
MFO = Low (+) = ≤ 0.3000, Moderate (++) = 0.3001-0.7000, High (+++) = ≥ 0.7001
GST = Low (+) = ≤ 0.0030, Moderate (++) = 0.0031-0.0070, High (+++) = ≥ 0.0071
AChE = Low (+) (homozygous susceptible, SS) = ≤ 30, Moderate (++) (heterozygous, RS) = 31-70, High (+++) (homozygous resistance, RR) = ≥ 70 (World Health Organization, 1998)

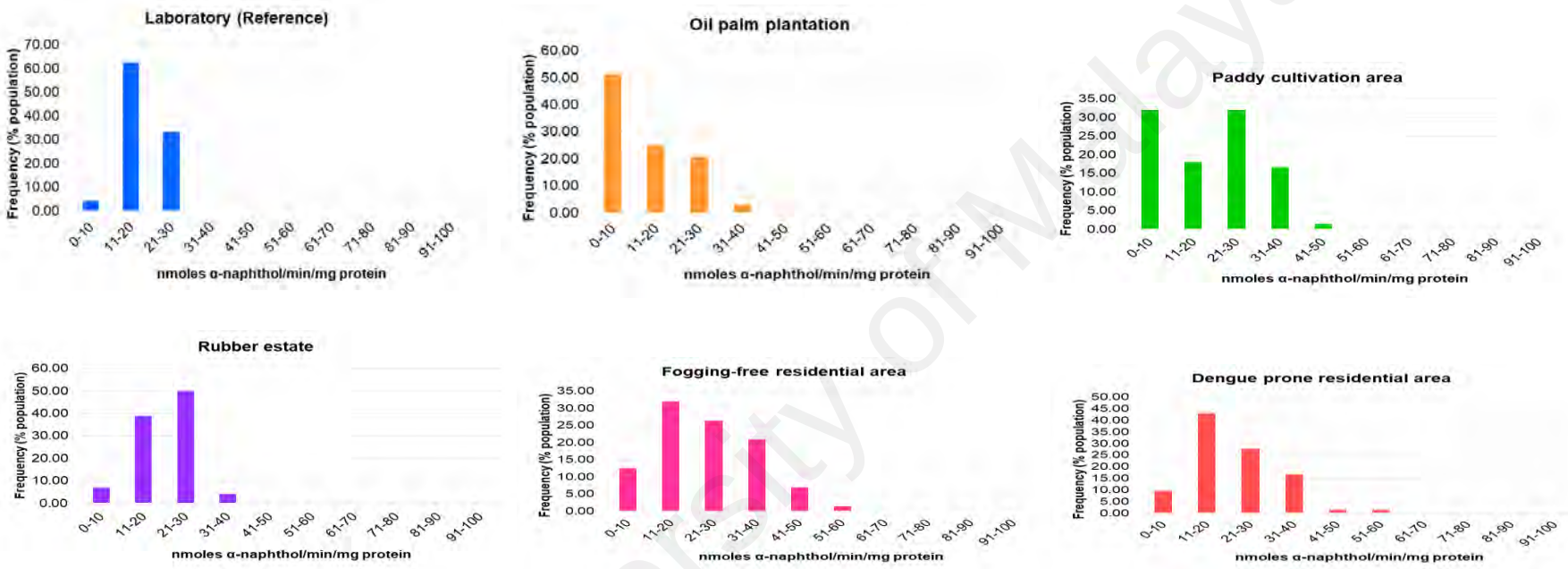

Figure 4.3: Non-specific α-esterases (α-EST) activity in *Aedes albopictus* larvae from different types of area.

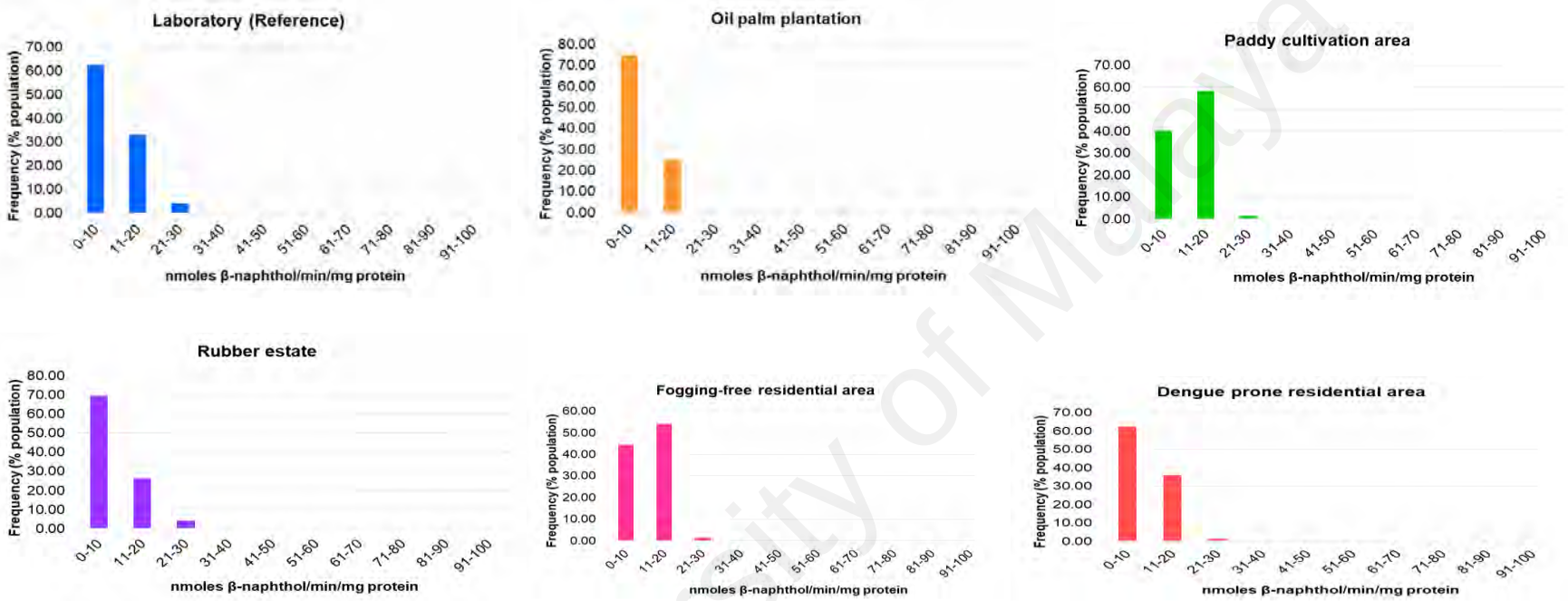

Figure 4.4: Non-specific β-esterases (β-EST) activity in *Aedes albopictus* larvae from different types of area.

Table 4.19: The distribution frequency of elevated enzyme activities in *Aedes albopictus* adults from different types of area.

Types of area	Study areas	Frequency (%) population														
		α-esterases			β-esterases			Mixed function oxidases			Glutathione-S-transferases			Acetylcholinesterase		
		Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)
Reference	Laboratory	100.00	0.00	0.00	100.00	0.00	0.00	4.17	25.00	70.83	100.00	0.00	0.00	4.17	95.83	0.00
Oil palm plantations	Kota Tinggi OP	100.00	0.00	0.00	100.00	0.00	0.00	30.56	48.61	20.83	100.00	0.00	0.00	0.00	94.44	5.56
	Klang OP															
	Temerloh OP															
Paddy cultivation areas	Kuala Selangor PD	97.22	2.78	0.00	100.00	0.00	0.00	8.33	43.06	48.61	50.00	50.00	0.00	5.56	90.28	4.17
	Kulim PD															
	Kuala Pilah PD															
Rubber estates	Sungai Buloh RB	95.83	4.17	0.00	100.00	0.00	0.00	13.89	61.11	25.00	100.00	0.00	0.00	1.39	94.44	4.17
	Temerloh RB															
	Kota Tinggi RB															
Fogging-free residential areas	Shah Alam FF	100.00	0.00	0.00	100.00	0.00	0.00	26.39	20.83	52.78	100.00	0.00	0.00	0.00	87.50	12.50
	Padang Serai FF															
	Temerloh FF															
Dengue prone residential areas	Kota Tinggi DEN	100.00	0.00	0.00	100.00	0.00	0.00	43.06	36.11	20.83	100.00	0.00	0.00	0.00	94.44	5.56
	Shah Alam DEN															
	Cheras DEN															

α-EST = Low (+) = ≤ 30, Moderate (++) = 31-70, High (+++) = ≥ 71
β-EST = Low (+) = ≤ 30, Moderate (++) = 31-70, High (+++) = ≥ 71
MFO = Low (+) = ≤ 0.3000, Moderate (++) = 0.3001-0.7000, High (+++) = ≥ 0.7001
GST = Low (+) = ≤ 0.0030, Moderate (++) = 0.0031-0.0070, High (+++) = ≥ 0.0071
AChE = Low (+) (homozygous susceptible, SS) = ≤ 30, Moderate (++) (heterozygous, RS) = 31-70, High (+++) (homozygous resistance, RR) = ≥ 70 (World Health Organization, 1998)

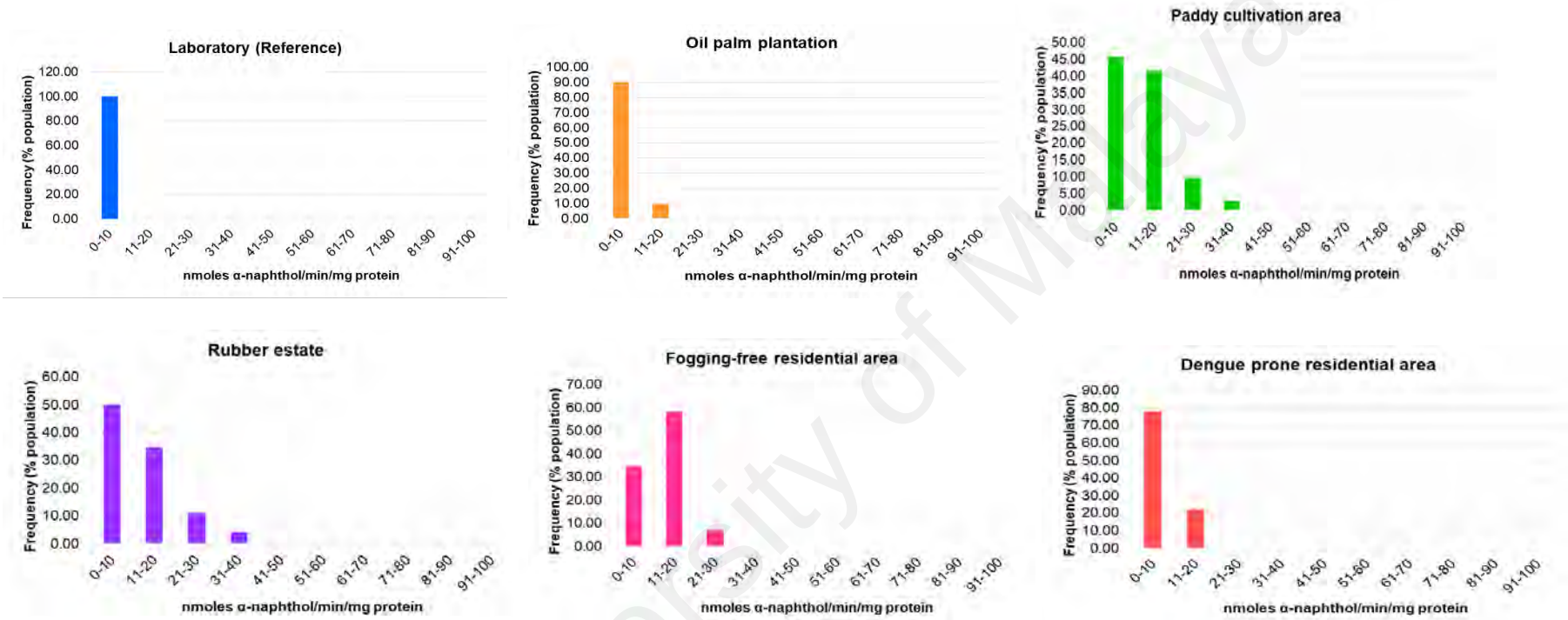

Figure 4.5: Non-specific α-esterases (α-EST) activity in *Aedes albopictus* adults from different types of area.

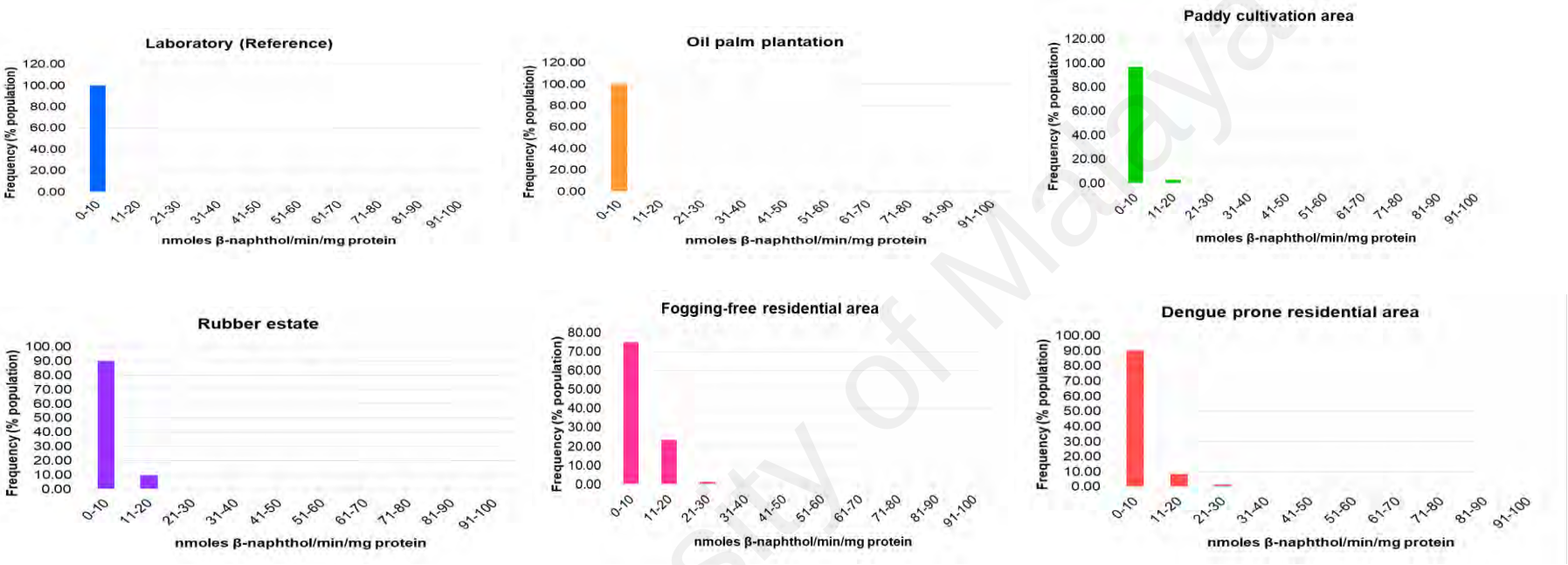

Figure 4.6: Non-specific β-esterases (β-EST) activity in *Aedes albopictus* adults from different types of area.

4.5.2 Mixed Function Oxidases (MFO) Enzyme Microassay

Normality Test conducted verified that data of mean elevated mixed function oxidases (MFO) activity was normally distributed ($P > 0.05$). One-way ANOVA revealed that there was no significant difference of elevated MFO activity among all larval and adult populations of *Ae. albopictus*. A significant difference in elevated MFO activity was observed via Post Hoc Test involving only *Ae. albopictus* larvae from oil palm plantations with fogging-free residential areas ($P \leq 0.05$). No significant increase of elevated MFO activity was displayed either at larval stage or adult stage of *Ae. albopictus* field populations as compared to the reference strain (Table 4.20 and Table 4.21). Resistance ratios (RR) calculated for all larvae and adults of *Ae. albopictus* from different types of area concerning to their MFO activities were all below 1.00 fold. All larvae and adults of *Ae. albopictus* subjected to MFO microassay were also grouped into either low (≤ 0.3000 nmoles cytochrome c/min/mg protein), moderate (0.3001-0.7000 nmoles cytochrome c/min/mg protein) or high (≥ 0.7001 nmoles cytochrome c/min/mg protein) activity. Surprisingly, even though there was no significant rise of MFO activity observed at both stages of *Ae. albopictus* from all types of area, these populations demonstrated mixed levels with a wide range of MFO activities (Table 4.18, Table 4.19, Figure 4.7 and Figure 4.8).

Table 4.20: Mean (± S.E.) values of mixed function oxidases (MFO) activity of *Aedes albopictus* larvae from different types of area at absorbance 630 nm.

Types of area	Study areas	Mean ± S.E. (nmoles cyt c/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	0.2929 ± 0.01	-
Oil palm plantations	Kota Tinggi OP	0.2927 ± 0.04[a]	1.00
	Klang OP		
	Temerloh OP		
Paddy cultivation areas	Kuala Selangor PD	0.2064 ± 0.04	0.70
	Kulim PD		
	Kuala Pilah PD		
Rubber estates	Sungai Buloh RB	0.1804 ± 0.06	0.62
	Temerloh RB		
	Kota Tinggi RB		
Fogging-free residential areas	Shah Alam FF	0.1707 ± 0.03[a]	0.58
	Padang Serai FF		
	Temerloh FF		
Dengue prone residential areas	Kota Tinggi DEN	0.2167 ± 0.07	0.74
	Shah Alam DEN		
	Cheras DEN		
One way ANOVA		F = 0.890 df = 15 P = 0.523	

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean mixed function oxidases of the field population / Mean mixed function oxidases of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean mixed function oxidases followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean mixed function oxidases of the field population was significantly different with mean mixed function oxidases of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.21: Mean (± S.E.) values of mixed function oxidases (MFO) activity of *Aedes albopictus* adults from different types of area at absorbance 630 nm.

Types of area	Study areas	Mean ± S.E. (nmoles cyt c/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	0.7411 ± 0.04	-
Oil palm plantations	Kota Tinggi OP	0.4802 ± 0.11	0.65
	Klang OP		
	Temerloh OP		
Paddy cultivation areas	Kuala Selangor PD	0.6852 ± 0.15	0.92
	Kulim PD		
	Kuala Pilah PD		
Rubber estates	Sungai Buloh RB	0.5526 ± 0.07	0.75
	Temerloh RB		
	Kota Tinggi RB		
Fogging-free residential areas	Shah Alam FF	0.7413 ± 0.22	1.00
	Padang Serai FF		
	Temerloh FF		
Dengue prone residential areas	Kota Tinggi DEN	0.4425 ± 0.08	0.60
	Shah Alam DEN		
	Cheras DEN		
One way ANOVA		F = 0.800 df = 15 P = 0.574	

$P > 0.05$ indicated no significant difference.
$P \leq 0.05$ indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean mixed function oxidases of the field population / Mean mixed function oxidases of the reference strain
$RR < 5$ = susceptible, $5 \leq RR \leq 10$ = moderate resistance, $RR > 10$ = high resistance as determined by WHO (2016a).
Mean mixed function oxidases followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean mixed function oxidases of the field population was significantly different with mean mixed function oxidases of the reference strain ($P \leq 0.05$) (Independent samples t-test).

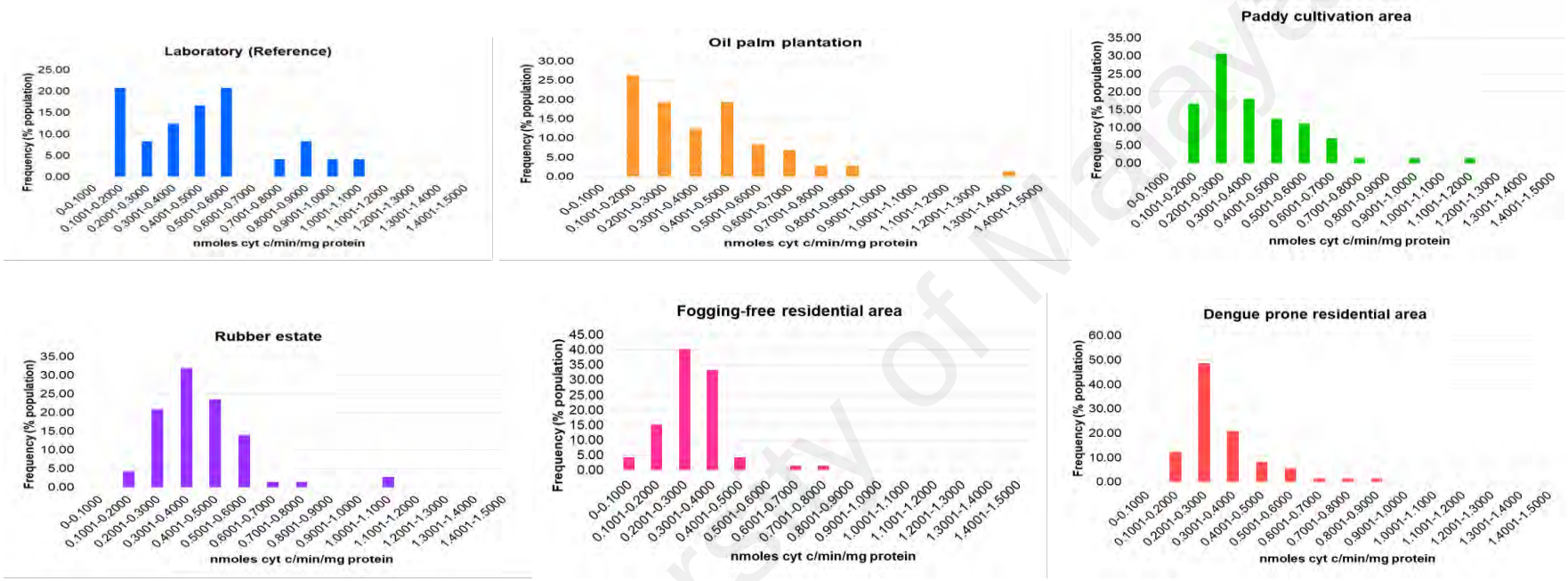

Figure 4.7: Mixed function oxidases (MFO) activity in *Aedes albopictus* larvae from different types of area.

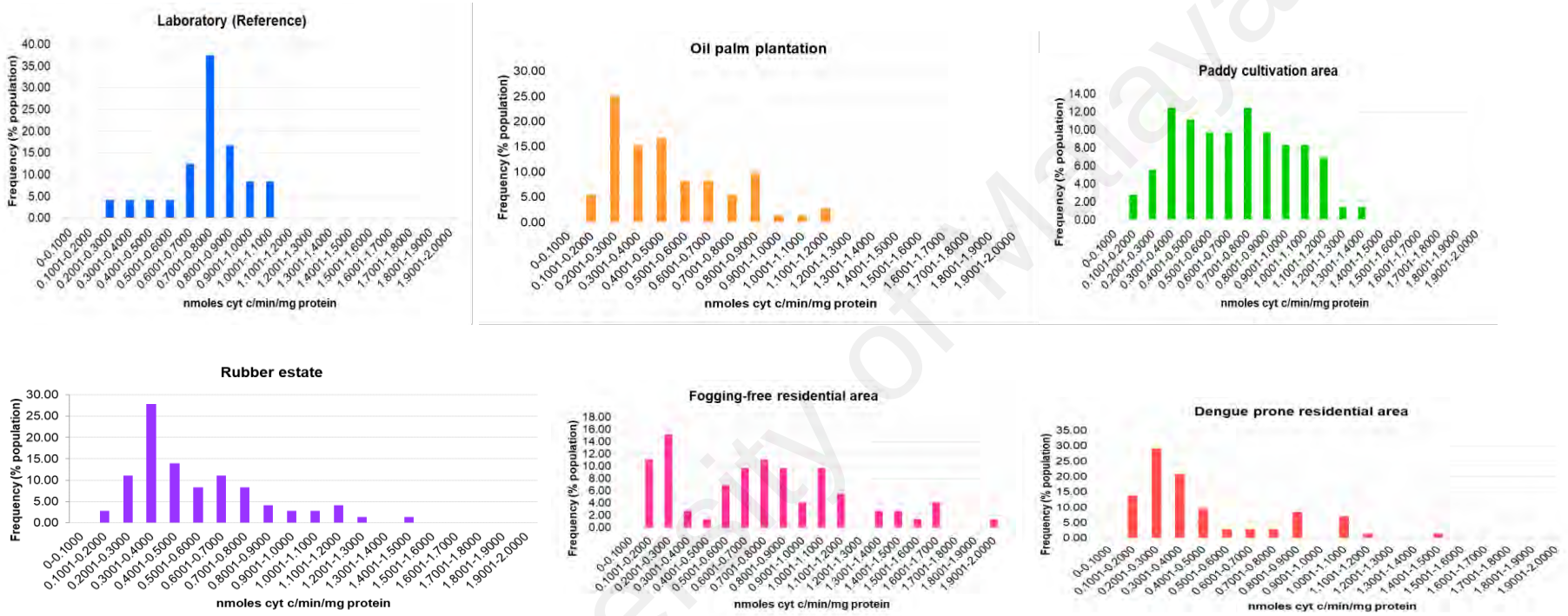

Figure 4.8: Mixed function oxidases (MFO) activity in *Aedes albopictus* adults from different types of area.

4.5.3 Glutathione-S-transferases (GST) Enzyme Microassay

Results of Normality Test confirmed that date of mean elevated glutathione-S-transferases (GST) activity was normally distributed ($P \leq 0.05$). One-way ANOVA showed that differences in mean elevated GST activity among *Ae. albopictus* from different types of area was significant at both larval and adult stage. Post Hoc Test displayed significant difference of mean elevated GST activity between *Ae. albopictus* from agricultural areas and non-agricultural areas at both developmental stages. In comparison with elevated mean GST activity in the reference strain of *Ae. albopictus* larvae, significant increase of GST activity was exhibited in *Ae. albopictus* larvae from all types of area except for oil palm plantations (Table 4.22). However, significant rise of GST activity at adult stage of *Ae. albopictus* was exhibited only in samples from paddy cultivation areas as compared to the reference strain (Table 4.23). The resistance ratios (RR) calculated for each population of *Ae. albopictus* tested ranged from 0.89 to 6.00 fold. All samples of GST microassay were also classified into either low (≤ 0.0030 mmoles CDNB/min/mg protein), moderate (0.0031-0.0070 mmoles CDNB/min/mg protein) or high (≥ 0.0071 mmoles CDNB/min/mg protein) activity. With the exception of samples from oil palm plantations, *Ae. albopictus* larvae from all types of area demonstrated low and moderate activities of GST (Table 4.18 and Figure 4.9). Alternatively, *Ae. albopictus* adults from all types of area showed low GST activity except for half of the population from paddy cultivation areas which possessed moderate GST activity (Table 4.19 and Figure 4.10).

Table 4.22: Mean (± S.E.) values of glutathione-S-transferases (GST) activity of *Aedes albopictus* larvae from different types of area at absorbance 410 nm.

Types of area	Study areas	Mean ± S.E. (mmoles CDNB/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	0.0009 ± 0.00	-
Oil palm plantations	Kota Tinggi OP	0.0008 ± 0.00[a]	0.89
	Klang OP		
	Temerloh OP		
Paddy cultivation areas	Kuala Selangor PD	*0.0026 ± 0.00[ab]	2.89
	Kulim PD		
	Kuala Pilah PD		
Rubber estates	Sungai Buloh RB	*0.0027 ± 0.00[a]	3.00
	Temerloh RB		
	Kota Tinggi RB		
Fogging-free residential areas	Shah Alam FF	*0.0018 ± 0.00	2.00
	Padang Serai FF		
	Temerloh FF		
Dengue prone residential areas	Kota Tinggi DEN	*0.0015 ± 0.00[b]	1.67
	Shah Alam DEN		
	Cheras DEN		
One way ANOVA		F = 3.432 df = 15 P = 0.046	

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean glutathione-S-transferases of the field population / Mean glutathione-S-transferases of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean glutathione-S-transferases followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean glutathione-S-transferases of the field population was significantly different with mean glutathione-S-transferases of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.23: Mean (± S.E.) values of glutathione-S-transferases (GST) activity of *Aedes albopictus* adults from different types of area at absorbance 410 nm.

Types of area	Study areas	Mean ± S.E. (mmoles CDNB/min/mg protein)	Resistance Ratio (RR)
Reference	Laboratory	0.0004 ± 0.00	-
Oil palm plantations	Kota Tinggi OP	0.0005 ± 0.00[a]	1.25
	Klang OP		
	Temerloh OP		
Paddy cultivation areas	Kuala Selangor PD	*0.0024 ± 0.00[ab]	6.00
	Kulim PD		
	Kuala Pilah PD		
Rubber estates	Sungai Buloh RB	0.0004 ± 0.00[b]	1.00
	Temerloh RB		
	Kota Tinggi RB		
Fogging-free residential areas	Shah Alam FF	0.0005 ± 0.00[b]	1.25
	Padang Serai FF		
	Temerloh FF		
Dengue prone residential areas	Kota Tinggi DEN	0.0004 ± 0.00[b]	1.00
	Shah Alam DEN		
	Cheras DEN		
One way ANOVA		F = 13.057 df = 15 P = 0.000	

$P > 0.05$ indicated no significant difference.
$P \leq 0.05$ indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean glutathione-S-transferases of the field population / Mean glutathione-S-transferases of the reference strain
$RR < 5$ = susceptible, $5 \leq RR \leq 10$ = moderate resistance, $RR > 10$ = high resistance as determined by WHO (2016a).
Mean glutathione-S-transferases followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean glutathione-S-transferases of the field population was significantly different with mean glutathione-S-transferases of the reference strain ($P \leq 0.05$) (Independent samples t-test).

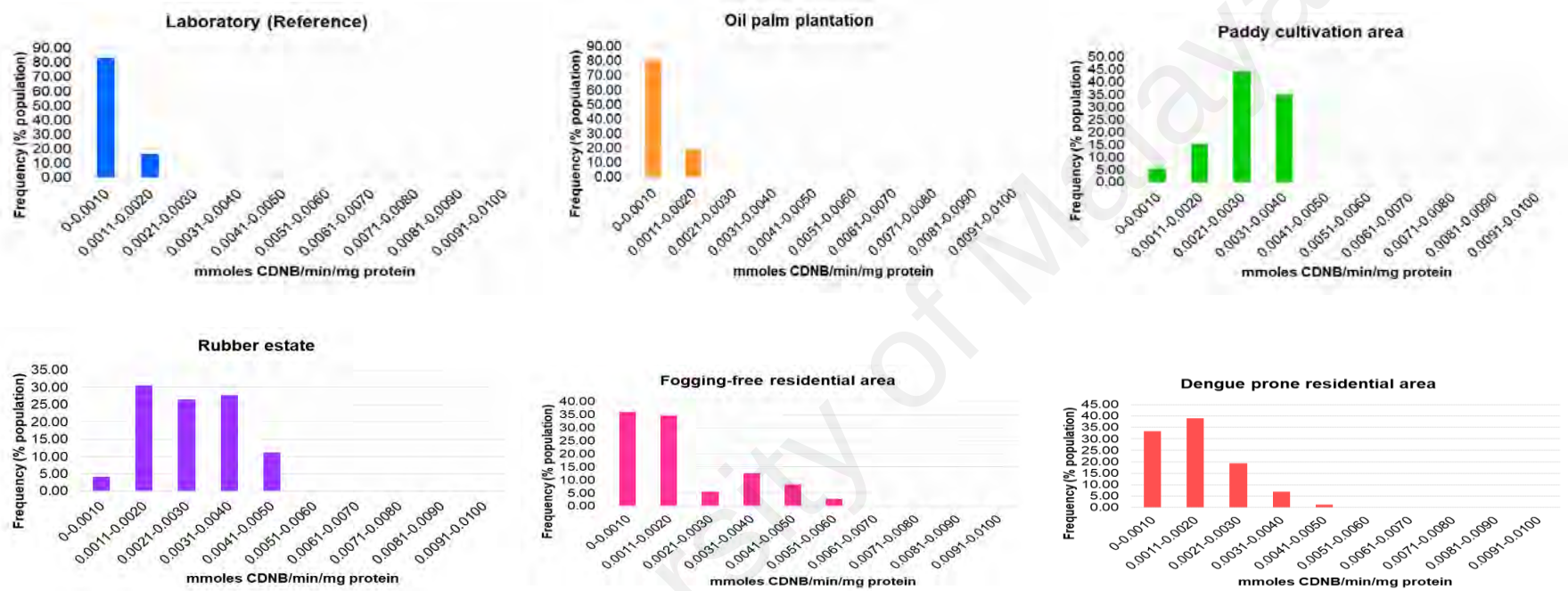

Figure 4.9: Glutathione-S-transferases (GST) activity in *Aedes albopictus* larvae from different types of area.

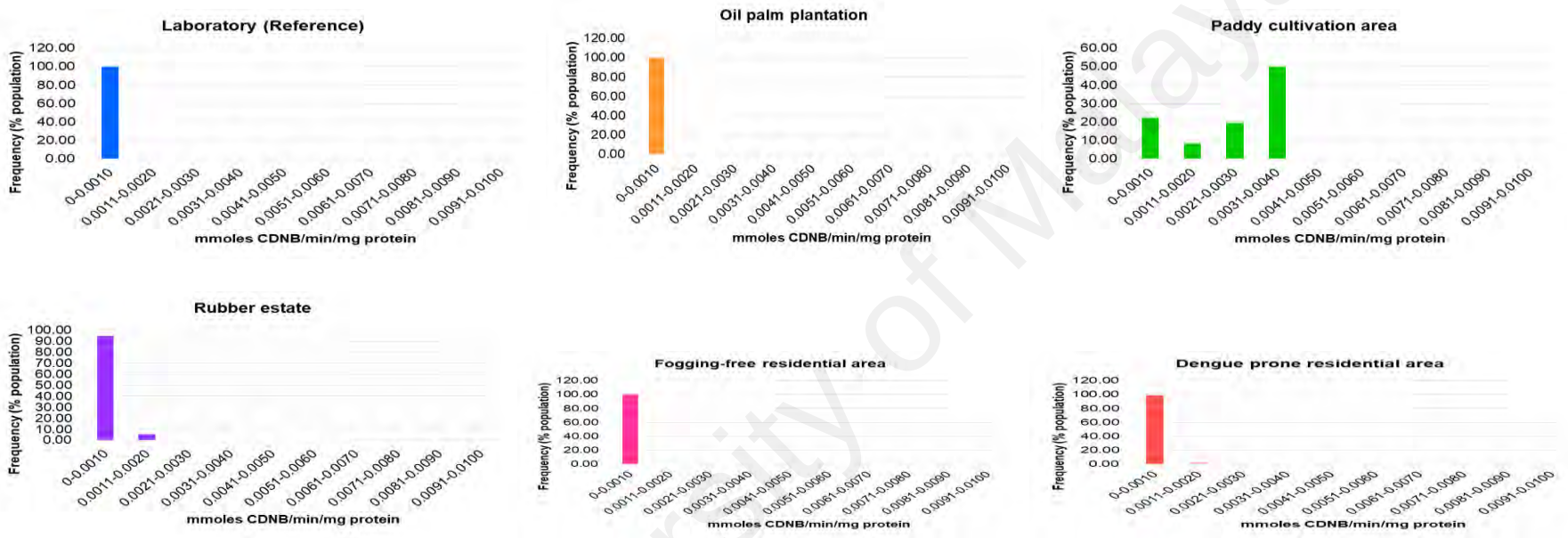

Figure 4.10: Glutathione-S-transferases (GST) activity in *Aedes albopictus* adults from different types of area.

4.5.4 Insensitive Acetylcholinesterase (AChE) Enzyme Microassay

Normality Test carried out verified that data of mean percent acetylcholinesterase (AChE) activity in propoxur-inhibited fraction was normally distributed ($P \leq 0.05$). Based on Paired samples t-test conducted, at larval stage of *Ae. albopictus*, lower AChE activity was observed significantly only in the reference strain, oil palm plantations and fogging-free residential areas populations when they were treated with propoxur which implied that AChE activity in these populations was still sensitive against propoxur (Table 4.24). In contrast, AChE enzyme in *Ae. albopictus* larvae from other field strains was insensitive against propoxur as indicated by the non-significant difference between their AChE activities in 5 mg/L propoxur and without any treatment of propoxur. Only mean percent of AChE activity in propoxur-inhibited fraction in *Ae. albopictus* larvae from oil palm plantations and fogging-free residential areas were significantly different with one another ($P \leq 0.05$). Resistance ratios (RR) of AChE activity recorded for all larval populations of *Ae. albopictus* were less than 1.00. Conversely, AChE activity in *Ae. albopictus* adults from all types of area was still sensitive to propoxur as denoted by significantly lower AChE enzyme in samples that had been treated with 1000 mg/L propoxur in comparison to those samples without the exposure to propoxur (Table 4.25). Mean percent of AChE activity of *Ae. albopictus* adults from oil palm plantations and paddy cultivation areas were significantly different with fogging-free residential areas populations ($P \leq 0.05$). Significant mean percent of AChE activity in propoxur-inhibited fraction was also noted in *Ae. albopictus* adults from rubber estates as well as both residential areas as compared to the reference strain. The resistance ratios (RR) of *Ae. albopictus* populations from all types of area ranged between 1.02 and 1.19. All samples of AChE microassay were also sorted into either low ($\leq$ 30% mean AChE activity in propoxur-inhibited fraction), moderate (31-70% mean AChE activity in propoxur-inhibited fraction) or high ($\geq$ 70% mean AChE activity in propoxur-inhibited

fraction) activity. More than 88% of larvae from each population of *Ae. albopictus* tested exhibited high AChE activity indicating that majority of them possessed homozygous resistance (RR) against insecticides associated with AChE enzyme (Table 4.18 and Figure 4.11). Meanwhile, at least 87% of *Ae. albopictus* adults from each of these populations were heterozygous (RS) against AChE-associated insecticides as shown by their moderate AChE activities (Table 4.19 and Figure 4.12).

Table 4.24: Mean (± S.E.) acetylcholinesterase activity in fractions with and without propoxur inhibition of *Aedes albopictus* larvae from different types of area at absorbance 410 nm.

Types of area	Study areas	Mean acetylcholinesterase activity (± S.E.)		Paired samples t-test	Mean percent acetylcholinesterase activity in propoxur-inhibited fraction (%)	Resistance Ratio (RR)
		ACTH + 5 mg/L propoxur	Control (without propoxur)			
Reference	Laboratory	0.0475 ± 0.00	0.0505 ± 0.00	P ≤ 0.05	94.22 ± 0.92	-
Oil palm plantations	Kota Tinggi OP	0.0492 ± 0.00	0.0541 ± 0.00	P ≤ 0.05	91.56 ± 0.50[a]	0.97
	Klang OP					
	Temerloh OP					
Paddy cultivation areas	Kuala Selangor PD	0.0527 ± 0.00	0.0622 ± 0.00	P > 0.05	88.21 ± 2.82	0.94
	Kulim PD					
	Kuala Pilah PD					
Rubber estates	Sungai Buloh RB	0.0494 ± 0.00	0.0597 ± 0.01	P > 0.05	88.81 ± 8.04	0.94
	Temerloh RB					
	Kota Tinggi RB					
Fogging-free residential areas	Shah Alam FF	0.0506 ± 0.00	0.0582 ± 0.00	P ≤ 0.05	88.52 ± 1.01[a]	0.94
	Padang Serai FF					
	Temerloh FF					
Dengue prone residential areas	Kota Tinggi DEN	0.0490 ± 0.00	0.0564 ± 0.00	P > 0.05	89.79 ± 2.11	0.95
	Shah Alam DEN					
	Cheras DEN					

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean percent acetylcholinesterase of the field population / Mean percent acetylcholinesterase of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean percent acetylcholinesterase followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean percent acetylcholinesterase of the field population was significantly different with mean percent acetylcholinesterase of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.25: Mean (± S.E.) acetylcholinesterase (AChE) activity in fractions with and without propoxur inhibition of *Aedes albopictus* adults from different types of area at absorbance 410 nm.

Types of area	Study areas	Mean acetylcholinesterase activity (± S.E.)		Paired samples t-test	Mean percent acetylcholinesterase activity in propoxur-inhibited fraction (%)	Resistance Ratio (RR)
		ACTH + 1000 mg/L propoxur	Control (without propoxur)			
Reference	Laboratory	0.0852 ± 0.00	0.1750 ± 0.01	P ≤ 0.05	50.74 ± 1.55	-
Oil palm plantations	Kota Tinggi OP	0.0936 ± 0.00	0.1807 ± 0.01	P ≤ 0.05	53.38 ± 1.55[a]	1.05
	Klang OP					
	Temerloh OP					
Paddy cultivation areas	Kuala Selangor PD	0.1006 ± 0.00	0.2029 ± 0.01	P ≤ 0.05	51.91 ± 2.02[b]	1.02
	Kulim PD					
	Kuala Pilah PD					
Rubber estates	Sungai Buloh RB	0.0932 ± 0.01	0.1695 ± 0.02	P ≤ 0.05	*56.50 ± 2.33	1.11
	Temerloh RB					
	Kota Tinggi RB					
Fogging-free residential areas	Shah Alam FF	0.0962 ± 0.00	0.1628 ± 0.01	P ≤ 0.05	*60.56 ± 1.97[ab]	1.19
	Padang Serai FF					
	Temerloh FF					
Dengue prone residential areas	Kota Tinggi DEN	0.1047 ± 0.01	0.1994 ± 0.02	P ≤ 0.05	*54.87 ± 4.55	1.08
	Shah Alam DEN					
	Cheras DEN					

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean percent acetylcholinesterase of the field population / Mean percent acetylcholinesterase of the reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean percent acetylcholinesterase followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
* = The increase of mean percent acetylcholinesterase of the field population was significantly different with mean percent acetylcholinesterase of the reference strain (P ≤ 0.05) (Independent samples t-test).

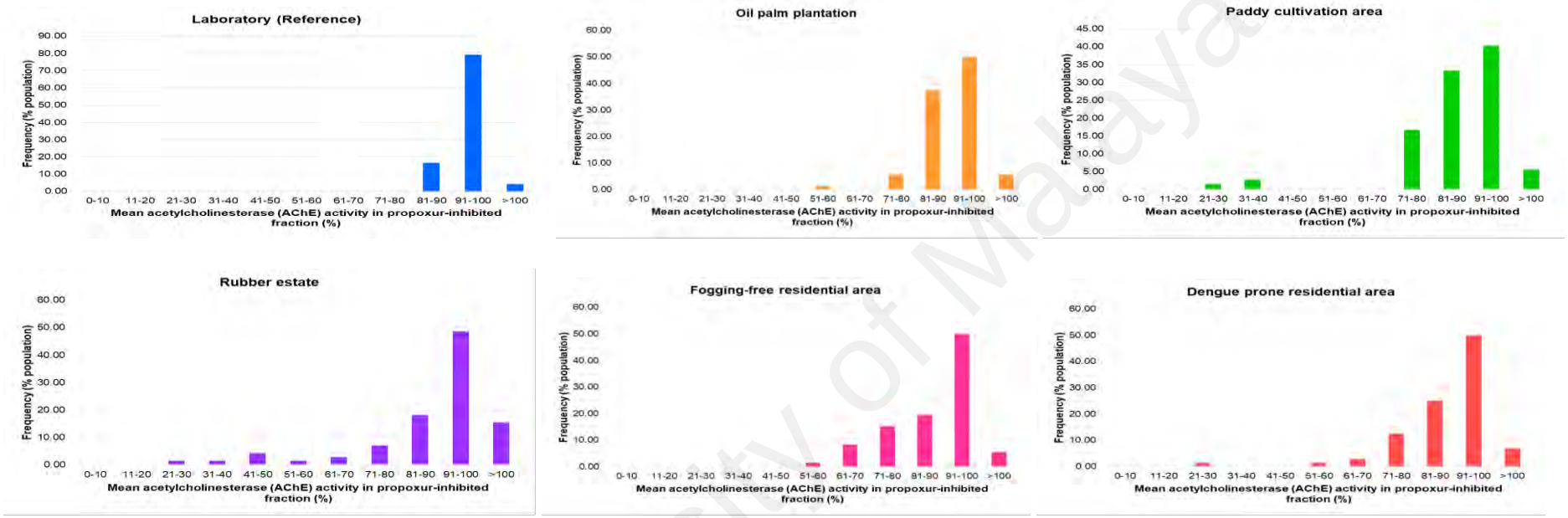

Figure 4.11: Mean acetylcholinesterase (AChE) activity in propoxur-inhibited fraction (%) in *Aedes albopictus* larvae from different types of area.

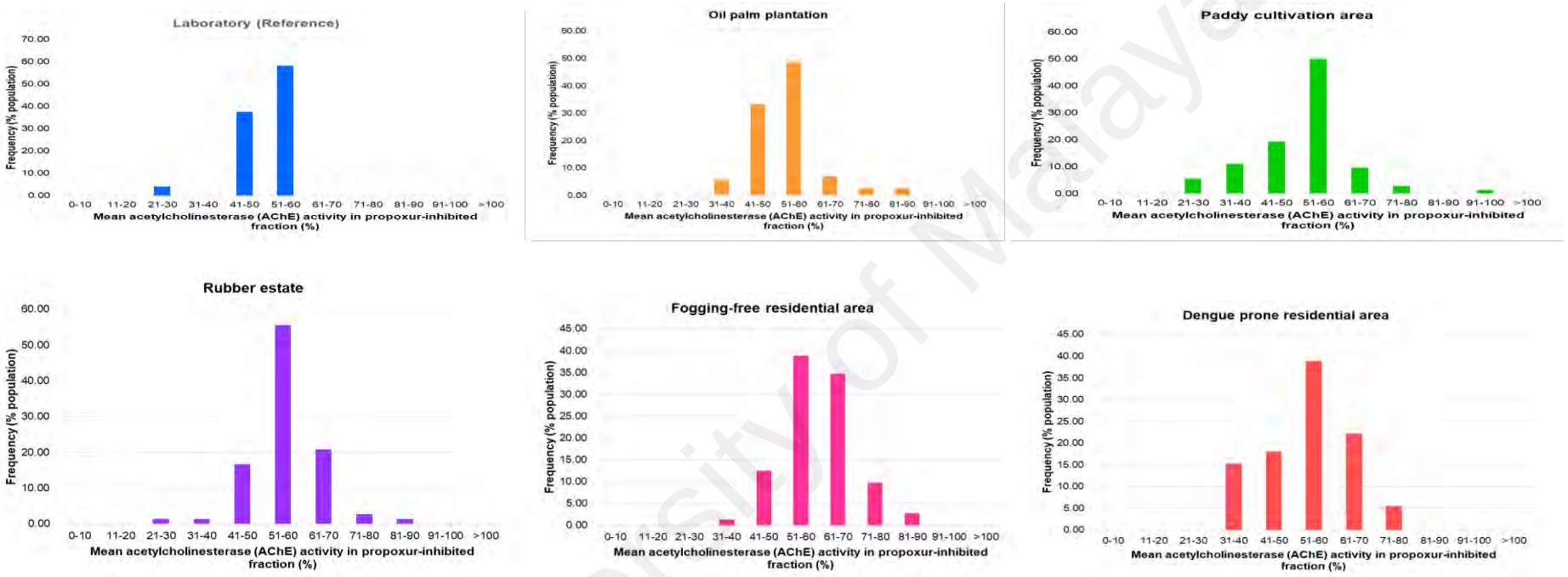

Figure 4.12: Mean acetylcholinesterase (AChE) activity in propoxur-inhibited fraction (%) in *Aedes albopictus* adults from different types of area.

4.5.5 Association between Different Detoxification Enzyme Activities in *Aedes albopictus* Larvae and Adults

The association between different detoxification enzyme activities in *Ae. albopictus* larvae and adults was determined by conducting the correlation analysis using Pearson Correlation Test. It is worth noting that only α-esterases and β-esterases were correlated with one another in *Ae. albopictus* larvae (r = 0.541, P = 0.030) (Table 4.26), whereas, no correlation was exhibited between all elevated enzyme activities in *Ae. albopictus* adults (Table 4.27). In terms of elevated enzyme activities in different stage of *Ae. albopictus*, only α-esterases activity displayed correlation between larval and adult stages (r = 0.497, P = 0.050) (Table 4.28).

Table 4.26: Correlation between different mean elevated enzyme activities for *Aedes albopictus* larvae.

Elevated enzyme activities	α-EST	β-EST	MFO	GST
β-EST	r = 0.541 P = 0.030			
MFO	r = -0.485 P = 0.057	r = 0.065 P = 0.812		
GST	r = 0.335 P = 0.205	r = 0.248 P = 0.354	r = -0.307 P = 0.247	
AChE	r = 0.035 P = 0.897	r = -0.388 P =0.137	r = -0.179 P = 0.506	r = -0.152 P = 0.574

Association between two elevated enzyme activities (Pearson Correlation Test) based on the correlation of mean elevated enzyme activities for two tested enzymes: r > 0.4 = Correlated (Two tested enzymes showed association between one another); r > 0.8 = Highly correlated (Two tested enzymes showed strong association between one another).
α-EST = α-esterases, β-EST = β-esterases, MFO = Mixed function oxidases, GST = Glutathione-S-transferases, AChE = Acetylcholinesterase.
P ≤ 0.05 = Significant

Table 4.27: Correlation between different mean elevated enzyme activities for *Aedes albopictus* adults.

Elevated enzyme activities	α-EST	β-EST	MFO	GST
β-EST	r = 0.415 P = 0.110			
MFO	r = 0.232 P = 0.387	r = -0.191 P = 0.478		
GST	r = 0.390 P = 0.135	r = 0.166 P = 0.540	r = 0.405 P = 0.119	
AChE	r = 0.382 P = 0.144	r = 0.447 P = 0.083	r = 0.185 P = 0.492	r = -0.017 P = 0.951

Association between two elevated enzyme activities (Pearson Correlation Test) based on the correlation of mean elevated enzyme activities for two tested enzymes: r > 0.4 = Correlated (Two tested enzymes showed association between one another); r > 0.8 = Highly correlated (Two tested enzymes showed strong association between one another).
α-EST = α-esterases, β-EST = β-esterases, MFO = Mixed function oxidases, GST = Glutathione-S-transferases, AChE = Acetylcholinesterase.
P ≤ 0.05 = Significant

Table 4.28: Correlation of mean elevated enzyme activities for *Aedes albopictus* between larval stage and adult stage.

Elevated enzyme activities	α-EST	β-EST	MFO	GST	AChE
α-EST	r = 0.497 P = 0.050				
β-EST		r = 0.462 P = 0.072			
MFO			r = -0.021 P = 0.940		
GST				r = 0.364 P = 0.165	
AChE					r = 0.097 P = 0.721

Association between the same elevated enzyme activity at larval and adult stage (Pearson Correlation Test) based on the correlation of specific mean elevated enzyme activity at larval stage with its activity at adult stage: r > 0.4 = Correlated (Specific elevated enzyme activity at larval stage was associated with its activity at adult stage); r > 0.8 = Highly correlated (Specific elevated enzyme activity at larval stage was strongly associated with its activity at adult stage).
α-EST = α-esterases, β-EST = β-esterases, MFO = Mixed function oxidases, GST = Glutathione-S-transferases, AChE = Acetylcholinesterase.
P ≤ 0.05 = Significant

4.5.6 Association between Susceptibility Status of *Aedes albopictus* Larvae and Adults with Their Level of Detoxification Enzyme Activities

The correlation analysis using Pearson Correlation Test was also performed to determine any association between the level of insecticide resistance ascertained from both larval and adult mosquito bioassays with enzyme activities. In this correlation test, data of percent mortality of *Ae. albopictus* larvae at 24 hours post-treatment using independent diagnostic dosages ($2xLC_{99}$) was used to be tested with data of all mean elevated enzyme activities of larvae while data of knockdown $time_{50}$ (KT_{50}) values of *Ae. albopictus* adults was tested with data of all mean elevated enzyme activities of *Ae. albopictus* adults. At larval stage, significant correlation was achieved only between percent mortality of *Ae. albopictus* larvae upon exposure to bendiocarb using the dosage of $2xLC_{99}$ of the reference strain at 24 hours post-treatment with acetylcholinesterase activity ($r = 0.542$, $P = 0.030$) (Table 4.29). On the other hand, at adult stage of *Ae. albopictus* tested, the significant correlation between KT_{50} values for propoxur ($r = 0.639$, $P = 0.008$) and bendiocarb ($r = 0.576$, $P = 0.019$) as well as deltamethrin ($r = 0.552$, $P = 0.027$), lambdacyhalothrin ($r = 0.613$, $P = 0.012$), cyfluthrin ($r = 0.549$, $P = 0.028$) and etofenprox ($r = 0.601$, $P = 0.014$) with β-esterases activity was observed (Table 4.30). There was also a significant correlation between KT_{50} values of malathion selection with glutathione-S-transferases activity ($r = 0.691$, $P = 0.003$) in *Ae. albopictus* adults of this study.

Table 4.29: Correlation of percent mortality of *Ae. albopictus* larvae at 24 hours post-treatment using independent diagnostic dosages ($2xLC_{99}$) with mean elevated enzyme activities at larval stage.

Elevated enzyme activities / Larvicides		α-EST	β-EST	GST	MFO	AChE
OC	DDT 0.8384 mg/L	r = -0.130 P = 0.632	r = -0.167 P = 0.537	r = 0.207 P = 0.442	r = 0.214 P = 0.425	r = -0.213 P = 0.428
	Dieldrin 0.3460 mg/L	r = 0.173 P = 0.522	r = 0.136 P = 0.614	r = 0.271 P = 0.311	r = 0.262 P = 0.326	r = -0.047 P = 0.863
OP	Malathion 5.0340 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.
	Fenitrothion 0.0540 mg/L	r = -0.160 P = 0.553	r = 0.001 P = 0.998	r = 0.012 P = 0.965	r = -0.016 P = 0.954	r = 0.094 P = 0.729
	Fenthion 0.0180 mg/L	r = -0.187 P = 0.487	r = -0.131 P = 0.629	r = 0.028 P = 0.917	r = 0.039 P = 0.887	r = -0.032 P = 0.905
	Temephos 0.0660 mg/L	r = 0.078 P = 0.774	r = 0.082 P = 0.763	r = 0.228 P = 0.397	r = -0.061 P = 0.821	r = 0.047 P = 0.862
	Chlorpyrifos 0.0160 mg/L	r = 0.331 P = 0.211	r = 0.140 P = 0.604	r = -0.131 P = 0.628	r = -0.083 P = 0.761	r = 0.153 P = 0.571
	Bromophos 0.2340 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.
CARB	Propoxur 4.8800 mg/L	r = -0.131 P = 0.628	r = -0.152 P = 0.574	r = -0.042 P = 0.877	r = -0.036 P = 0.896	r = 0.057 P = 0.833
	Bendiocarb 4.0760 mg/L	r = -0.020 P = 0.941	r = -0.196 P = 0.467	r = -0.071 P = 0.793	r = -0.071 P = 0.794	r = 0.542 P = 0.030
PY	Permethrin 0.0580 mg/L	r = -0.188 P = 0.486	r = -0.039 P = 0.886	r = 0.190 P = 0.480	r = 0.264 P = 0.323	r = -0.071 P = 0.793
	Deltamethrin 0.0460 mg/L	r = 0.051 P = 0.852	r = 0.020 P = 0.942	r = 0.317 P = 0.231	r = -0.246 P = 0.358	r = -0.171 P = 0.528
	Lambdacyhalothrin 0.0880 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.
	Cyfluthrin 0.0740 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.
	Etofenprox 0.1520 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.

Association between susceptibility status of larvae against various larvicides and elevated enzyme activities (Pearson Correlation Test) based on the correlation of percent mortality of larvae at 24 hours post-treatment using independent diagnostic dosages ($2xLC_{99}$) with mean elevated enzyme activities at larval stage: r > 0.4 = Correlated (Two tested parameters showed association between one another); r > 0.8 = Highly correlated (Two tested parameters showed strong association between one another).
N.D. = Not Determined due to 100% mortality at 24 hours post-treatment.
OC = Organochlorines; OP = Organophosphates; CARB = Carbamates; PY = Pyrethroids.
α-EST = α-esterases, β-EST = β-esterases, MFO = Mixed function oxidases, GST = Glutathione-S-transferases, AChE = Acetylcholinesterase.
$P \leq 0.05$ = Significant

Table 4.30: Correlation of KT_{50} values of *Ae. albopictus* adults with mean elevated enzyme activities at adult stage.

Elevated enzyme activities / Adulticides		α-EST	β-EST	GST	MFO	AChE
OC	DDT 4%	r = 0.099 P = 0.715	r = 0.386 P = 0.140	r = 0.061 P = 0.822	r = -0.061 P = 0.822	r = 0.027 P = 0.921
	Dieldrin 4%	N.D.	N.D.	N.D.	N.D.	N.D.
OP	Malathion 5%	r = -0.079 P = 0.771	r = 0.174 P = 0.520	r = 0.691 P = 0.003	r = 0.158 P = 0.558	r = -0.101 P = 0.711
	Fenitrothion 1%	N.D.	N.D.	N.D.	N.D.	N.D.
CARB	Propoxur 0.1%	r = -0.006 P = 0.983	r = 0.639 P = 0.008	r = 0.002 P = 0.994	r = -0.031 P = 0.910	r = 0.165 P = 0.542
	Bendiocarb 0.1%	r = -0.033 P = 0.903	r = 0.576 P = 0.019	r = 0.045 P = 0.870	r = -0.053 P = 0.846	r = 0.006 P = 0.983
PY	Permethrin 0.75%	r = -0.129 P = 0.634	r = 0.350 P = 0.184	r = 0.223 P = 0.407	r = 0.188 P = 0.486	r = 0.138 P = 0.610
	Deltamethrin 0.05%	r = -0.149 P = 0.581	r = 0.552 P = 0.027	r = 0.026 P = 0.924	r = 0.163 P = 0.546	r = 0.308 P = 0.247
	Lambdacyhalothrin 0.05%	r = -0.033 P = 0.905	r = 0.613 P = 0.012	r = 0.061 P = 0.823	r = 0.271 P = 0.310	r = 0.357 P = 0.175
	Cyfluthrin 0.15%	r = -0.079 P = 0.772	r = 0.549 P = 0.028	r = 0.073 P = 0.789	r = 0.158 P = 0.558	r = 0.240 P = 0.370
	Etofenprox 0.5%	r = -0.217 P = 0.420	r = 0.601 P = 0.014	r = -0.311 P = 0.241	r = -0.365 P = 0.165	r = 0.296 P = 0.265

Association between susceptibility status of adult mosquitoes against various adulticides and elevated enzyme activities (Pearson Correlation Test) based on the correlation of KT_{50} values of adult mosquitoes with mean elevated enzyme activities at adult stage: r > 0.4 = Correlated (Two tested parameters showed association between one another); r > 0.8 = Highly correlated (Two tested parameters showed strong association between one another).

N.D. = Not Determined due to no knockdown throughout the exposure period.

OC = Organochlorines; OP = Organophosphates; CARB = Carbamates; PY = Pyrethroids.

α-EST = α-esterases, β-EST = β-esterases, MFO = Mixed function oxidases, GST = Glutathione-S-transferases, AChE = Acetylcholinesterase.

$P \leq 0.05$ = Significant

Summary of the insecticide resistance occurrence and the underlying metabolic enzyme activities detected at both larval and adult stages of all *Ae. albopictus* populations tested in this study are presented in Table 4.31 and Table 4.32, respectively. Different level of resistance was demonstrated against various insecticides of different classes by *Ae. albopictus* from each type of study area. In general, *Ae. albopictus* larvae and adults from all types of area were susceptible to pyrethroids except for *Ae. albopictus* larvae from rubber estates and dengue prone residential areas that developed incipient resistance against permethrin. *Aedes albopictus* larvae from all types of area were also susceptible to organochlorines except for *Ae. albopictus* larvae from dengue prone residential areas which demonstrated moderate resistance against DDT. Mixed level of resistance against organophosphates and carbamates was displayed among *Ae. albopictus* larvae and adults from each type of study area.

The presence of elevated metabolic enzyme activities was also diversified among *Ae. albopictus* of different types of area and at both larval and adult stages. Significant increased activities of α-esterases and glutathione-S-transferases were discovered at larval stage while significant elevated activities of α-esterases, β-esterases, glutathione-S-transferases and acetylcholinesterase were demonstrated at adult stage of *Ae. albopictus* from different types of area. No significant increased activity of mixed function oxidases was exhibited either at larval or adult stage of *Ae. albopictus* from all types of area.

Table 4.31: Summary of insecticide resistance status based on the independent diagnostic dosage of larvicides and the underlying metabolic mechanisms detected in all types of area in which *Aedes albopictus* larvae were collected.

Status of area	Categories of area	Types of area	Insecticide resistance															Elevated enzyme activities				
			Organochlorines		Organophosphates						Carbamates		Pyrethroids									
			DDT	DIE	MAL	FENI	FEN	TEM	CHL	BRO	PRO	BEN	PER	DEL	LAM	CYF	ETO	α-EST	β-EST	MFO	GST	AChE
Fogging-free areas	Agricultural areas	Oil palm plantations	S	S	S	S	S	M	M	S	S	S	S	S	S	S	S	-	-	-	-	-
		Paddy cultivation areas	S	S	S	S	M	M	R	S	M	R	S	S	S	S	S	-	-	-	+	-
		Rubber estates	S	S	S	M	S	M	R	S	M	R	M	S	S	S	S	-	-	-	+	-
	Non-agricultural areas	Fogging-free residential areas	S	S	S	S	M	R	R	S	M	R	S	S	S	S	S	+	-	-	+	-
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	M	S	S	R	R	R	R	S	R	R	M	S	S	S	S	+	-	-	+	-

DIE = Dieldrin; MAL = Malathion; FENI = Fenitrothion; FEN = Fenthion; TEM = Temephos; CHL = Chlorpyrifos; BRO = Bromophos; PRO = Propoxur; BEN = Bendiocarb; PER = Permethrin; DEL = Deltamethrin; LAM = Lambdacyhalothrin; CYF = Cyfluthrin; ETO = Etofenprox; α-EST = α-esterases; β-EST = β-esterases; MFO = mixed function oxidases; GST = glutathione-S-transferases; AChE = acetylcholinesterase; R = resistant; M = moderate resistance; S = susceptible; + = presence of mechanism; - = absence of mechanism.

Table 4.32: Summary of insecticide resistance status based on percent mortality at 24 hours post-treatment and the underlying metabolic mechanisms detected in all types of area in which *Aedes albopictus* adults were collected.

Status of area	Categories of area	Types of area	Insecticide resistance											Elevated enzyme activities				
			Organochlorines		Organophosphates		Carbamates		Pyrethroids									
			DDT	DIE	MAL	FENI	PRO	BEN	PER	DEL	LAM	CYF	ETO	α-EST	β-EST	MFO	GST	AChE
Fogging-free areas	Agricultural areas	Oil palm plantations	R	S	M	R	S	M	S	S	S	S	S	+	-	-	-	-
		Paddy cultivation areas	R	M	M	R	R	R	S	S	S	S	S	+	+	-	+	-
		Rubber estates	M	M	M	R	R	R	S	S	S	S	S	+	+	-	-	+
	Non-agricultural areas	Fogging-free residential areas	R	S	M	R	M	R	S	S	S	S	S	+	+	-	-	+
Dengue prone areas	Non-agricultural areas	Dengue prone residential areas	R	M	R	R	R	R	S	S	S	S	S	+	+	-	-	+

DIE = Dieldrin; MAL = Malathion; FENI = Fenitrothion; PRO = Propoxur; BEN = Bendiocarb; PER = Permethrin; DEL = Deltamethrin; LAM = Lambdacyhalothrin; CYF = Cyfluthrin; ETO = Etofenprox; α-EST = α-esterases; β-EST = β-esterases; MFO = mixed function oxidases; GST = glutathione-S-transferases; AChE = acetylcholinesterase; R = resistant; M = moderate resistance; S = susceptible; + = presence of mechanism; - = absence of mechanism.

4.6 Synergistic Effect of Piperonyl Butoxide (PBO) in *Aedes albopictus* Adults against Organochlorines and Pyrethroids

The role of a synergist, piperonyl butoxide (PBO), in enhancing the efficacy of vectors control insecticides was investigated in this study. Synergism study was carried out by exposing *Ae. albopictus* adults from all types of area to PBO for an hour before being subjected to an insecticide of pyrethroids or organochlorines for another one hour. Results from synergist assays were compared with the results of WHO adult mosquito bioassays as both testings were conducted simultaneously so that any significant decrease of knockdown time for *Ae. albopictus* adults could be revealed.

The combination of PBO with DDT reduced the KT_{50} values of each field population of *Ae. albopictus* adults by 1.21 to 1.40 times (Table 4.33). However, significant decline of KT_{50} values was shown only in *Ae. albopictus* populations from oil palm plantations, paddy cultivation areas and fogging-free residential areas. Meanwhile, any significant decrease of KT_{50} values upon the exposure of these adult populations of *Ae. albopictus* to PBO + dieldrin could not be determined due to zero knockdown or mortality recorded throughout the exposure period (Table 4.33).

As for pyrethroids, significant decline of KT_{50} values was observed in *Ae. albopictus* adults from oil palm plantations, paddy cultivation areas and fogging-free residential areas after the exposure of PBO + permethrin (Table 4.34). Meanwhile, for the selection of PBO + deltamethrin, significant reduction of KT_{50} values was demonstrated in *Ae. albopictus* populations from oil palm plantations, rubber estates and fogging-free residential areas (Table 4.35). Significant decrease of KT_{50} values was displayed among similar *Ae. albopictus* populations exposed to PBO + lambdacyhalothrin at 1.32 to 1.65 times (Table 4.36). Furthermore, the selection of PBO + cyfluthrin had significantly reduced the KT_{50} values of almost all field populations of *Ae. albopictus* by 1.30 to 1.70 fold (Table 4.37). However, the combination of PBO with etofenprox only significantly

lessened the KT_{50} values of *Ae. albopictus* adults from oil palm plantations and fogging-free residential areas by 1.36 and 1.58 times, respectively (Table 4.38).

The knockdown percentage at 60 minutes and 30 minutes of the exposure time was also calculated for the selection of PBO + organochlorines and PBO + pyrethroids, respectively. These results were then been compared with the mortality percentage at 24 hours post-treatment of the same exposure. For DDT exposure, the resistance status for *Ae. albopictus* adults of the reference strain and rubber estates had been downgraded from highly resistant without the use of PBO to moderately resistant with the pre-exposure to PBO (Table 4.39). In fact, after 24 hours of the holding period, all field strains except from the oil palm plantations that had been exposed to PBO prior to DDT had either improved or retained their resistance status as moderately resistant to DDT. Contrarily, as mentioned previously, there was no knockdown or mortality displayed throughout the exposure period of either dieldrin alone or PBO + dieldrin (Table 4.40). Nonetheless, at 24 hours post-treatment, the pre-exposure of PBO before subjected to dieldrin had transformed *Ae. albopictus* adults from all types of area to become or remain susceptible to dieldrin in comparison to similar populations that had not been pre-exposed to PBO.

As for pyrethroids, at 30 minutes of exposure period, *Ae. albopictus* populations exposed to permethrin alone developed high resistance against permethrin (Table 4.41). However, the pre-exposure of PBO had upgraded their susceptibility against dieldrin to moderately resistant and even fully susceptible for *Ae. albopictus* population from paddy cultivation areas. No obvious differences could be observed between the exposure of permethrin alone and PBO + permethrin at 24 hours post-treatment since full mortalities were demonstrated in all *Ae. albopictus* populations.

Similar findings were obtained upon the selection of deltamethrin alone and PBO + deltamethrin. The high resistance status of all tested *Ae. albopictus* populations

achieved at 30 minutes of deltamethrin exposure was promoted to either incipient resistance or almost fully susceptible when PBO was utilized prior to deltamethrin exposure (Table 4.42). Only *Ae. albopictus* adults from dengue prone residential areas retained their high resistance status against deltamethrin even after the use of PBO but at higher percent knockdown. Conversely, complete mortalities were exhibited after 24 hours of holding in all *Ae. albopictus* populations exposed either to deltamethrin alone or PBO + deltamethrin.

In addition, the use of PBO prior to lambdacyhalothrin selection did not clearly improve the high resistance status of tested *Ae. albopictus* populations against lambdacyhalothrin at 30 minutes of the exposure time except for the reference strain and oil palm plantations populations (Table 4.43). Total mortalities were attained in all *Ae. albopictus* populations at 24 hours post-treatment of both lambdacyhalothrin alone and also PBO + lambdacyhalothrin.

For the cyfluthrin selection, at 30 minutes of the exposure time, *Ae. albopictus* adults from different types of area were either tolerance or highly resistant to cyfluthrin (Table 4.44). With the aid of PBO, most of these populations had either remained or enhanced their susceptibility against cyfluthrin to either susceptible or tolerance to cyfluthrin. Little improvement of the knockdown rate was displayed in *Ae. albopictus* adults from dengue prone residential areas even after the pre-exposure of PBO which made the population remain highly resistant to cyfluthrin. Nonetheless, all tested populations of *Ae. albopictus* exhibited full mortalities at 24 hours post-treatment of either cyfluthrin alone or PBO + cyfluthrin.

Furthermore, without the use of PBO, *Ae. albopictus* adults from all types of area developed high resistance against etofenprox at 30 minutes of the exposure period (Table 4.45). The pre-exposure of PBO prior to etofenprox enhanced the susceptibility status of the reference strain and fogging-free residential areas populations to moderate

resistance against etofenprox at the same exposure time while the rest of the populations retained their high resistance status against etofenprox due to only the slight increase of knockdown recorded when PBO was utilized. Complete mortalities were achieved in all *Ae. albopictus* populations tested at 24 hours post-treatment of either etofenprox alone or PBO + etofenprox.

Normality Test performed confirmed that all data derived from both WHO adult bioassay and mixed function oxidases (MFO) microassay conducted in this synergism study were normally distributed ($P > 0.05$). Any differences in the level of elevated MFO activity upon the pre-exposure of PBO was also investigated. Overall, a significant decrease of MFO activity level was observed in *Ae. albopictus* adults from all types of area that had been pre-exposed to PBO as compared to similar population samples that had not been pre-subjected to PBO (Table 4.46). Similar to MFO enzyme microassay conducted earlier on non-exposed *Ae. albopictus* adult mosquito samples, all PBO-exposed *Ae. albopictus* adult mosquito samples from all types of area that were subjected to MFO microassay were also grouped into either low (≤ 0.3000 nmoles cytochrome c/min/mg protein), moderate (0.3001-0.7000 nmoles cytochrome c/min/mg protein) or high (≥ 0.7001 nmoles cytochrome c/min/mg protein) activity. A similar trend could generally been observed in all populations of *Ae. albopictus* adults whereby all these populations showed a drastic reduction in the percentage of adult mosquitoes with high level of MFO activity upon the PBO pre-exposure (Table 4.47 and Figure 4.13). Moreover, all populations demonstrated increased percentages in both low level and moderate level of MFO activity confirming the significant suppression effect of PBO on MFO activity in *Ae. albopictus* adults from all types of area. Nevertheless, no significant correlation was demonstrated between KT_{50} values of PBO-exposed *Ae. albopictus* adults upon selection to organochlorines or pyrethroids with the MFO

activity in the same population of *Ae. albopictus* adults exposed to PBO alone (Table 4.48).

Table 4.33: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to DDT alone, PBO + DDT, dieldrin alone and PBO + dieldrin.

Types of area	Insecticides / Study areas	DDT 4%	PBO + DDT 4%	Synergistic Ratio (SR)	t-test	Dieldrin 4%	PBO + Dieldrin 4%	Synergistic Ratio (SR)	t-test
		KT_{50} (min) 95% C.L.	KT_{50} (min) 95% C.L.			KT_{50} (min) 95% C.L.	KT_{50} (min) 95% C.L.		
Reference	Laboratory	55.29 (54.11-56.68)	31.87 (31.20-32.55)	1.73	P > 0.05	N.D.	N.D.	N.D.	N.D.
Oil palm plantations	Kota Tinggi OP	66.93 ± 4.44 (61.28-75.68)	50.82 ± 0.33 (50.35-51.45)	1.32 ± 0.09	P ≤ 0.05	N.D.	N.D.	N.D.	N.D.
	Klang OP								
	Temerloh OP								
Paddy cultivation areas	Kuala Selangor PD	67.50 ± 6.48 (55.65-77.97)	48.31 ± 1.82 (46.00-51.90)	1.40 ± 0.15	P ≤ 0.05	N.D.	N.D.	N.D.	N.D.
	Kulim PD								
	Kuala Pilah PD								
Rubber estates	Sungai Buloh RB	52.73 ± 5.49 (42.97-61.95)	43.27 ± 3.72 (37.06-49.93)	1.21 ± 0.03	P > 0.05	N.D.	N.D.	N.D.	N.D.
	Temerloh RB								
	Kota Tinggi RB								
Fogging-free residential areas	Shah Alam FF	74.40 ± 4.10 (70.03-82.59)	57.04 ± 4.46 (48.30-62.93)	1.32 ± 0.11	P ≤ 0.05	N.D.	N.D.	N.D.	N.D.
	Padang Serai FF								
	Temerloh FF								
Dengue prone residential areas	Kota Tinggi DEN	68.24 ± 4.03 (62.06-75.81)	56.03 ± 4.03 (50.35-63.81)	1.22 ± 0.05	P > 0.05	N.D.	N.D.	N.D.	N.D.
	Shah Alam DEN								
	Cheras DEN								

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.

Table 4.34: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to permethrin alone and PBO + permethrin.

Types of area	Insecticides / Study areas	Permethrin 0.75% KT_{50} (min) 95% C.L.	PBO + Permethrin 0.75% KT_{50} (min) 95% C.L.	Synergistic Ratio (SR)	t-test
Reference	Laboratory	26.20 (25.54-26.81)	15.64 (15.29-15.97)	1.68	P > 0.05
Oil palm plantations	Kota Tinggi OP	24.40 ± 0.98 (23.11-26.33)	17.78 ± 1.94 (15.83-21.66)	1.39 ± 0.09	P ≤ 0.05
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	24.29 ± 3.69 (18.56-31.19)	14.60 ± 1.51 (11.59-16.23)	1.66 ± 0.14	P ≤ 0.05
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	21.40 ± 1.52 (18.94-24.17)	15.92 ± 1.83 (12.91-19.23)	1.36 ± 0.06	P > 0.05
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	25.93 ± 0.65 (24.63-26.66)	17.12 ± 1.76 (13.62-19.26)	1.56 ± 0.20	P ≤ 0.05
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	26.11 ± 3.10 (20.11-30.47)	17.88 ± 2.73 (13.76-23.04)	1.48 ± 0.10	P > 0.05
	Shah Alam DEN				
	Cheras DEN				

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.

Table 4.35: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to deltamethrin alone and PBO + deltamethrin.

Types of area	Insecticides / Study areas	Deltamethrin 0.05% KT_{50} (min) 95% C.L.	PBO + Deltamethrin 0.05% KT_{50} (min) 95% C.L.	Synergistic Ratio (SR)	t-test
Reference	Laboratory	26.06 (25.58-26.52)	13.91 (13.62-14.20)	1.87	P > 0.05
Oil palm plantations	Kota Tinggi OP	24.11 ± 1.17 (21.79-25.54)	18.22 ± 1.73 (15.67-21.51)	1.35 ± 0.14	P ≤ 0.05
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	23.85 ± 3.94 (17.53-31.09)	15.70 ± 1.62 (12.61-18.08)	1.50 ± 0.11	P > 0.05
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	21.38 ± 1.30 (19.03-23.53	16.52 ± 1.06 (15.24-18.63)	1.29 ± 0.04	P ≤ 0.05
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	30.22 ± 1.37 (27.65-32.33)	19.71 ± 1.85 (16.03-21.76)	1.58 ± 0.23	P ≤ 0.05
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	28.15 ± 1.95 (24.26-30.35)	21.60 ± 3.14 (16.47-27.31)	1.36 ± 0.23	P > 0.05
	Shah Alam DEN				
	Cheras DEN				

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.

Table 4.36: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to lambdacyhalothrin alone and PBO + lambdacyhalothrin.

Types of area	Insecticides / Study areas	Lambdacyhalothrin 0.05% KT_{50} (min) 95% C.L.	PBO + Lambdacyhalothrin 0.05% KT_{50} (min) 95% C.L.	Synergistic Ratio (SR)	t-test
Reference	Laboratory	29.94 (29.54-30.33)	17.98 (17.30-18.60)	1.67	P > 0.05
Oil palm plantations	Kota Tinggi OP	27.00 ± 0.56 (26.10-28.04)	19.94 ± 1.81 (17.52-23.48)	1.37 ± 0.10	P ≤ 0.05
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	28.97 ± 4.50 (20.76-36.28)	20.38 ± 2.39 (15.79-23.84)	1.42 ± 0.14	P > 0.05
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	26.42 ± 0.89 (25.32-28.19)	20.37 ± 1.90 (16.63-22.85)	1.32 ± 0.12	P ≤ 0.05
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	36.56 ± 1.28 (34.27-38.68)	23.70 ± 3.86 (16.94-30.32)	1.65 ± 0.34	P ≤ 0.05
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	34.17 ± 2.28 (29.62-36.76)	26.35 ± 3.09 (20.53-31.04)	1.34 ± 0.22	P > 0.05
	Shah Alam DEN				
	Cheras DEN				

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.

Table 4.37: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to cyfluthrin alone and PBO + cyfluthrin.

Types of area	Insecticides / Study areas	Cyfluthrin 0.15% KT_{50} (min) 95% C.L.	PBO + Cyfluthrin 0.15% KT_{50} (min) 95% C.L.	Synergistic Ratio (SR)	t-test
Reference	Laboratory	23.08 (22.69-23.48)	13.25 (12.97-13.53)	1.74	$P > 0.05$
Oil palm plantations	Kota Tinggi OP	20.78 ± 0.49 (20.24-21.76)	14.70 ± 2.09 (11.42-18.59)	1.46 ± 0.18	$P \leq 0.05$
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	21.88 ± 3.32 (17.63-28.43)	14.71 ± 1.59 (11.67-17.07)	1.48 ± 0.12	$P \leq 0.05$
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	19.59 ± 0.78 (18.06-20.58)	14.28 ± 1.00 (12.94-16.24)	1.38 ± 0.06	$P \leq 0.05$
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	26.41 ± 2.62 (21.38-30.19)	16.92 ± 2.74 (11.58-20.68)	1.70 ± 0.46	$P \leq 0.05$
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	25.33 ± 1.54 (22.36-27.54)	20.06 ± 2.40 (16.45-24.61)	1.30 ± 0.19	$P > 0.05$
	Shah Alam DEN				
	Cheras DEN				

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test ($P \leq 0.05$) = $P > 0.05$ indicated no significant difference; $P \leq 0.05$ indicated significant difference.

Table 4.38: Knockdown time values at 50% (KT_{50}) and synergistic ratio (SR) of *Aedes albopictus* adults from different types of area exposed to etofenprox alone and PBO + etofenprox.

Types of area	Insecticides / Study areas	Etofenprox 0.5% KT_{50} (min) 95% C.L.	PBO + Etofenprox 0.5% KT_{50} (min) 95% C.L.	Synergistic Ratio (SR)	t-test
Reference	Laboratory	25.53 (25.04-26.01)	23.11 (22.40-23.85)	1.10	P > 0.05
Oil palm plantations	Kota Tinggi OP	30.94 ± 0.94 (29.76-32.79)	22.93 ± 1.65 (19.86-25.51)	1.36 ± 0.07	P ≤ 0.05
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	26.31 ± 1.69 (23.56-29.38)	21.11 ± 1.84 (18.45-24.64)	1.25 ± 0.03	P > 0.05
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	29.92 ± 4.29 (21.75-36.26)	19.57 ± 1.18 (17.97-21.88)	1.53 ± 0.19	P > 0.05
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	31.81 ± 1.69 (28.61-34.34)	20.43 ± 1.51 (17.83-23.05)	1.58 ± 0.18	P ≤ 0.05
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	36.46 ± 2.99 (30.92-41.16)	26.91 ± 5.22 (20.94-37.31)	1.42 ± 0.20	P > 0.05
	Shah Alam DEN				
	Cheras DEN				

PBO = piperonyl butoxide (synergist).
C.L. = Confidence Limit
Synergistic Ratio (SR) = KT_{50} of the adulticide / KT_{50} of PBO + adulticide
N.D. = Not Determined due to no knockdown.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.

Table 4.39: Percent knockdown at 60 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against DDT alone and PBO + DDT.

Types of area	Insecticides / Study areas	Percent knockdown at 60 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		DDT 4%	PBO + DDT 4%	DDT 4%	PBO + DDT 4%
Reference	Laboratory	^{R}58.00 ± 8.87	^{M}97.00 ± 1.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{R}33.33 ± 7.75^{a}	^{R}61.33 ± 2.40^{a}	^{R}75.33 ± 12.81	^{R}86.67 ± 6.77
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}45.00 ± 11.27	^{R}80.67 ± 8.84	^{R}82.00 ± 4.04^{b}	^{M}92.67 ± 4.48
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{R}63.67 ± 10.11^{ac}	^{M}91.00 ± 4.73^{ac}	^{M}93.33 ± 4.81	^{M}96.00 ± 3.06
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}27.67 ± 2.85^{c}	^{R}63.33 ± 3.93^{c}	^{R}73.33 ± 0.88^{b}	^{M}95.33 ± 2.33
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}32.33 ± 6.84	^{R}58.33 ± 9.82^{c}	^{R}82.00 ± 9.07	^{M}96.67 ± 1.86
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 2.721 df = 15 P = 0.084	F = 4.669 df = 15 P = 0.019	F = 1.313 df = 15 P = 0.333	F = 0.955 df = 15 P = 0.488

Percent knockdown at 60 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.
N.D. = Not Determined due to no knockdown.

Table 4.40: Percent knockdown at 60 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against dieldrin alone and PBO + dieldrin.

Types of area	Insecticides / Study areas	Percent knockdown at 60 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Dieldrin 4%	PBO + Dieldrin 4%	Dieldrin 4%	PBO + Dieldrin 4%
Reference	Laboratory	N.D.	N.D.	[S]100.00 ± 0.00	[S]100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	N.D.	N.D.	[S]100.00 ± 0.00	[S]99.33 ± 0.67
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	N.D.	N.D.	[M]96.33 ± 3.67	[S]98.33 ± 1.67
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	N.D.	N.D.	[M]97.67 ± 2.33	[S]99.67 ± 0.33
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	N.D.	N.D.	[S]100.00 ± 0.00	[S]100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	N.D.	N.D.	[M]95.33 ± 4.67	[S]98.33 ± 1.67
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		N.D.	N.D.	F = 0.475 df = 15 P = 0.787	F = 0.424 df = 15 P = 0.822

Percent knockdown at 60 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
N.D. = Not Determined due to no knockdown.

Table 4.41: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against permethrin alone and PBO + permethrin.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Permethrin 0.75%	PBO + Permethrin 0.75%	Permethrin 0.75%	PBO + Permethrin 0.75%
Reference	Laboratory	^{R}76.00 ± 6.73	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{R}81.67 ± 0.33^{a}	^{M}95.00 ± 2.31^{a}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}75.67 ± 17.25	^{S}100.00 ± 0.00^{ab}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{R}84.33 ± 4.70	^{M}94.00 ± 6.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}68.33 ± 4.10^{a}	^{M}96.33 ± 2.03	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}70.00 ± 10.02	^{M}93.00 ± 5.51^{b}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 0.450 df = 15 P = 0.804	F = 0.467 df = 15 P = 0.792	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.42: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against deltamethrin alone and PBO + deltamethrin.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Deltamethrin 0.05%	PBO + Deltamethrin 0.05%	Deltamethrin 0.05%	PBO + Deltamethrin 0.05%
Reference	Laboratory	^{R}71.00 ± 15.95	^{S}99.00 ± 1.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{R}85.00 ± 2.31^{a}	^{M}97.33 ± 2.19	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}81.00 ± 13.05	^{M}97.00 ± 1.73	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{R}88.33 ± 5.17^{c}	^{M}93.33 ± 3.33	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}50.33 ± 12.42^{ac}	^{M}94.67 ± 2.91	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}58.67 ± 5.17^{ac}	^{R}79.00 ± 13.45	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 4.142 df = 15 P = 0.027	F = 1.177 df = 15 P = 0.385	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.43: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against lambdacyhalothrin alone and PBO + lambdacyhalothrin.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Lambdacyhalothrin 0.05%	PBO + Lambdacyhalothrin 0.05%	Lambdacyhalothrin 0.05%	PBO + Lambdacyhalothrin 0.05%
Reference	Laboratory	^{R}49.00 ± 17.92	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{R}66.33 ± 3.38^{a}	^{M}90.67 ± 5.33	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}57.33 ± 19.20	^{R}86.67 ± 6.67	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{R}69.00 ± 5.51^{c}	^{R}85.33 ± 7.88	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}27.00 ± 5.13^{ac}	^{R}80.33 ± 14.71	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}34.00 ± 7.77^{ac}	^{R}65.67 ± 14.84	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 2.946 df = 15 P = 0.069	F = 0.842 df = 15 P = 0.549	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.44: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against cyfluthrin alone and PBO + cyfluthrin.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Cyfluthrin 0.15%	PBO + Cyfluthrin 0.15%	Cyfluthrin 0.15%	PBO + Cyfluthrin 0.15%
Reference	Laboratory	^{M}90.00 ± 7.57	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{M}90.00 ± 3.61^{a}	^{S}98.67 ± 0.88	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}82.33 ± 14.31	^{S}98.33 ± 1.20	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{M}92.33 ± 1.86^{c}	^{M}96.00 ± 4.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}69.00 ± 8.19^{c}	^{M}95.33 ± 1.86	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}67.67 ± 4.18^{ac}	^{R}89.67 ± 4.41	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 1.832 df = 15 P = 0.194	F = 1.420 df = 15 P = 0.297	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.45: Percent knockdown at 30 minutes of the exposure time and percent mortality at 24 hours post-treatment for *Aedes albopictus* adults from different types of area against etofenprox alone and PBO + etofenprox.

Types of area	Insecticides / Study areas	Percent knockdown at 30 minutes of the exposure time (%)		Percent mortality after 24 h (%)	
		Etofenprox 0.5%	PBO + Etofenprox 0.5%	Etofenprox 0.5%	PBO + Etofenprox 0.5%
Reference	Laboratory	^{R}70.00 ± 4.16	^{M}91.00 ± 7.72	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{R}48.00 ± 7.37^{a}	^{R}77.00 ± 5.69	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	^{R}64.33 ± 8.21^{b}	^{R}86.00 ± 10.02	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	^{R}53.67 ± 14.84	^{R}86.33 ± 4.91	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	^{R}41.67 ± 3.76^{a}	^{M}94.00 ± 2.31	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	^{R}30.67 ± 7.13^{b}	^{R}65.67 ± 22.92	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 1.954 df = 15 P = 0.172	F = 0.723 df = 15 P = 0.622	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent knockdown at 30 minutes of the exposure time (%) = Mean of knockdown adult mosquitoes + Standard Error (S.E.)
Percent mortality after 24 h (%) = Mean of mortality adult mosquitoes + Standard Error (S.E.)
S = susceptible, M = moderate resistance, R = high resistance as determined by WHO (2016a).
Percent knockdown or percent mortality followed by different letter indicated significant difference between one another ($P \leq 0.05$) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b}= Significantly different with paddy cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4.46: Mean (± S.E.) values of mixed function oxidases (MFO) activity of non-exposed and PBO-exposed *Aedes albopictus* adults from different types of area at absorbance 630 nm.

Types of area	Study areas	Non-exposed		PBO-exposed	t-test
		Mean ± S.E. (nmoles cyt c/min/mg protein)	Resistance Ratio (RR)	Mean ± S.E. (nmoles cyt c/min/mg protein)	
Reference	Laboratory	0.7411 ± 0.04	-	0.4616 ± 0.05	P ≤ 0.05
Oil palm plantations	Kota Tinggi OP	0.4802 ± 0.11	0.65	0.3728 ± 0.06	P ≤ 0.05
	Klang OP				
	Temerloh OP				
Paddy cultivation areas	Kuala Selangor PD	0.6852 ± 0.15	0.92	0.3691 ± 0.01[b]	P ≤ 0.05
	Kulim PD				
	Kuala Pilah PD				
Rubber estates	Sungai Buloh RB	0.5526 ± 0.07	0.75	0.4017 ± 0.04[c]	P ≤ 0.05
	Temerloh RB				
	Kota Tinggi RB				
Fogging-free residential areas	Shah Alam FF	0.7413 ± 0.22	1.00	0.2855 ± 0.02[bc]	P ≤ 0.05
	Padang Serai FF				
	Temerloh FF				
Dengue prone residential areas	Kota Tinggi DEN	0.4425 ± 0.08	0.60	0.3108 ± 0.03	P ≤ 0.05
	Shah Alam DEN				
	Cheras DEN				
One way ANOVA		F = 0.800 df = 15 P = 0.574		F = 1.841 df = 15 P = 0.192	

P > 0.05 indicated no significant difference.
P ≤ 0.05 indicated significant difference.
S.E. = Standard Error
Resistance Ratio (RR) = Mean mixed function oxidases of the non-exposed field population / Mean mixed function oxidases of the non-exposed reference strain
RR < 5 = susceptible, 5 ≤ RR ≤ 10 = moderate resistance, RR > 10 = high resistance as determined by WHO (2016a).
Mean mixed function oxidases followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b]= Significantly different with paddy cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.
Independent samples t-test (P ≤ 0.05) = P > 0.05 indicated no significant difference; P ≤ 0.05 indicated significant difference.
* = The increase of mean mixed function oxidases of the field population was significantly different with mean mixed function oxidases of the reference strain (P ≤ 0.05) (Independent samples t-test).

Table 4.47: The distribution frequency of elevated mixed function oxidases (MFO) activities in non-exposed and PBO-exposed *Aedes albopictus* adults from different types of area.

Types of area	Study areas	Frequency (%) population					
		Mixed function oxidases of non-exposed adult mosquitoes			Mixed function oxidases of PBO-exposed adult mosquitoes		
		Low (+)	Moderate (++)	High (+++)	Low (+)	Moderate (++)	High (+++)
Reference	Laboratory	4.17	25.00	70.83	29.17	50.00	20.83
Oil palm plantations	Kota Tinggi OP	30.56	48.61	20.83	45.83	47.22	6.94
	Klang OP						
	Temerloh OP						
Paddy cultivation areas	Kuala Selangor PD	8.33	43.06	48.61	47.22	48.61	4.17
	Kulim PD						
	Kuala Pilah PD						
Rubber estates	Sungai Buloh RB	13.89	61.11	25.00	25.00	70.83	4.17
	Temerloh RB						
	Kota Tinggi RB						
Fogging-free residential areas	Shah Alam FF	26.39	20.83	52.78	59.72	38.89	1.39
	Padang Serai FF						
	Temerloh FF						
Dengue prone residential areas	Kota Tinggi DEN	43.06	36.11	20.83	61.11	36.11	2.78
	Shah Alam DEN						
	Cheras DEN						

MFO = Low (+) = ≤ 0.3000, Moderate (++) = 0.3001-0.7000, High (+++) = ≥ 0.7001

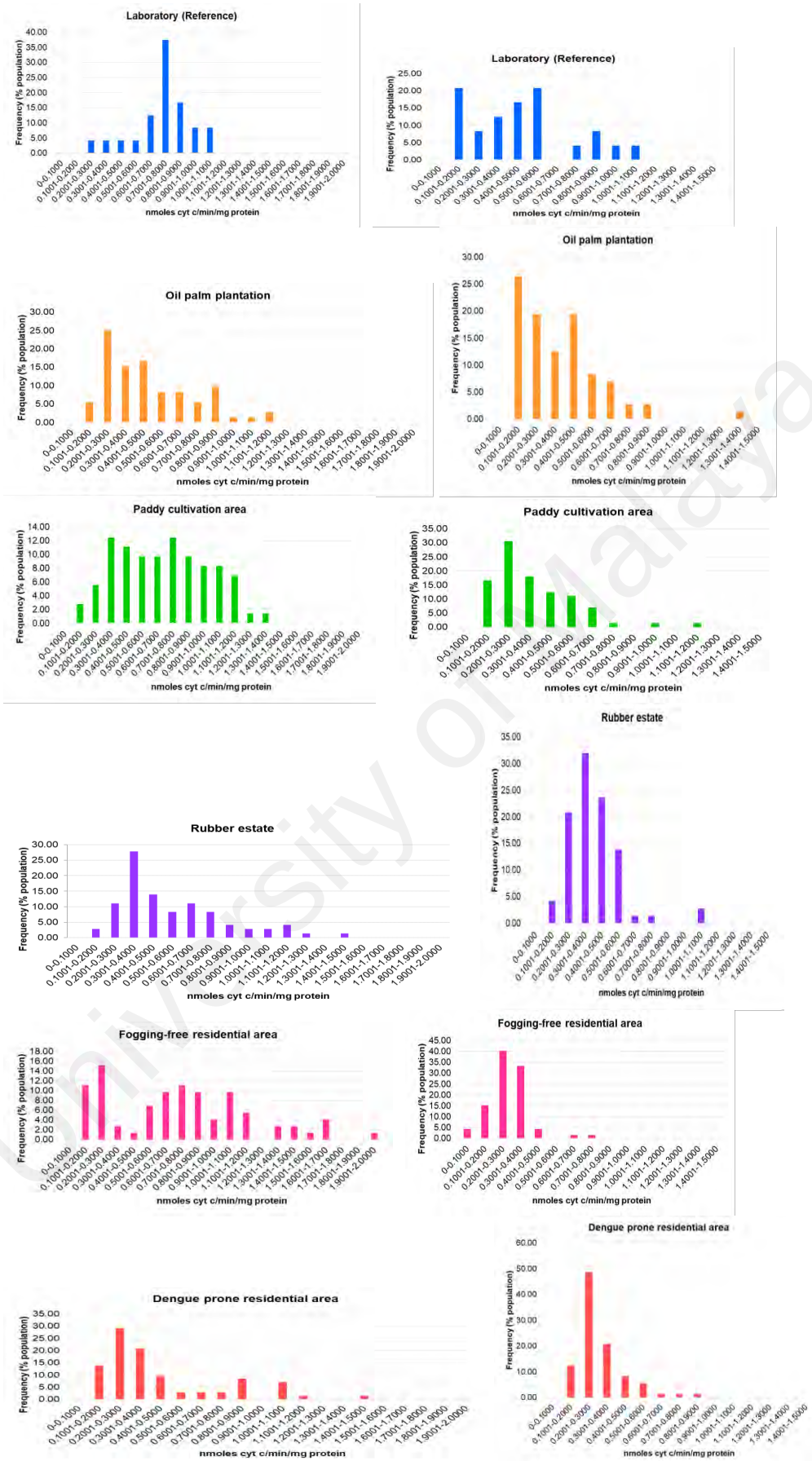

Figure 4.13: Mixed function oxidases (MFO) activity in non-exposed (left side) and PBO-exposed (right side) *Ae. albopictus* adults from different types of area.

Table 4.48: Correlation of KT_{50} values of *Ae. albopictus* adults exposed to PBO + organochlorine and PBO + pyrethroid, with mean elevated mixed function oxidases (MFO) activity of PBO-exposed *Ae. albopictus* adults.

Elevated MFO activity / Insecticides		MFO of PBO-exposed *Ae. albopictus* adults
OC	DDT 4%	r = -0.616 P = 0.011
	Dieldrin 4%	N.D.
PY	Permethrin 0.75%	r = -0.014 P = 0.959
	Deltamethrin 0.05%	r = -0.301 P = 0.257
	Lambdacyhalothrin 0.05%	r = -0.163 P = 0.546
	Cyfluthrin 0.15%	r = -0.184 P = 0.495
	Etofenprox 0.5%	r = -0.143 P = 0.597

Association between reduced KT_{50} values due to pre-exposure of PBO prior to organochlorines and pyrethroids selection, and lower elevated MFO activity in PBO-exposed *Ae. albopictus* adults (Pearson Correlation Test) based on the correlation of KT_{50} values of *Ae. albopictus* adults exposed to PBO + organochlorine and PBO + pyrethroid with mean elevated MFO activity of PBO-exposed *Ae. albopictus* adults: r > 0.4 = Correlated (Two tested parameters showed association between one another); r > 0.8 = Highly correlated (Two tested parameters showed strong association between one another).
N.D. = Not Determined due to no knockdown throughout the exposure period.
OC = Organochlorines; PY = Pyrethroids.
MFO = Mixed function oxidases
$P \leq 0.05$ = Significant

CHAPTER 5: DISCUSSION

5.1 Ovitrap Surveillance of *Aedes* Mosquitoes in Selected Agricultural and Non-agricultural Areas in Peninsular Malaysia

Vector surveillance using ovitraps is mainly targeting *Aedes* immatures particularly *Ae. aegypti* and *Ae. albopictus* which is attributable to their breeding preferences in water-holding containers. Conversely, rather than only *Ae. aegypti* and *Ae. albopictus* larvae, ovitrap surveillance conducted in fifteen study areas selected for this study had resulted in the capture of immatures of other species as well including *Cx. quinquefasciatus*, *Ar. subalbatus* and *Uranotaenia* sp. In Malaysia, *Ae. aegypti* has been commonly discovered indoors (Rudnick, 1986). *Ae. aegypti* has high preferences to inhabit both natural and man-made breeding sites especially in urban and suburban areas (Lima-Camara et al., 2016). On the other hand, *Ae. albopictus* is ordinarily found outdoors (Hawley, 1988). *Ae. albopictus* prefers peripheral forest habitats as well as disturbed and intense vegetation habitats (Rudnick et al., 1986). Nevertheless, *Ae. aegypti* and *Ae. albopictus* are also known as sympatric species which allow them to inhabit similar habitats (Klowden, 1993).

The existence of ideal breeding habitats in an area influences the population density of mosquito vectors. In Thailand, the distribution of *Ae. aegypti* during dry and wet seasons were significantly influenced by the number of water storage containers present indoors and outdoors in the study areas (Boonklong & Bhumiratana, 2016). As mentioned in Chapter 3 of this thesis, all study areas are well-equipped with proper water supply system. Hence, there is no need for the residents in these areas to store water for their daily use. With the presence of very few man-made water holding containers inside and outside of the houses in all study areas, the potential breeding

habitats of *Ae. aegypti* as an indoor and man-made container breeder were also lessened. This scenario had consequently reduced the population density of *Ae. aegypti* in all study areas which caused the collection of *Ae. aegypti* in ovitraps became difficult as well. Besides, the difficulty of capturing *Ae. aegypti* immatures could also be due to the fact that *Ae. aegypti* is a substantially low-density species even in localities with blood source richness due to high human population densities (Nordin et al., 2013). Other than that, the control of *Ae. aegypti* indoors is generally very successful in Malaysia for now due to continuous health education and rigorous law enforcement by the Ministry of Health Malaysia (H. L. Lee, personal communication, August 12, 2019).

Furthermore, the obliteration of vegetation and forests due to development of human habitations and other infrastructures has led to direct sunlight exposure on mosquito breeding habitats which could explain the fewer mosquito species and populations in residential areas (Zahouli et al., 2016). Findings from this study clearly presented *Ae. albopictus* as the most prevalent species in all study areas which signifies its important role in the spread of *Aedes*-borne diseases in these areas. The presence of cultivated industrial crops, cash crops, trees, ornamental plants, shrubs and dense vegetation especially in all types of agricultural areas selected for this study that are also located in suburban or rural zones offered ideal oviposition and resting habitats particularly for *Ae. albopictus*. Meanwhile, trees and ornamental plants nurtured by human dwellers as well as shrubs and vegetation within residential areas that are located in urban or suburban zone granted similar functions of potential breeding grounds for both *Ae. aegypti* and *Ae. albopictus*. Studies by Santana et al. (2006) in Brazil demonstrated that oviposition by *Ae. albopictus* in ovitraps was significantly greater in areas with vegetation in spite of human existence. In contrast, oviposition by *Ae. aegypti* in ovitraps was significantly higher in areas without vegetation but with plentiful human populations. Not only that, the presence of water-bearing containers inside and outside houses mostly in rural areas

contributed to the existence and escalated populations of *Aedes* (Aziz et al., 2012). In the meantime, the presence of *Ae. aegypti* immatures in two dengue prone residential areas in this study indicated its role in the spread of arboviruses in both study areas whereas its existence in one oil palm plantation and two rubber estates exhibited its possibility in transmitting similar diseases within these areas sooner or later.

Culex quinquefasciatus was also discovered in eight study areas of this study. Positive breeding of *Cx. quinquefasciatus* was only detected in two residential areas located in suburban and rural areas, respectively, while the rest were agricultural areas that are located in suburban and rural areas. As revealed by Low et al. (2012), the distribution of *Cx. quinquefasciatus* was pervasive covering urban, suburban, rural and remote zones. *Culex quinquefasciatus* larvae are commonly found in foul water such as in paddy fields, drains and open cesspits (Paramanik et al., 2012). *Culex quinquefasciatus* also has a preference to inhabit water bodies with organic richness such as tanks, puddles, tyre tracks and pools (Mwangangi et al., 2009). In this study, the presence of drainage and irrigation system in agricultural areas with certain parts of them inadequately managed and clogged with waste and leaf debris offered ideal breeding grounds with organic content richness for the growth of *Cx. quinquefasciatus* larvae.

Armigeres subalbatus was also collected in ovitraps placed in all three rice cultivation areas, a rubber estate (Kota Tinggi RB) and a dengue prone residential area (Kota Tinggi DEN). *Armigeres subalbatus* is generally found near to human habitations with improper sanitation (Thankachan & Gopinath, 2017). Hence, it was not surprising to encounter *Ar. subalbatus* in all three rice cultivation areas as villagers' houses were scattered within these areas which made the proper management of disposal and sanitation system difficult.

Moreover, *Uranotaenia* sp. was collected only in ovitraps placed in Kota Tinggi RB. Shady and gloomy surroundings under rubber trees, edge forest and condensed vegetation in Kota Tinggi RB were proposed as preferred oviposition and resting grounds for *Uranotaenia* sp. At present, *Uranotaenia* sp. is not considered as a medically important mosquito species locally. In Malaysia, there is no comprehensive study on *Uranotaenia* sp. that has been undertaken thus far. As reported by Thongsripong et al. (2013), *Uranotaenia* sp. is a common mosquito species in the forest but hardly to be identified.

To the best of my knowledge, this study is the initial attempt of dengue vector surveillance which comprises different types of agricultural areas. In general, the results of this study revealed the presence of no less than three species of mosquito larvae in each type of agricultural area. Between all types of agricultural areas, *Ae. albopictus* larvae were encountered at highest numbers in rubber estates. As highlighted by Sumodan (2012), rubber estates offer breeding grounds for *Ae. albopictus* in which this mosquito species was able to deposit eggs in latex cups.

Not only that, rice fields and drainage ditches around these areas act as permanent and transient breeding grounds for many mosquito species depending on the paddy developmental stages (Forattini et al., 1993). In Goa, *Ae. albopictus*, *Ar. subalbatus* and *Cx. quinquefasciatus* populations were spotted in rice fields, plant containers and also domestic or peridomestic receptacles (Kulkarni & Naik, 1989). Studies by Jacob et al. (2006) in Kenya displayed rice fields as the most significant breeding grounds for *Culex* mosquitoes including *Cx. quinquefasciatus* which was also observed in the present study. Thongsripong et al. (2013) reported that mosquito populations in the forest habitats were more diverse than in the rice cultivation area. However, in the present study, the diversity of mosquito species was more remarkable in agricultural areas as compared to non-agricultural areas. This scenario could be due to the source richness in

agricultural areas which allow different mosquito compositions to breed and survive within the same environment. Furthermore, the presence of many natural and artificial mosquito breeding receptacles also initiates the existence of different species of mosquito populations in study areas. For example, according to Chatterjee et al. (2015), it was found that coconut shells which usually contain high nutrient contents provided ideal breeding habitats for adult female mosquitoes. Similar justification could be applied for all rice cultivation areas used in this study as many discarded coconut shells could be observed within these study areas.

Numerous studies on dengue vector surveillance by ovitrapping had been conducted previously worldwide. Ovitrap surveillance conducted in Batticaloa district, Sri Lanka uncovered 57% and 43% of *Ae. aegypti* and *Ae. albopictus* larvae, respectively (Dharshini et al., 2011). In contrast, *Aedes* surveillance using ovitraps in Sonitpur district, Assam, India recorded 99.3% of *Ae. albopictus* larvae rather than *Ae. aegypti* larvae (Das et al., 2014).

Owing to the importance of *Aedes* mosquitoes in spreading several arbovirus diseases locally, *Aedes* surveillance using ovitraps was also carried out regularly in Malaysia. Ovitrap surveillance performed by Lim et al. (2010) in two settlements within Pulau Ketam, Selangor, Malaysia revealed *Ae. aegypti* as the predominant species in both study sites. The abundance of *Ae. aegypti* in both sites could be due to less vegetation available, gloomy and humid conditions of most houses and also the location of Pulau Ketam which is nearby seaport where *Ae. aegypti* was first introduced in Malaysia in early days. On the other hand, *Aedes* surveillance using ovitraps by Wan-Norafikah et al. (2012) indicated an equal role of *Ae. aegypti* and *Ae. albopictus* in transmitting dengue in all localities selected even though *Ae. albopictus* was more prevalent in Kg. Paya Rambai, Kelantan and Sepanggar-Karamunsing, Sabah while *Ae. aegypti* was more dominant in Kg, Ladang-Pasir Panjang, Terengganu. Furthermore,

dengue vector surveillance was conducted in two suburban residential sites within Kampar, Perak, Malaysia for thirteen weeks (Ho et al., 2014). *Ae. albopictus* was found to be the dominant species in both study sites with OI of nearly 95.00%. Ovitrap surveillance was also performed by Rozilawati et al. (2015) in thirteen study sites within Selangor, Kuala Lumpur and Penang Island. Once again, *Aedes albopictus* was found to be the predominant vector species in all study sites except in Taman Paling Jaya, Selangor and Sentul Utama Flat, Kuala Lumpur. *Aedes* surveillance using ovitraps was also carried out by Noor Afizah et al. (2015b) in two villages within Carey Island, Selangor, Malaysia. Complete dominance of *Ae. albopictus* was demonstrated in ovitraps placed indoors and outdoors with OI ranging from 62.5% to 88.0%. These findings showed the capacity of *Ae. albopictus* to breed in any habitats available.

As stated by Lee (1992b), an area with OI of more than 10% for *Aedes* species possesses a possible risk of dengue outbreak. As exhibited in this study, the OI documented for all study areas ranged from 64.00% to 96.00%. Hence, human inhabitants of all study areas were highly vulnerable to the transmission of dengue viruses. According to the Sector of Vector-borne Disease, Disease Control Division, Ministry of Health Malaysia (2005), an area with the OI of 30% or more is classified as level 3. At this point, inspection of breeding habitats, law enforcement, source reduction, fogging and health consciousness promotion are compulsory to be carried out.

The invasion of any species of mosquito larvae collected in this study inside or outside premises will not be discussed as ovitraps utilized in all study areas of this study were placed randomly without any discrimination between indoors and outdoors. Nevertheless, the phenomenon of species invasion of *Aedes* mosquitoes from their usual environment to recent settings had been emphasized by previous researchers. For instance, Bagny et al. (2009) reported that for more than half a century ago, in Reunion

Island, *Ae. aegypti* was commonly co-bred with *Ae. albopictus* in rock holes. However, by the twentieth century, an ecological succession of *Ae. albopictus* had caused the diminution of *Ae. aegypti* populations in the island. In Malaysia, *Aedes* surveillance using ovitraps was carried out by Mohiddin et al. (2015) in three dengue sites in Penang Island. The most abundant species was *Ae. albopictus* (92.4%) while the rest were *Ae. aegypti* (7.6%). The ovitrap index for all study sites ranged from 8% to 77%. Even though *Ae. albopictus* is acknowledged as a rural mosquito, this mosquito species was found to breed indoors at higher rates than *Ae. aegypti* in all study sites. This scenario showed that *Ae. albopictus* has been adapting to urban settings and creating overlap populations with *Ae. aegypti*. The detection of *Ae. albopictus* breeding indoors could also be either because of the absolute absence of *Ae. aegypti* or the ability of *Ae. albopictus* to oviposit in a wide range of containers (Noor Afizah et al., 2015b). The destructive effect of *Ae. aegypti* populations due to the invasion of *Ae. albopictus* could alter the transmission dynamics of mosquito-borne pathogens.

In addition, both residential areas and agricultural areas share the similarity in terms of the use of chemicals either for management of crop pest or vector control approaches. Hence, the consequences of the extensive and intensive use of pesticides which include herbicides, insecticides and fungicides in agricultural areas as well as insecticides for the control of mosquito populations in residential areas had been highlighted by few researchers. The use of agrochemicals influences the physicochemical characteristics of aquatic breeding grounds and thus, will indirectly affect the selection of breeding habitats by larvae and adult mosquitoes (Kipyab et al., 2015). For example, containers with fertilizer treatments attracted *Cx. quinquefasciatus* female mosquitoes to lay eggs in these containers while the development of *Cx. quinquefasciatus* larvae and pupae in agricultural pesticide-treated containers was slower although their emergence to adult stage became faster (Kibuthu et al., 2016). Therefore, the presence or absence of a

mosquito species in both agriculture and residential areas could be influenced by the utilization of chemicals as well as the frequency and dosage of these pesticides or insecticides.

Ovitraps are useful in determining the abundance and breeding behaviour of container-breeder mosquito species especially *Aedes*. Dibo et al. (2005) proved the efficiency of ovitraps in detecting *Aedes* vectors as some of their ovitraps were positive as early as on the first week of deployment. In Malaysia, Wan-Norafikah et al. (2009) confirmed the sensitivity and consistency of ovitraps in detecting *Aedes* populations in the environment even at low frequency by the utilization of only ten ovitraps in a study site. Fifty ovitraps were placed at each study locality of the present study. As indicated by Chen et al. (2006b), ovitraps should be deployed in not less than 10% of the number of houses at each study area. Based on the observation made in each study area, the number of houses in each of these study areas was far less than 500 houses. Hence, the number of ovitraps utilized in each study area of this research work was more than the minimum number of ovitraps required to be deployed in order to obtain significant results.

Not only that, the use of attractants such as hay infusion in ovitraps rather than the plain tap water induces gravid female mosquitoes to lay eggs in these ovitraps. The foul smelling hay infusion augmented the number of eggs collected (Reiter et al., 1991). Studies by Polson et al. (2002) in Cambodia demonstrated the efficacy of ovitraps enhanced with hay infusion in attracting the egg laying of *Ae. aegypti* in spite of the placement of these ovitraps indoors or outdoors. Gopalakrishnan et al. (2012) also showed higher egg density per trap in hay infusion (623.6 ± 41) compared to egg density per trap in tap water (144.5 ± 61.5) for *Ae. albopictus*. Increased number of collected *Ae. albopictus* eggs due to the use of hay infusion was also confirmed by Velo et al. (2016) in Tirana, Albania.

A total of 570 ovitraps placed in all study areas were positive with the breeding of mosquito immatures. From this number, 490 of them were exclusively colonized by *Ae. albopictus*. These findings confirmed *Ae. albopictus* as the most abundant species in all study areas. *Aedes albopictus* is eminent with its plasticity behaviour which permits it to tolerate and survive within various types of breeding grounds. Alternatively, *Cx. quinquefasciatus* only occurred singly in two ovitraps in Klang OP indicating its preference for different types of breeding habitats.

Mixed breeding of more than one species of mosquito immatures within the same ovitrap indicates the competency of mosquito larvae to share similar environmental conditions that provide sufficient biotic and abiotic factors needed by each larval species. Several elements had been suggested by former researchers as main reasons of mixed infestation among different species of mosquito larvae which include temporal and spatial variation, fast and massive urbanization as well as differences in fecundity and life cycle period of each species (Chan et al., 1971; Leisnham & Juliano, 2009).

Mixed breeding of different species of mosquito immatures discovered during surveillance had been reported by many previous researchers across the globe. In Calcutta city, although *Ae. aegypti* and *Ae. albopictus* were found to show preference to breed indoors and outdoors, respectively, mixed breeding of both species was also demonstrated within the same area (Tandon & Ray, 2000). Shared breeding of *Ae. aegypti* and *Ae. albopictus* within the same breeding habitats was also reported in Cameroon (Simard et al., 2005). Moreover, mixed breeding of *Ae. aegypti* and *Ae. albopictus* was displayed in ovitraps deployed indoors (1 - 6%) and outdoors (4 - 15%) for nine months in Batticaloa, Sri Lanka (Dharshini et al., 2011). Co-infestation of *Aedes*, *Culex* and *Anopheles* in various types of artificial receptacles was also demonstrated in Makkah City, Saudi Arabia (Aziz et al., 2012). In Cote d'Ivoire, mixed

breeding of *Ae. aegypti* and *Cx. quinquefasciatus* was encountered particularly in tyres and unused containers (Zahouli et al., 2017).

Mixed breeding of several species of mosquito immatures had also been highlighted in local surveillance studies. Co-breeding of *Ae. aegypti* and *Ae. albopictus* were observed in 55.4% of positive ovitraps deployed in Georgetown, Penang Island, Malaysia (Yap & Thiruvengadam, 1979) while shared breeding of similar species of larvae was demonstrated at 9% in urban housing areas and 4.5% in vacant lands in Sibu town (Seng & Jute, 1994). After more than a decade, mixed breeding of *Ae. aegypti* and *Ae. albopictus* in ovitraps was demonstrated again in four dengue endemic areas in Kuala Lumpur and Selangor which ranged from 10% to 32% (Chen et al., 2006a). Five years later, ovitrap surveillance performed in Bentong, Pahang, Malaysia by Norzahira et al. (2011) still discovered shared breeding of *Ae. aegypti* and *Ae. albopictus* indoors (7.95% - 29.67%) and outdoors (5.52% - 44.95%).

Larval surveillance using dipping method had also been performed by Low et al. (2012) in Malaysia. During the surveillance, they encountered co-occurrence of *Cx. quinquefasciatus* with *Ar. subalbatus* (1.28% - 3.77%) and also with the other three species which were not discovered in this study. Mixed breeding of *Ae. aegypti* and *Ae. albopictus* in ovitraps was observed again in 2012 by Wan-Norafikah et al. in 15.22% to 31.82% of the total productive ovitraps placed in three selected localities in Kelantan, Terengganu and Sabah, Malaysia. In Penang Island, Malaysia Saifur et al. (2013) reported that shared breeding of *Ae. aegypti* and *Ae. albopictus* was found mainly in urban and suburban sites. In line with this, *Aedes* surveillance by ovitrapping carried out by Rozilawati et al. (2015) few years later in Selangor, Kuala Lumpur and Penang Island also demonstrated mixed breeding of *Ae. aegypti* and *Ae. albopictus* indoors or outdoors or both. Meanwhile, data of ovitrap surveillance performed in Kuala Selangor PD in this study were also supported by a larval surveillance study undertaken at similar

study area which indicated numerous natural and artificial breeding habitats that were positive with *Ae. albopictus* and *Cx. quinquefasciatus* (Wan-Norafikah et al., 2017b).

From the ovitrap surveillance conducted, it is worthwhile to note that mosquito vectors particularly *Ae. albopictus* could dominantly breed on its own within one breeding receptacle or even co-breed with other mosquito species within the same breeding habitat. In fact, to the extent of my knowledge, mixed breeding of *Ae. albopictus* with *Ar. subalbatus* and also co-breeding of *Ae. albopictus* with *Uranotaenia* sp. discovered in the present study are novel and initially reported in Malaysia. This mixed infestation scenario is a challenging hindrance in the vector control programmes since each control method to be applied should cover all species of mosquito vectors occupying the same breeding sites.

5.2 Susceptibility of *Aedes albopictus* Larvae against WHO Diagnostic Dosage of Larvicides

Aedes albopictus larvae from different types of area were exposed to two organochlorine and six organophosphate larvicides at WHO recommended dosages. All larval populations exhibited high resistance against DDT, temephos, chlorpyrifos and bromophos while moderate to high resistance were demonstrated among majority of these larval populations upon selection to dieldrin, malathion, fenitrothion and fenthion. In general, these findings suggested that more volume of larvicides and frequent larviciding activities are needed for effective controls of *Ae. albopictus* larval populations in all types of area if the WHO recommended dosages are used as the diagnostic dosages in the basic preparation of these larvicides in the laboratory before these values are increased many times to obtain the operational dosages to be applied in the field. However, it is worth noting that the utilization of greater volume of larvicides and frequent larviciding activities could worsen the insecticide resistance development

among all *Ae. albopictus* larval populations. Moreover, the use of WHO recommended diagnostic dosages which are very low, in determining the susceptibility status of local mosquito larval populations could also lead to an overestimation and misinterpretation of the susceptibility status of these larval populations. The idea of utilizing the WHO recommended dosages of larvicides as the diagnostic dosages for local mosquito larval strains should be carefully decided as these recommended dosages are too general, whereas *Aedes* larval populations from different areas in Malaysia and even in other countries experienced different history of insecticide exposures which then instigated various levels of susceptibility against each larvicide.

Different larvicides are employed for larval control strategies in Malaysia and other countries over time. Both DDT and dieldrin which belong to organochlorine group of insecticides are persistent organic pollutants (Rahman, 2013) and have been extensively used worldwide in public health and agricultural sector. In old days, DDT had been utilized in the control of *Ae. aegypti* in Malaysia until 1957 before it was substituted with dieldrin (Macdonald, 1958; Nazni et al., 2009). However, since both insecticides are slowly degraded in nature (Jorgenson, 2001; Ahmed et al., 2015), they could remain in the environment for such a very long time. Thus, it is not surprising to spot the presence of resistance phenotype against any of these insecticides among local mosquito species including *Ae. albopictus*.

Owing to the suspension of organochlorines in the vector control activities, the era of the utilization of organophosphates in controlling mosquito vectors had taken place. Organophosphates were believed to be safer than organochlorines since their degradation processes in the environment are faster than the latter insecticide group (Hertz-Picciotto et al., 2018). In Malaysia, temephos and malathion are the recommended organophosphates used for the control of mosquito larvae and adult mosquitoes, respectively (Vythilingam et al., 1992). Even though the use of malathion

for dengue control in Malaysia had actually been relieved by pyrethroids since 1996 (Teng & Singh, 2001), it is still being used in the local space spraying operations in rotation with pyrethroids until now (J. Nor-Jaiza, personal communication, January 15, 2019). Hence, the resistance against both temephos and malathion among Malaysian *Aedes* larvae and adults should be expected.

Other than that, fenitrothion and fenthion exposures have significant effects on both larval and adult stages of mosquitoes (Thomas, 1962; Sulaiman et al., 1999). However, these insecticides are more frequently used as adulticides in Malaysia (Loke et al., 2015, Ong, 2016). Furthermore, chlorpyrifos was found to be effective in eliminating *Anopheles* larvae from the Malaysian paddy fields for at least two to seven days (Yap & Ho, 1977). Apart from that, chlorpyrifos is mostly being utilized in agricultural pest management and also to counter the infestation of pyrethroid-resistant German cockroaches in local food preparation retailers (Ismail & Ngan, 2005; Chai & Lee, 2010). As for bromophos, no report on its field application in Malaysia has been documented so far. In fact, across the globe, only one field trial using bromophos was conducted and reported in Nigeria to control *An. gambiae* and *An. funestus* which was effective for at least five months in the Lagos area and only a month in the Kaduna area (Pant & Self, 1966).

Meanwhile, cross resistance among four larvicides of organophosphates as well as cross resistance between an organochlorine and two organophosphates had been determined. Although not all larvicides tested were utilized in the public health operations, cross resistance involving these larvicides could be due to their selection for agricultural pest control. Thus, it is important to prevent the application of larvicides involved in the cross resistance to minimize the insecticide resistance development against these insecticides among *Ae. albopictus* larvae from all study areas.

There were only limited preceding studies on the susceptibility status of *Ae. albopictus* larvae against WHO recommended diagnostic doses of larvicides as compared to the same testings on *Ae. aegypti*. Moreover, most of tested *Ae. albopictus* populations were subjected only to temephos at former WHO recommended dose of 0.020 mg/L and fewer to the rest of WHO recommended larvicides. For instance, *Ae. albopictus* larvae collected from four different topographies in South Andaman were subjected to WHO recommended doses of temephos (0.020 mg/L), malathion (1 mg/L) and fenthion (0.05 mg/L). These larvae displayed high resistance to temephos but almost fully susceptible against the other two larvicides (Sivan et al., 2015). In Rawalpindi, Pakistan, *Ae. albopictus* larvae collected from four study sites developed tolerance to temephos at WHO recommended dose (0.020 mg/L). These results were not surprising as temephos was the only larvicide utilized in Rawalpindi, Pakistan for the control of malaria and dengue since 1969 (Arslan et al., 2016). The larval bioassays were also undertaken by Bharati & Saha (2017) in India to evaluate the susceptibility status of nine strains of *Ae. albopictus* larvae against temephos at WHO recommended dose (0.020 mg/L) as well as at the dose recommended by the India government (0.0125 mg/L). From the results obtained, Nagrakata strain was tolerant to both recommended doses while Siliguri strain was moderately resistant only to the latter recommended dose.

In Malaysia, Chen et al. (2005a) reported that at the revised WHO recommended diagnostic dose of 0.012 mg/L, *Ae. albopictus* larvae from four localities within Kuala Lumpur and Selangor displayed high resistance against temephos with mortality percentage ranging from 6.40% to 59.50% at 24 hours post-treatment which were much higher than the mortality percentage obtained for *Ae. albopictus* larvae in this study. Meanwhile, in recent studies by Elia-Amira et al. (2018), all strains of *Ae. albopictus* larvae collected from eight districts in Sabah, Malaysia exhibited resistance to WHO

recommended diagnostic doses of DDT, malathion and temephos with zero mortality recorded for the two former larvicides. Chlorpyrifos and dieldrin were the most effective larvicides for almost all strains of *Ae. albopictus* larvae collected within Sabah, Malaysia. Exposure to both fenitrothion and fenthion demonstrated a more than 70% mortality in *Ae. albopictus* larvae from two divisions of Sabah, Malaysia. Besides, a wide range of mortality percentage was noted upon the bromophos selection to *Ae. albopictus* larvae from various districts of Sabah, Malaysia. Hence, these two local findings clearly showed that each larvicide applied at WHO recommended diagnostic dose was not necessarily effective against *Ae. albopictus* populations in all sites even though they are within the same state or country which basically utilize similar procedures of vector control approaches.

5.3 Susceptibility of *Aedes albopictus* Field Strains Larvae against Independent Diagnostic Dosage of Larvicides Established from *Aedes albopictus* Reference Strain Larvae

Besides the exposure to WHO recommended doses of organochlorines and organophosphates, *Ae. albopictus* larvae from all types of area were also subjected to independent diagnostic dosages of organochlorines, organophosphates, carbamates and pyrethroids. In order to achieve this, the reference strain of *Ae. albopictus* larvae was exposed to each of these larvicides at a broad range of concentrations in which at least five concentrations that caused the mortality between 5% to 95% after 24 hours of recovery period were selected to construct the regression line for each larvicide. The double dose of 99% lethal concentration (LC_{99}) values for the reference strain ($2xLC_{99}$) was acknowledged as the revised independent diagnostic dosages of these larvicides for *Ae. albopictus* larvae.

In this research work, all newly established independent diagnostic doses of these larvicides were much higher than the WHO recommended diagnostic doses except for fenthion. According to Macoris et al. (2005), if the WHO recommended diagnostic dosages which were lower than the revised independent diagnostic dosages are used in the resistance monitoring testings, an overestimation of resistance among these *Ae. albopictus* population is highly possible. In contrast, for fenthion, if the WHO recommended diagnostic dose that was higher than the established independent diagnostic dose is utilized upon these *Ae. albopictus* larvae, there is a chance of underestimating the resistance in these populations and also discriminating resistant individuals but not distinguishing a decrease in the susceptibility since the susceptibility status of the reference strain was not exploited as a comparison.

Overall, diversified level of susceptibility was presented by *Ae. albopictus* larvae from different types of agricultural and non-agricultural areas against each larvicide at independent diagnostic doses established from the reference strain of the same species. These results indirectly revealed the miscellaneous history of insecticide exposures and diverse selection frequency in different types of area which thereby suggesting different larvicides to be used in each of these study areas. Based on the independent diagnostic doses acquired, findings of this study showed the suitability of malathion and bromophos as the larvicides of choice in all types of area. The utilization of both fenitrothion and fenthion as larvicides were still acceptable in several agricultural areas but definitely not recommended for the use in dengue prone residential areas. Meanwhile, the plan of employing either temephos or chlorpyrifos in any of the study areas needs to be carefully determined since moderate to high resistance were recorded against both larvicides among all larval populations. On the other hand, regardless of the susceptibility status exhibited among almost all *Ae. albopictus* larval populations against both DDT and dieldrin, both larvicides were still not to be selected as the

larvicides of choice for all study areas as their use in local vector control strategies had already been prohibited.

In comparison between the results of the WHO larval bioassay at WHO recommended dosages and the WHO larval bioassay at independent discriminated diagnostic dosages, moderate to high resistance were exhibited among *Ae. albopictus* larvae from almost all types of area against organochlorines and organophosphates in the earlier bioassays. However, dissimilar patterns of susceptibility among these larval populations against all classes of insecticides were demonstrated in the WHO larval bioassay conducted using the independent discriminated diagnostic dosages established from the reference strain susceptibility data. High susceptibility had been observed among *Ae. albopictus* larvae from various types of area against certain insecticides while some of them were either tolerance or highly resistant to the rest of the insecticides. Overturned findings were also observed for certain organochlorine and organophosphate larvicides tested at both WHO recommended dosages and $2xLC_{99}$ values of the reference strain. As such, for DDT, *Ae. albopictus* larvae from all types of area were classified as resistant when subjected to WHO recommended dose of 0.012 mg/L. However, this scenario was to the contrary when almost all these populations were categorized as susceptible to DDT at 0.8384 mg/L of the established independent discriminated diagnostic dosage. Similar situation was observed for the susceptibility testings of these *Ae. albopictus* larvae against malathion and bromophos.

Hence, even though the findings obtained from the WHO larval bioassays using WHO recommended doses and $2xLC_{99}$ values of the reference strain as outlined by WHO are beneficial in determining the susceptibility status of mosquito larvae, it is strongly suggested that each field population of mosquito larvae is subjected to at least five concentrations of the respective larvicide that will cause mortality between 5% to 95% at 24-hours post-treatment in order to attain the individual LC_{50} value for every

population. This LC_{50} value which will be more specific for one particular population will allow the Health Department and local authorities to precisely verify the susceptibility status of each mosquito species population from that particular area and assist them in the selection of the most suitable larvicide to be applied at the respective locality.

Referring to previous studies conducted in other parts of the world, most researchers carried out larval bioassays using the LC_{50} values instead of establishing their own $2xLC_{99}$ values as outlined by WHO. For instance, fifteen field populations of *Ae. albopictus* larvae collected in Italy were susceptible to temephos with LC_{50} values between 0.0026 and 0.0085 mg/L that were much lower than the WHO recommended dose for temephos (0.012 mg/L) (Romi et al., 2003). In southern India, *Ae. albopictus* immatures collected from two international airports were susceptible to temephos (0.020 mg/L), fenthion (0.05 mg/L), malathion (1.0 mg/L) and fenitrothion (0.06 mg/L) (Sharma et al., 2004).

Furthermore, three separate larval bioassays were also conducted in China. In Haikou city, China, Chen et al. (2016) reported that four of five strains of *Ae. albopictus* larvae were resistant to deltamethrin and beta-cypermethrin while two of these populations were also resistant to permethrin. In Guangzhou, China, the resistance level of four strains of *Ae. albopictus* larvae were higher against pyrethroids as compared to organochlorines, carbamates and organophosphates with resistance ratios against all these insecticides ranged from 2.2 to 275 (Yiguan et al., 2017). In addition, the LC_{50} values against deltamethrin obtained for six strains of *Ae. albopictus* larvae from urban, suburban and rural areas of southern China ranged between 0.011 and 0.038 mg/L (Li et al., 2018). Meanwhile, larval bioassays conducted by Ishak et al. (2015) in Malaysia showed higher LC_{50} for temephos in *Ae. albopictus* from Penang (0.020 mg/L) and

Kuala Lumpur (0.015 mg/L) as compared to *Ae. aegypti* from similar study sites (0.006 – 0.008 mg/L).

Up till now, only two accessible former studies reported on the revised independent diagnostic dosages of larvicides using their reference strain of either *Ae. aegypti* or *Ae. albopictus* larvae but only covered between two and three common larvicides. Hence, the present study is the first attempt of establishing independent diagnostic doses of all classes of larvicides using the reference strain of *Ae. albopictus* larvae. In Brazil, Macoris et al. (2005) reported that the diagnostic doses of fenitrothion, malathion and temephos for their *Ae. aegypti* Rockefeller strain were 0.0100 mg/L, 0.200 mg/L and 0.0080 mg/L, respectively, in which all these concentrations were much lower than the diagnostic doses of similar larvicides obtained in the current study. On the other hand, Rahim et al. (2016) performed almost similar larval bioassays to determine the discriminating diagnostic doses of temephos and malathion for *Ae. albopictus* susceptible strain reared at the Vector Control Research Unit (VCRU), Universiti Sains Malaysia (USM), Penang, Malaysia. They reported that the revised discriminating diagnostic doses of temephos and malathion for their reference strain were 0.020 mg/L and 0.200 mg/L, respectively. Their revised discriminating diagnostic dose of temephos was similar to the previous WHO recommended diagnostic dose of temephos while their revised discriminating diagnostic dose of malathion was higher than WHO recommended diagnostic dose of malathion but lower than the discriminating diagnostic dose of malathion obtained in the present study. All their five field strains collected from Penang showed either incipient resistance or high resistance against both larvicides. Rahim et al. (2017) also suggested revised diagnostic doses of malathion, permethrin and deltamethrin for Malaysian *Ae. albopictus* adults which were either much lower (for malathion) or much higher (for permethrin and deltamethrin) than the WHO recommended doses for *Ae. aegypti* adults. These results indicated the differences

and significance of attaining the local diagnostic dosages in order to accurately determine the susceptibility status of local mosquito populations against insecticides. Nevertheless, the process of obtaining the revised diagnostic dosages for all commonly used insecticides is time-consuming, labour intensive and requires a large number of mosquito samples.

According to Lee et al. (1997), the differences in the diagnostic dosages could be due to genetical backgrounds of the mosquito populations. Moreover, since the diagnostic dose is closely related to sensitivity and specificity, the decrease of diagnostic dose could indicate an escalation of sensitivity but with the possibility of picking up either the susceptible strain or the resistant strain (Macoris et al., 2005).

Temephos is one of the most common larvicides utilized in the vector control strategies in Malaysia. As mentioned by Chen et al. (2005b), the operational dose of temephos for larviciding activity in Malaysia is 1 mg/L. Even though all field strains employed in the current study showed either incipient resistance or high resistance against temephos at 2xLC_{99} value of 0.0660 mg/L, the percentage mortality demonstrated by all these populations was at least 84%. Thus, it is expected that total mortality could be achieved in these field populations when temephos is applied in the field at the operational dose of 1 mg/L. However, environmental parameters such as rain could also diminish the effectiveness of the insecticides (Rahim et al., 2016). Not only that, the migration of either susceptible or resistant mosquitoes could also affect the proportion of susceptible and resistant individuals in the field populations (Lee et al., 1997) which will indirectly influence the efficacy of the insecticides.

Since the independent diagnostic doses of larvicides for the susceptibility testings of field strain larvae are established from the reference strain of the same species, it is crucial to ensure that the susceptibility of the local reference strain against all insecticides is maintained at the very maximum level in order to sustain its reference

status. Furthermore, the establishment of local diagnostic dosages based on our own Malaysian reference strain is important in order to obtain a more reliable, significant and convincing findings on the susceptibility status of local mosquito vectors against all commonly used insecticides. The susceptibility level of the reference strain should be utilized as a guidance or an indication in the bioassay performed upon the field strain mosquitoes (Macoris et al., 2005). Even though results of the susceptibility tests obtained for field strain mosquitoes that had been calculated using the results of the susceptibility tests of the local reference strain will be more useful to only one particular country where the testings were conducted, findings obtained are still comparable with reports of susceptibility testings from other countries that follow the same techniques. Hence, special attention and efforts should be given to ensure that the local laboratory strain used as a reference strain in the study is well-maintained in the laboratory for many generations with no compromise on any insecticide selection either purposely or unintentionally. Continuous monitoring on the susceptibility of the reference strain against all insecticides should be carried out to prevent the resistance development against any insecticides and thus, maintaining its status as a dependable reference strain in all mosquito studies. Researchers in other laboratories across the world also utilized several well-recognized laboratory susceptible strains such as New Orleans (NO) strain, Bora Bora strain or Rockefeller strain of *Ae. aegypti* as the reference strain of their studies. However, not all laboratories including entomology laboratories available in Malaysia have the access to these foreign laboratory susceptible strains which require various import procedures and legislations. Furthermore, the reference strain of *Ae. albopictus* used in this study originated from the Medical Entomology Unit, Institute for Medical Research (IMR) Malaysia. The Institute for Medical Research (IMR) is the research and diagnostic centre of the Ministry of Health (MOH) Malaysia in which all decisions on insecticides to be employed or any other approaches to be performed in the

local vector control activities will be based on the research findings by researchers of IMR. Moreover, the use of local laboratory susceptible strains in determining the diagnostic dosages of insecticides before being compared with the field populations of the same species will reduce the differences between these strains to obtain more accurate data since all strains possess relatively similar genetical backgrounds (Lee et al., 1997). Hence, the employment of local laboratory susceptible strain especially from IMR, Malaysia remains the best option for now.

Other than that, the cross resistance between larvicides from the same insecticide class was exhibited in organochlorines, organophosphates and carbamates, whereas the cross resistance between larvicides from different insecticide classes involved all four classes tested in this study. Cross resistance among larvicides from the same and different insecticide classes are not solely due to vector control activities since not all larvicides tested were employed in Malaysian public health, but also because of their extensive application in the agricultural practice. Hence, it is crucial for the Health Department and the local authorities to ensure that only larvicides that were not involved in the cross resistance detected are used in all study localities to combat the breeding of *Ae. albopictus* larvae.

5.4 Susceptibility of *Aedes albopictus* Adults against WHO Diagnostic Dosage of Adulticides

Aedes albopictus adults from three types of agricultural areas as well as fogging-free and dengue prone residential areas were subjected to eleven insecticides. Different levels of susceptibility against all selected adulticides were presented by these *Ae. albopictus* populations. Referring to the KT_{50} values, the fastest knockdown effects were exerted by permethrin 0.75%, deltamethrin 0.05% and cyfluthrin 0.15%. Contrarily, both dieldrin 4% and fenitrothion 1% required the longest time to act on all

Ae. albopictus adults tested as zero knockdown was recorded for all populations throughout the one hour exposure period.

With the exception of dieldrin 4% and fenitrothion 1% exposures, at least 27.00% to 97.00% knockdown was achieved at 60 minutes of the exposure time for organochlorine, organophosphate and carbamates tested. Overall, the exposure of propoxur 0.1% displayed highest percent knockdown in almost all *Ae. albopictus* populations at 60 minutes of the exposure time. On the other hand, by 30 minutes of the exposure time for pyrethroids, higher range of percent knockdown was recorded for *Ae. albopictus* adults from different types of agricultural area as compared to the percent knockdown for *Ae. albopictus* adults from fogging-free and dengue prone residential areas. These findings showed that *Ae. albopictus* populations from agricultural areas were generally more susceptible to pyrethroids than *Ae. albopictus* populations from non-agricultural areas.

After 24 hours of recovery period, dieldrin exerted the best lethal effects to all populations of *Ae. albopictus* adults compared to any other employed organochlorines, organophosphates and carbamates. However, since the use of organochlorines is prohibited in Malaysia, the utilization of malathion, propoxur and fenitrothion is generally the better options for the control of *Ae. albopictus* adults in all agricultural and non-agricultural areas selected for this study. Meanwhile, complete mortalities were achieved at 24 hours post-treatment among all *Ae. albopictus* populations upon the selection to five pyrethroids. These findings indicated pyrethroids as the best adulticides to be applied for the control of *Ae. albopictus* populations in all types of area selected for this study especially the agricultural areas.

In comparison between different types of agricultural areas, *Ae. albopictus* adults from both oil palm plantations and rubber estates generally required lesser time to achieve 50% knock down of their populations than *Ae. albopictus* adults from paddy

cultivation areas. This scenario indicated the possibility of higher and more frequent insecticides exposures among *Ae. albopictus* in paddy cultivation areas as compared to the selection pressure received by *Ae. albopictus* populations in oil palm plantations and rubber estates. The insecticide exposures within all agricultural areas derived mainly from the agricultural activities and being worsened by the use of household insecticides by the communities of these agricultural areas and also by the vector control operations of public health in the future when there is any occurrence of mosquito-borne diseases within these areas.

On the other hand, *Ae. albopictus* adults from both fogging-free and dengue prone residential areas generally needed more time to attain 50% knockdown as compared to *Ae. albopictus* populations from agricultural areas. Moreover, the mortality percentages recorded for *Ae. albopictus* adults from non-agricultural areas were often lower than the mortality percentages of *Ae. albopictus* populations from agricultural areas upon the exposure to organochlorines, organophosphates and carbamates. The least knockdown and mortality percentages of *Ae. albopictus* adults from dengue prone residential areas after having subjected to all selected adulticides could be due to regular mosquito control activities conducted in these localities especially during the occurrence of any reported dengue cases or during dengue outbreak. Conversely, the preceding use of insecticides or other pesticides in the crop pest management prior to the development of these fogging-free residential areas could contribute to the reduction of susceptibility of *Ae. albopictus* adults against public health insecticides. This is because all fogging-free residential areas selected for this study had been developed in less than 10 years. Based on the informal interview with the representative of the developer company of Shah Alam FF residential area as well as the residents who have stayed in these three fogging-free residential areas since the opening of these localities, Shah Alam FF was formerly fostered as an oil palm plantation while Padang Serai FF and Temerloh FF

were previously established as rubber estates prior to the residential development. Other than that, the use of domestic insecticides which are usually available in the supermarket and commercial outlets could also trigger the decrease of susceptibility level of *Ae. albopictus* from fogging-free and dengue prone residential areas against tested adulticides. Furthermore, since the agricultural areas in Malaysia are located mostly in suburban and rural regions, the accessibility of larvicides and adulticides by residents in non-agricultural areas at the nearby supermarket and commercial outlets is higher as compared to residents in human habitations within agricultural areas. Commercial household insecticides in Malaysia consisting both of larvicides and adulticides are usually comprised of active ingredients from organophosphates and pyrethroids. Hence, the development of resistance among tested *Ae. albopictus* populations against organochlorines and carbamates could be due to the former utilization of organochlorines like DDT and dieldrin in Malaysia decades ago as well as the cross resistance between carbamates with organophosphates.

In addition, correlation tests were performed to determine any cross resistance between all adulticides employed in the present study. Cross resistance was observed within carbamates, within pyrethroids, between organophosphates and carbamates as well as between carbamates and pyrethroids. These outcomes indicated the possibility of rapid resistance development among tested *Ae. albopictus* populations against all classes of insecticides. Therefore, the rotational use of insecticides with different modes of actions in the vector control strategies and crop pest control activities conducted within these agricultural and non-agricultural areas is truly essential.

Resistance development among *Ae. albopictus* adults against numerous adulticides at similar or different concentration and exposure time have been documented across the world. Sharma et al. (2004) revealed that *Ae. albopictus* adults from Thiruvananthapuram and Cochin of India developed resistance to DDT and dieldrin but

susceptible to malathion, fenitrothion, propoxur, permethrin, deltamethrin and lambdacyhalothrin. In another study conducted in India as well, *Ae. albopictus* adults collected from five localities were subjected to DDT 4%, permethrin 0.75% and deltamethrin 0.05% (Kushwah et al., 2015a). Different levels of resistance were demonstrated by all strains against DDT ranging from 61% to 92% mortalities which were about the same range with the results obtained in the present study. Additionally, only Kerala and Delhi strains displayed moderate resistance against permethrin and deltamethrin, respectively, while the remaining populations were susceptible to these two pyrethroids. Furthermore, *Ae. albopictus* adults collected from different topographies in South Andaman demonstrated various level of resistance against DDT 4%, fenitrothion 1%, bendiocarb 0.1%, permethrin 0.75%, lambdacyhalothrin 0.05% and cyfluthrin 0.15% (Sivan et al., 2015). Latest research work by Chatterjee et al. (2018) showed that *Ae. albopictus* adults collected from three districts of West Bengal, India were highly resistant to DDT but susceptible to both malathion and deltamethrin.

Meanwhile, four wild populations of *Ae. albopictus* adults in Thailand were moderately resistant to permethrin while another field strain of the same mosquito species developed high resistance against the same pyrethroid with their mortality percentages ranging from 80% to 97% and less than 80%, respectively (Chuaycharoensuk et al., 2011). However, all these five field strains of *Ae. albopictus* were susceptible to both deltamethrin and lambdacyhalothrin.

In Cameroon, *Ae. albopictus* adults collected from Yaounde, Buea and Bertoua were highly resistant to DDT 4% with mortality percentage of 36.3%, 47.0% and 80.5%, respectively (Kamgang et al., 2011). At the same time, Yaounde *Ae. albopictus* population was also highly resistant to deltamethrin 0.06% with mortality percentage of 83.3%. However, all three field populations of *Ae. albopictus* adults tested were susceptible against propoxur 0.3% and fenitrothion 0.5%. Six years later, Kamgang et

al. (2017) once again reported on resistance development among *Ae. albopictus* adults from three localities in Yaounde, Cameroon against deltamethrin 0.05%, bendiocarb 0.1% and DDT 4% but fully susceptible to malathion 5%.

Besides that, two adult mosquito populations of *Ae. albopictus* from Florida, United States of America developed resistance to DDT and malathion with mortality percentages ranging from 54% to 75% and 80% to 86%, respectively (Marcombe et al., 2014). Seven strains of *Ae. albopictus* adults from North Carolina were also found to be either developing resistance or highly resistant to malathion and also chlorpyrifos but all these strains were susceptible to permethrin and deltamethrin (Richards et al., 2018). Alternatively, two from almost twenty wild strains of *Ae. albopictus* which were collected from permethrin-treated areas in Italy exhibited resistance to permethrin 0.75% but all tested populations were susceptible to deltamethrin 0.05% (Pichler et al., 2018).

In Bangui, Africa, five from six populations of *Ae. albopictus* adults developed resistance against DDT (Ngoagouni et al., 2016). Moreover, two of these populations were resistant to deltamethrin, whereas one of them developed resistance against propoxur while the other one population was resistant to fenitrothion. Another study carried out in Rawalpindi, Pakistan also described on high resistance among four strains of *Ae. albopictus* adults against DDT (37% to 53 % mortality), malathion (61% to 76% mortality), bendiocarb (64% to 71% mortality) and permethrin (62% to 74% mortality) (Arslan et al., 2016). Not only that, moderate resistance was also observed among these populations against deltamethrin (90.33% to 95% mortality) and lambdacyhalothrin (90% to 96 % mortality).

In addition, *Ae. albopictus* adults from Guangzhou and Shenzhen, China demonstrated KT_{50} values of 18.2 and 24.7 minutes, respectively, upon exposure to deltamethrin 0.05% (Xu et al., 2016). After 24 hours of recovery period, both strains

showed incipient resistance to deltamethrin with mortality rates of 96.1% and 90.1%, respectively. A year later, Yiguan et al. (2017) also reported on high resistance against deltamethrin and DDT displayed among *Aedes albopictus* adults collected from nine sites in China and suggested a cross resistance between these two insecticides. Besides, six strains of *Ae. albopictus* from urban, suburban and rural areas of Southern China were found to be susceptible to malathion (Li et al., 2018). However, one urban strain was resistant to deltamethrin and propoxur while two urban strains and one rural strain were resistant to DDT.

In Kuala Lumpur, Malaysia, *Ae. albopictus* adults from four dengue prone residential areas that were subjected to permethrin 0.75% exhibited lethal time for 50% population (LT_{50}) values ranging from 19.39 minutes to 20.65 minutes (Wan-Norafikah et al., 2013b) which were much lower than the KT_{50} values obtained for all *Ae. albopictus* populations employed in the present study. Furthermore, two strains of *Ae. albopictus* adults collected from residential areas in Kampar, Perak, Malaysia were also more susceptible to permethrin 0.75% and deltamethrin 0.05% (Ho et al., 2014) as compared to all *Ae. albopictus* populations of the current study. However, both Kampar strains of *Ae. albopictus* developed resistance to malathion 5% with KT_{50} values of 48.46 minutes and 62.69 minutes, respectively whereby these KT_{50} values were not much different with the KT_{50} values obtained for *Ae. albopictus* populations from agricultural and non-agricultural areas tested in the present study. Additionally, both Kampar strains of *Ae. albopictus* adults also developed high resistance to fenitrothion 1% which was also in line with the current study.

Other than that, Ishak et al. (2015) conducted the WHO adult bioassays on *Ae. albopictus* adults from four dengue areas of Peninsular Malaysia. *Ae. albopictus* adults from Kuala Lumpur and Kota Bharu were highly resistant to DDT while Penang strain was fully susceptible to DDT. *Aedes albopictus* adults from Penang, Kuala Lumpur and

Johor Bahru were also resistant to dieldrin and bendiocarb while resistance against malathion was detected only among *Ae. albopictus* adults from Kuala Lumpur and Johor Bahru. Nevertheless, *Ae. albopictus* populations from all study sites were completely susceptible to both permethrin and deltamethrin except for Kuala Lumpur strain which showed a moderate resistance against both pyrethroids.

Two years later, Rahim et al. (2017) in Penang, Malaysia established revised diagnostic doses of malathion, permethrin and deltamethrin for *Ae. albopictus* adults which were 2.4%, 0.95% and 0.28%, respectively. The revised diagnostic doses for permethrin and deltamethrin were higher than the WHO recommended doses and the doses of impregnated papers used in the present study. Therefore, complete mortalities obtained in this study upon exposure to both permethrin and deltamethrin at 0.75% and 0.05% proved that *Ae. albopictus* adults from all agricultural and non-agricultural areas of the current study were truly susceptible to these pyrethroids. Conversely, although the malathion 5% used in the present study was far higher than the revised diagnostic dose of malathion suggested by Rahim et al. (2017), *Ae. albopictus* populations from all agricultural and non-agricultural areas had already developed incipient to high resistance against malathion 5%. These findings clearly indicated the increasing malathion resistance in local populations of *Ae. albopictus*.

In the present study, it is worth noting that the dose of permethrin implemented was 0.75% which is three times higher than the WHO recommended dose for *Aedes* species (0.25%). Higher doses of impregnated papers of dieldrin, malathion, deltamethrin and lambdacyhalothrin were also utilized in the present study as compared to their WHO recommended doses. This is because the impregnated papers of these insecticides that follow the WHO recommended diagnostic doses were not available for purchase at the WHO Collaborating Centre; Vector Control Research Unit (VCRU), Universiti Sains Malaysia, Penang during this study. Hence, adult mosquito bioassays using papers

impregnated with the WHO recommended doses of these insecticides should be conducted in the future to assess the susceptibility status of these mosquito populations against these WHO recommended doses. Nevertheless, the use of higher doses of these insecticides than their WHO recommended dosages in this study still provides valuable information that could also be considered as an early indication of insecticide resistance development in tested mosquitoes. As such, for malathion, even by the utilization of impregnated paper with higher dose than the WHO recommended dose, all tested *Ae. albopictus* field strains had already exhibited moderate to high resistance against malathion. This scenario had indirectly showed the rapid development of malathion resistance in these *Ae. albopictus* populations.

Overall, all five pyrethroids selected in the present study were the most effective for the control of *Ae. albopictus* adults from all agricultural and non-agricultural areas. Nevertheless, regular monitoring on the susceptibility status of these *Ae. albopictus* populations against public health insecticides as well as the rotational utilization of insecticides with different resistance mechanisms are necessary to be conducted in order to prevent the development of higher resistance level or irreversible gene mutations at the target site of insecticides within these *Ae. albopictus* populations.

5.5 Characterization of Biochemical Enzyme Mechanisms Contributing to Insecticide Resistance in *Aedes albopictus* Larvae and Adults

Metabolic resistance is generally associated with elevated activities of one or several detoxification enzymes in the mosquito biochemical pathways of insecticide mechanism (Araujo et al., 2016). The detoxification enzymes that typically associated with insecticide resistance in insects include carboxylesterases, mixed function oxidases and glutathione-S-transferases (Zhou et al., 2015). Elevated carboxylesterases activity is linked to the resistance to organophosphates, carbamates and pyrethroids (Jones et al.,

2013; Pereira et al., 2014; Kudom et al., 2015). Mixed function oxidases are associated with resistance to pyrethroids, organophosphates and carbamates (Alvarez et al., 2013). Elevated level of glutathione-S-transferases activity is correlated with resistance to organochlorines particularly DDT, organophosphates and pyrethroids (Che-Mendoza et al., 2009). Alternatively, the target site mutations of acetylcholinesterase are closely related to organophosphate and carbamate resistance in mosquitoes (Essandoh et al., 2013).

In the present study, enzyme microassays had been performed to detect the presence and activity level of non-specific esterases, mixed function oxidases, glutathione-S-transferases and insensitive acetylcholinesterases in *Ae. albopictus* populations from agricultural and non-agricultural areas. Generally, different activity level of each enzyme was expressed at both stages of *Ae. albopictus*. For non-specific esterases, the role of both α-EST and β-EST in the resistance mechanism was more visible in *Ae. albopictus* adults. Whilst the significant involvement of α-EST could still be perceived among *Ae. albopictus* larval populations from fogging-free and dengue prone residential areas, any significant engagement of β-EST in the resistance mechanism of tested *Ae. albopictus* larvae was unclear. Nevertheless, all populations of *Ae. albopictus* larvae and adults were homogeneous with low EST activities.

Contrarily, despite many *Ae. albopictus* larval and adult populations from agricultural and non-agricultural areas that were already progressing from homogeneous population with low MFO activity to heterogeneous population in MFO activity, the significant role of MFO in the resistance mechanism of these *Ae. albopictus* populations was not detected. These findings undoubtedly supported the results of WHO larval and adult mosquito bioassays conducted that indicated the nearly full susceptibility of *Ae. albopictus* populations from both agricultural and non-agricultural areas against all pyrethroids employed in this study. In other words, mixed function oxidases did not

play any significant role in the resistance mechanisms of *Ae. albopictus* from all agricultural and non-agricultural areas against organochlorines, organophosphates and carbamates tested.

Meanwhile, the function of glutathione-S-transferases in the resistance mechanism of *Ae. albopictus* populations from agricultural and non-agricultural areas was in contrast to non-specific esterases. Whilst all *Ae. albopictus* larval and adult populations were homogeneous with low GST activity, the significant role of GST in the resistance mechanism of these mosquito populations was more conspicuous at larval stage instead of adult stage as observed in non-specific esterases. Glutathione-S-transferases was significantly involved in the resistance mechanism of *Ae. albopictus* larvae and adults from paddy cultivation areas but its presence in *Ae. albopictus* populations of oil palm plantations was irrelevant for their resistance mechanism at larval and adult stages.

In line with the involvement of acetylcholinesterase in the resistance of organophosphates and carbamates (Menozzi et al., 2004), the efficacy of propoxur as a larvicide and adulticide among *Ae. albopictus* populations from different agricultural areas as well as fogging-free and dengue prone residential areas was evaluated. Based on the significant reduction of acetylcholinesterase activity upon the addition of propoxur, this carbamate was still effective as an adulticide in combating *Ae. albopictus* adults in all types of agricultural and non-agricultural areas in this study. However, propoxur was only effective to be applied for larviciding activity in oil palm plantations and fogging-free residential areas. Furthermore, the mean percent acetylcholinesterase activity in propoxur-inhibited fraction (%) recorded for all agricultural and non-agricultural areas was high especially at adult stage but these values were almost equivalent with the values of the reference strain. At larval stage, *Ae. albopictus* from all types of area were homogeneous with high AChE activity, whereas all these populations were heterogeneous in AChE activity at adult stage.

In regard to the use of propoxur in the acetylcholinesterase (AChE) microassay, it was suggested by World Health Organization (1998b) that the concentration of propoxur should be set as it should allow us to clearly differentiate between susceptible individuals, heterozygote and homozygote resistants via several methods. Since these procedures of establishing the appropriate concentration require many samples and are time-consuming, it was decided that the concentration of propoxur used in the AChE microassay of this study followed the same concentrations employed in the WHO larval and adult bioassays, respectively, so that findings from all these testings would be comparable to one another.

Overall, significant roles of elevated level of detoxification enzyme activities were more noticeable in *Ae. albopictus* adult populations tested rather than the larval populations. Substantial elevated levels of α-EST, β-EST, GST and AChE activities were found in *Ae. albopictus* adults while only elevated levels of α-EST and GST activities were notably involved in the resistance mechanism of these larval populations. Nevertheless, the association among these enzymes at both stages was difficult except for the α-esterases activity. Furthermore, these findings generally indicated the role of α-EST and GST in the resistance mechanisms of all tested *Ae. albopictus* larvae especially against fenitrothion, fenthion, temephos and chlorpyrifos as well as propoxur and bendiocarb. On the other hand, α-EST, β-EST, GST and AChE generally played crucial roles in the resistance mechanisms of all *Ae. albopictus* adult populations against organochlorines, organophosphates and carbamates selected as adulticides for this study.

Research work on the role of detoxification enzymes in the insecticide resistance development in mosquitoes had also been previously conducted across regions. However, most of these enzyme microassays were performed on *Ae. aegypti* instead of *Ae. albopictus*. In Venezuela, organophosphate resistance detected in *Ae. aegypti*

collected from two states was triggered by nonspecific esterase activity while mixed function oxidases were involved in carbamate resistance among the same mosquito populations (Mazzarri & Georghiou, 1995). On the other hand, *Ae. aegypti* from Santiago de Cuba which exhibited different levels of resistance against fenthion, malathion, deltamethrin, temephos, methyl-pirimifos, cypermethrin and chlorpyrifos displayed elevated level of activities for only esterases and glutathione-S-transferases (Rodriguez et al., 1999). About six years later, Flores et al. (2005) revealed a significant role of α-esterases in the permethrin resistance among four field strains of *Ae. aegypti* from Baja California, Mexico. However, for permethrin and deltamethrin resistance observed in *Ae. aegypti* of Bandung-West Java strain, both mixed function oxidases and esterases were found to be involved in the resistance mechanisms occurred (Ahmad et al., 2007). Meanwhile, temephos resistance detected in *Ae. aegypti* from Nakhon Sawan 2 was conferred by the elevated level of esterases (Poupardin et al., 2014), whereas significant elevated level of glutathione-S-transferases activity was detected in one field strain of *Ae. aegypti* adults from Singapore that developed resistance against DDT (Koou et al., 2014b).

As for *Ae. albopictus*, glutathione-S-transferases activity was found to be associated with DDT resistance in this mosquito species collected from United States of America while β-esterases activity was significantly correlated with possible malathion resistance developed among the same mosquito populations (Marcombe et al., 2014). Moreover, Ngoagouni et al. (2016) reported on the increased activity of α-esterases, β-esterases, glutathione-S-transferases and mixed function oxidases following the insecticide resistance development in several strains of *Ae. albopictus* from Bangui, Africa. However, the association between the elevated activity of these enzymes with their various resistance level against DDT, deltamethrin, propoxur and fenitrothion was unclear. In India, higher level of esterases in *Ae. albopictus* captured from nine study

sites as compared to the level of mixed function oxidases in the same mosquito population showed the more crucial role of esterases in temephos resistance detected in these mosquitoes (Bharati & Saha, 2017).

Current studies by Li et al. (2018) in southern China found that glutathione-S-transferases levels were significantly higher in four of six populations of *Ae. albopictus* adults that exhibited resistance to DDT, deltamethrin and propoxur while esterases level was significantly higher in only one of those populations. Additionally, in India, two from eight field strains of *Ae. albopictus* larvae were highly resistant to temephos while three *Ae. albopictus* adult populations were moderately resistant to cyfluthrin (Rath et al., 2018). At larval stage, significant elevated activities of α-esterases, β-esterases and glutathione-S-transferases were observed in these populations. On the other hand, at adult stage, significant elevated activities of mixed function oxidases and glutathione-S-transferases were displayed in certain populations tested while both α-esterases and β-esterases activities were clearly high in all populations.

In Malaysia, Lee & Chong (1995) demonstrated elevated glutathione-S-transferases activity in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. maculatus*. However, no correlation was discovered between the elevated glutathione-S-transferases activity with DDT susceptibility in all mosquitoes employed. Fifteen years later, Selvi et al. (2010) also reported that there was no association between the susceptibility level of *Ae. albopictus* against malathion with the elevated level of non-specific esterases activity either at larval or adult stage.

As for *Ae. albopictus* collected from Kuala Lumpur, despite significant association exhibited between LT_{50} values of four permethrin-exposed populations with mixed function oxidases level, the small decrease in the level of mixed function oxidases activity in these populations upon the PBO + permethrin exposure suggested the involvement of other detoxification enzymes in the susceptibility reduction of *Ae.*

albopictus adults against permethrin (Wan-Norafikah et al., 2013b). Furthermore, Chen et al. (2013a) demonstrated that there was no correlation between temephos resistance of *Ae. albopictus* adults from two localities in Selangor and Kuala Lumpur, Malaysia with the esterases, mixed function oxidases and acetylcholinesterase activities which indicated the possibility of multiple insecticide resistance in these populations which were elicited by both metabolic pathways and target site mutation. Meanwhile, recent study by Rasli et al. (2018) using *Ae. aegypti* permethrin-selected strain that displayed high resistance to four pyrethroids exhibited high level of mixed function oxidases activity but low level of esterases and acetylcholinesterase activities.

Although each detoxification enzyme could confer resistance to more than one class of insecticides, the elevated level of enzyme activity is not always detected in all mosquito strains and species. This scenario indicates that each detoxification enzyme or altered target site gene is not necessarily involved and significantly expressed at all developmental stages of each mosquito population and species although resistance to a specific insecticide was exhibited in more than one growing stage. As such, molecular studies using *Ae. aegypti* showed that upon permethrin exposure, cytochrome c gene was expressed throughout all developmental stages but cytochrome c protein was detected only at adult stage (Zhao et al., 2008). This is because resistance mechanisms could confer resistance in immature and/or mature stages of mosquitoes and other insects. For instance, Kumar et al. (2002) demonstrated dissimilar response to deltamethrin at larval and adult stage of deltamethrin-selected of *Ae. aegypti*. Despite constant deltamethrin selection for 40 generations during both larval and adult stages, deltamethrin resistance was expressed only at the larval stage but not at the adult stage. Another study by Kasai et al. (2014) also confirmed this phenomenon whereby a high permethrin resistance level was displayed only at larval stage even though the selection pressure of permethrin was performed at adult stage. Hence, different elevated level of

enzymes activities involved in the resistance mechanisms should be expected in each mosquito population and species which depend on the expression of the resistance genes in these mosquitoes.

The correlation analysis was conducted in order to determine any cross resistance between two insecticides from the same and different classes as well as to verify the association between the resistance development and the involvement of detoxification enzymes or modified target site genes in tested mosquitoes. Generally, the correlation between the insecticide resistance with the elevated level of one specific enzyme activity detected within a mosquito population is hardly to be achieved as demonstrated by many previous studies due to variation of other known and unknown detoxification enzymes as well as target site gene mutations that could also significantly involve in the resistance mechanisms detected. In larval bioassay, the correlation analysis using the percent mortality between insecticides used at double dosage of LC_{99} of the reference strain at 24 hours post-treatment was performed. However, correlation involving certain insecticides namely malathion, bromophos, lambdacyhalothrin, cyfluthrin and etofenprox was not able to be determined due to complete mortalities achieved for these insecticides. There were no lethal concentration values at 50% (LC_{50}) of these insecticides available to be used in the correlation analysis in this study. This is because the WHO larval bioassays carried out in this study were based on the method outlined by WHO whereby only LC_{50} and LC_{99} values of the reference strain are needed to determine which later drive the calculation of double dosage of LC_{99} ($2xLC_{99}$) values that were used for the susceptibility testings of the field strains. Moreover, most of these five insecticides that were not able to be analysed for the correlation test are normally being used as adulticides in the local vector control activities, whereas temephos is frequently used as a larvicide in the dengue control operations and was included in the correlation analysis performed.

On the other hand, the correlation analysis conducted for adults was based on the KT_{50} values. This is because most of the insecticides used as adulticides possess the knockdown effects of adults instead of killing effects seen in larvicides. Even though the correlation analysis involving dieldrin and fenitrothion were not being able to be tested since there was no knockdown and mortality observed throughout the exposure period, insecticides that are regularly employed as adulticides in the vector control operations in Malaysia such as all the pyrethroids listed in this study were included in the correlation analysis performed.

Cross resistance commonly happens between insecticides with similar mode of action or target (Casida, 2017). Interestingly, resistance development against one specific insecticide could sometimes be supplemented with high resistance development against other insecticides which occurs not only through similar resistance mechanisms causing cross resistance but also through different resistance mechanisms causing multiple cross resistance (Hidayati et al., 2011). Some similarities in cross resistance between insecticides were discovered between larval and adult stages of *Ae. albopictus* tested in the present study. Cross resistance between propoxur and bendiocarb in which both of them belong to the same class of carbamates was demonstrated at larval and adult stages of *Ae. albopictus*. Furthermore, cross resistance between different classes of insecticides was also presented at both larval and adult stages of *Ae. albopictus* involving the cross resistance between permethrin of pyrethroids with both propoxur and bendiocarb of carbamates, respectively. Cross resistance between certain insecticide classes such as between pyrethroids like permethrin and organochlorines such as DDT have been frequently reported (Dadzie et al., 2017). As for insecticides like DDT and pyrethroids which possess similar resistance mechanism, close monitoring on the resistance development among mosquito populations to these insecticides should be continuously performed.

In addition, there were only eight associations between insecticide exposure and enzyme activities that had been verified at both larval and adult stage of *Ae. albopictus* populations employed in this study. At larval stage of *Ae. albopictus*, only acetylcholinesterase activity was found significantly linked to the percent mortality due to bendiocarb exposure at double dosage of LC_{99} of the reference strain. The significant role of elevated level of β-esterases activity in the reduction of susceptibility to carbamates and almost all pyrethroid adulticides at adult stage of *Ae. albopictus* was relatively demonstrated. Besides, the involvement of elevated level of glutathione-S-transferases activity in the malathion resistance of *Ae. albopictus* adults was also validated. Nevertheless, the presence of significant elevated enzyme activities particularly α-esterases, β-esterases, glutathione-S-transferases and insensitive acetylcholinesterase either at the larval, adult or both stages of *Ae. albopictus* confirmed the various level of resistance development against insecticides namely organochlorines, organophosphates and carbamates observed in these mosquito populations as indicated in both larval and adult mosquito bioassays. Significant increase of enzyme activities was more apparent in adults than larvae of *Ae. albopictus* from both agricultural and non-agricultural areas. The absence of significant increased activity of mixed function oxidases confirmed the approximately complete susceptibility *of Ae. albopictus* larvae and adults from almost all types of area against pyrethroids.

Few earlier studies had also highlighted the difficulties in obtaining the association between these two parameters. According to Siegfried & Scott (1992), the resistance level does not always correlate with the elevated enzyme level since the metabolic pathways of an enzyme could involve different forms of that particular enzyme. Lee & Chong (1995) revealed on increased glutathione-S-transferases activity in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. maculatus*. However, no correlation was discovered between the elevated glutathione-S-transferases activity with DDT susceptibility in all

Malaysian mosquitoes employed. In Singapore, similar results were displayed whereby no correlation was found between the resistance ratio of temephos and the esterases activity as well as between the pyrethroid resistance and any enzyme activities detected in field populations of *Ae. aegypti* larvae (Koou et al., 2014a).

5.6 Synergistic Effect of Piperonyl Butoxide (PBO) in *Aedes albopictus* Adults against Organochlorines and Pyrethroids

Adulticides comprising active ingredients of the combination of a synergist, piperonyl butoxide (PBO) with pyrethroids, are widely and commercially available in the local and foreign market. Hence, the efficacy of the pre-exposure of PBO prior to any pyrethroid exposure on *Ae. albopictus* adults from both agricultural and non-agricultural areas was investigated in this study. Since the cross resistance between organochlorines and pyrethroids was frequently reported throughout the world, the potency of PBO utilization in combination with organochlorines in combatting the resistance development among *Ae. albopictus* populations selected in this study had also been assessed. Furthermore, any association between resistance development after the use of PBO in combination with organochlorines and pyrethroids with the activity of mixed function oxidases (MFO) enzyme had also been tested as MFO activity had been found to be closely associated with the resistance occurrence against these two classes of insecticides.

In the present study, significant decrease of KT_{50} values was exhibited in *Ae. albopictus* populations from several types of area. The employment of PBO in combination with any organochlorines or pyrethroids was significantly effective for at least two types of area. The combination of PBO with all tested organochlorines and pyrethroids could efficiently control the *Ae. albopictus* populations in the oil palm plantations and fogging-free residential areas as demonstrated by significant decrease of

KT_{50} values. Based on the same parameter, the combination of PBO with either DDT, permethrin or cyfluthrin was significantly effective in the paddy cultivation areas while the pre-selection of PBO prior to deltamethrin, lambdacyhalothrin or cyfluthrin exposure was significant to combat *Ae. albopictus* adults in rubber estates.

As a synergist, PBO inhibits the oxidising activity of enzymes like mixed function oxidases to breakdown or metabolize the insecticide so that the efficacy of the insecticide is enhanced (Gunasekaran et al., 2016) which will consequently increase the mortality percentage of mosquitoes. The combination of PBO and DDT had improved the mortality percentage after 24 hours of recovery period in all types of area with the minimum of 86.67% mortality. Although KT_{50} values of dieldrin and PBO + dieldrin were not been able to be calculated due to zero knockdown and mortality throughout the exposure period, the pre-exposure of PBO prior to dieldrin was effective against all *Ae. albopictus* populations tested as indicated by the mortality percentage of more than 98.00% achieved at 24 hours post-treatment.

For the combination of PBO and pyrethroids, since complete mortalities were displayed in all populations of *Ae. albopictus* adults exposed to pyrethroids alone and PBO + pyrethroids at 24 hours post-treatment, it was decided for this study that the knockdown percentage should be measured at the half time of the exposure period so that any differences arising from the use of the synergist could be observed. With the exception of dengue prone residential areas, at 30 minutes of the exposure period, at least 77.00% knockdown was achieved in *Ae. albopictus* populations from different types of area upon the exposure of PBO in combination with either one of the pyrethroids. For dengue prone residential areas, the utilization of PBO prior to the selection of pyrethroids improved the range of percent knockdown for *Ae. albopictus* adults at 30 minutes of the exposure time from between 30.67% and 70.00% to between 65.67% and 93.00%. These findings supported the significant role of PBO in enhancing

the potency of adulticides particularly pyrethroids in which less exposure time needed to gain higher mortalities.

The use of a synergist will only be helpful if the insecticide resistance in mosquitoes is implicated by the metabolic enzyme pathways (Koou et al., 2014a). The role of mixed function oxidases (MFO) in resistance mechanism could be ascertained by the pre-exposure of synergist like PBO prior to selection pressure of insecticides (Chang et al., 2014b). In this study, the pre-exposure of PBO had driven significant decrease of MFO activities in *Ae. albopictus* populations from all agricultural and non-agricultural areas. This scenario indicated the engagement of MFO in the reduced susceptibility of *Ae. albopictus* adults from all types of area against organochlorines and pyrethroids but could still be significantly suppressed by the use of PBO. Nevertheless, the significant involvement of other detoxification enzymes like non-specific esterases (EST), glutathione-S-transferases (GST) and insensitive acetylcholinesterase (AChE) detected in the enzyme microassays in the decreased susceptibility against organochlorines and pyrethroids should be further investigated in the future with the aid of other synergists.

The efficacy of PBO as a synergist in combination with organochlorines and pyrethroids against different mosquito vectors had been evaluated by many researchers with less attention given to *Ae. albopictus*. In India, the combination use of PBO and deltamethrin showed significant involvement of mixed function oxidases in deltamethrin resistance in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* larvae (Kumar et al., 1991). Fakoorziba et al. (2009) also reported that *Ae. aegypti* from Mysore, India showed 95.23% suppression after the exposure of PBO + deltamethrin. In Vietnam, the combination of PBO and deltamethrin had significantly increased the mortality percentage to 98% and 100% in susceptible and Nha Trang resistant strains of *Ae. aegypti* (Bingham et al., 2011). Later in 2013, Darriet & Chandre demonstrated that the pyrethroid-resistant strain of *Ae. aegypti* from Vietnam showed only 17.3%

mortality after the exposure to deltamethrin. However, the combination of PBO with deltamethrin had triggered the synergistic effect which increased the mortality rate to 43.2%.

On the other hand, in Singapore, the addition of PBO prior to the exposure of pyrethroids failed to improve the efficacy of pyrethroids indicating the role of target site resistance in field populations of *Ae. aegypti* adults (Koou et al., 2014b). Similar phenomenon was observed in Thailand in which the combination of PBO and permethrin did not give any significant impact on moderate resistance to permethrin among Nakhon Sawan 2 (NS2) strain of *Ae. aegypti*. This situation showed that the permethrin resistance occurred in NS2 strain was conferred by the mutation of *kdr* gene (Poupardin et al., 2014). Meanwhile, the pre-exposure to PBO had verified the involvement of MFO in the deltamethrin and bendiocarb resistance in both *Ae. aegypti* and *Ae. albopictus* collected from Yaounde, Cameroon. However, the mortality rate of both species after the exposure to PBO + DDT was not increased which implicates the role of other resistance mechanisms in the resistance development against DDT (Kamgang et al., 2017).

In Malaysia, the pre-exposure of PBO had proved the role of MFO in the permethrin resistance in *Ae. albopictus* adults (Nazni et al., 2000). The combination of PBO with permethrin was also tested upon *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* adults from Kuala Lumpur. The positive correlation between the reduction of MFO activities with the decrease of LT_{50} values observed in most of the field strains of these mosquito species indicated the significant involvement of MFO in the permethrin resistance presented in these populations (Wan-Norafikah et al., 2010; Wan-Norafikah et al., 2013a; Wan-Norafikah et al., 2013b). Furthermore, the predominant role of MFO in pyrethroid resistance in *Ae. aegypti* adults from Penang and Johor Bahru was also exhibited after the pre-exposure to PBO prior to pyrethroids (Ishak et al., 2015).

However, only a partial recovery was achieved in *Ae. aegypti* Kuala Lumpur strain exposed to PBO + permethrin (26% mortality) and PBO + deltamethrin (71% mortality) as well as in *Ae. aegypti* Penang strain exposed to PBO + DDT (55% mortality). As for *Ae. albopictus*, 99% mortality was achieved in Penang strain exposed to PBO + DDT while only partial recovery was obtained in Kuala Lumpur strain (52% mortality). The scenario of partial recovery demonstrated in the respective *Ae. aegypti* and *Ae. albopictus* populations showed the involvement of other metabolic enzyme mechanisms or gene mutations in the insecticide target sites.

Findings gained from the synergism study performed showed the substantial use of PBO together with adulticides especially pyrethroids to prolong the efficacy of these insecticides. Further investigations should be performed involving other synergists and classes of insecticides which are to be tested on both larval and adult stages of different mosquito species. These findings should then be simultaneously compared with results obtained from bioassays, enzyme microassays and also molecular analysis. The ability to gather and correlate these data will ensure that sufficient and accurate information on the best combination of the synergist and insecticide to be implemented in the respective area will be acquired.

5.7 Insecticide Resistance Studies in Mosquitoes: Challenges and Limitations

Despite several approaches suggested by World Health Organization for vector control strategies, chemical control using insecticides continues as the most preferred and economical method in many countries. Nevertheless, the widespread and persistent use of insecticides has conferred to insecticide resistance development among mosquito vectors. Furthermore, the employment of pesticides in the agricultural sector in which most of them are similar or share mode of actions or target sites with public health

insecticides has been reported to aggravate this situation. Mosquito susceptibility studies linked to this issue have been reported in other countries especially from African region in which agriculture is the main industrial sector for these countries and the malaria incidences are their primary public health concerns. However, no such inclusive mosquito susceptibility studies with association to pesticide use in agricultural pest management that has been conducted in Malaysia. Hence, the present study was undertaken to determine the degree of seriousness with regard to the susceptibility of *Ae. albopictus* populations from top three Malaysian industrial crops growing areas upon indirect exposure from the agricultural sector. The underlying metabolic mechanisms of the resistance among tested *Ae. albopictus* populations were also investigated.

WHO larval and adult mosquito bioassays are the most frequently used diagnostic assays worldwide to determine the susceptibility status of mosquitoes against insecticides. WHO susceptibility kits provide a sign of resistance occurrence and trends (Selvi et al., 2010). The preparation of the technical grade of insecticide solutions by the Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang, Malaysia that acts as the World Health Organization Collaborating Centre minimizes the differences in the testing results of researchers due to the presence of other minor substances in the insecticide solutions. The availability of insecticide impregnated papers at recommended dosages for specific mosquito species supplied by VCRU as well permit the standardization of method among researchers so that most research outcomes could be equally compared to one another. Although the larval bioassay is naturally more sensitive than the adult bioassay in detecting the change of susceptibility level in mosquitoes, both bioassays should be conducted simultaneously for each mosquito strain as resistance is not restricted to only one specific stage of mosquito (Nazni et al., 2005).

Nevertheless, results of WHO larval and adult mosquito bioassays from numerous researchers are still sometimes difficult to compare. This is because certain researchers were not aware of the latest recommended diagnostic dosages of insecticides published by WHO. For instance, the recommended diagnostic dose of temephos had been revised from 0.02 mg/L to 0.012 mg/L in 1992 but few susceptibility studies are still utilizing the former concentration (Ranson et al., 2010). Furthermore, as described previously, although carbamates and pyrethroids are also employed in larviciding activities in certain countries, the WHO recommended diagnostic dosages for larvae only cover larvicides of organoclorines and organophosphates. This is because most carbamates and pyrethroids are moderately and highly toxic to fish and other non-target aquatic organisms (Ghazala et al., 2014; Prusty et al., 2015).

Apart from that, results of WHO larval and adult mosquito bioassays are usually presented in the form of LC_{50} (and/or LC_{95}) values for larvae, KT_{50} (and/or KT_{95}) or LT_{50} (and/or LT_{95}) values for adults, percent mortality for both larvae and adults to indicate the resistance level as classified by World Health Organization (2016a) as well as resistance ratios. Findings of WHO larval and adult mosquito bioassays that are exhibited in the first three forms are still comparable (Ranson et al., 2010). However, the comparison of results became difficult when several literature references provided only resistance ratios which were solely based on the LC_{50}, KT_{50} or LT_{50} values of their reference strain whereby there will be high possibility that the interpretation of these results will be confusing and misleading (Ranson et al., 2010). All four parameters mentioned earlier were illustrated in the present study except for the LC_{50} values for field populations of *Ae. albopictus* larvae as the WHO larval bioassay procedure was followed which requires the determination of LC_{50} values for the reference strain only.

Furthermore, a mosquito population is classified susceptible if its resistance ratio is below five, moderately resistant if the resistance ratio falls between five and ten, and

highly resistant if the resistance ratio is more than ten (Mazzarri & Georghiou, 1995; World Health Organization, 2016a). As indicated in this research work, resistance ratios obtained in the WHO adult mosquito bioassays and enzyme microassays were all below five. Thus, if the susceptibility status was determined mainly and solely by their resistance ratios, *Ae. albopictus* populations from all agricultural and non-agricultural areas could all then be classified as susceptible to all insecticides tested. Nevertheless, these findings would turn out to be totally different when the susceptibility status of these *Ae. albopictus* populations were established based on the percent mortality as performed in this study. As stated previously, the calculation of resistance ratio is closely related to the resistance ratio of the reference strain which will definitely be different among all susceptibility studies conducted worldwide while the calculation of percent mortality is much simpler, straight forward and directly follows the resistance classification by WHO. Hence, the determination of the susceptibility status of the mosquito population according to the percent mortality is more accurate and comparable. In fact, as observed in this study, cross resistance among certain tested insecticides was also demonstrated in spite of susceptible resistance ratios obtained for all *Ae. albopictus* populations. This scenario once again showed that the resistance classification by percent mortality is more precise. In other words, the interpretation of results should be very conscientiously performed if the researchers decided to classify the resistance status of their tested populations according to resistance ratios to avoid the underestimation. At the same time, this situation should actually alert the researchers in the sense that even with low resistance ratios, moderate to high resistance and cross resistance between insecticides could still be reached, so more if the resistance ratios are beyond five or ten fold. Other than that, the heterogeneity of the reference strain should also be taken into account if resistance ratio alone is selected for the resistance classification. Heterogeneous population of the reference strain affects their

susceptibility against insecticides whereby full susceptibility to all insecticides is difficult to be achieved. Consequently, the accuracy of the resistance classification based on the resistance ratio alone could also be challenged.

Several shortcomings of WHO susceptibility testings have driven towards the development of biochemical assays. Biochemical assays are beneficial in signifying the involvement of metabolic resistance (Djouaka et al., 2008). Biochemical assays involve the detection and qualitative analysis of important detoxification enzymes in mosquitoes (Selvi et al., 2010). Alterations of few wide classes of enzymes happening in the test population in comparison to the reference population could be indicated by biochemical assays (Macoris et al., 2018). Biochemical assays should be performed together with the conventional but well-established and standardized bioassays as well as the molecular and proteomic approaches if necessary in order to further understand the resistance mechanisms involved in the tested mosquito populations. This is because in certain settings, biochemical assays could only partially support the bioassay results using the synergists since not all elevated activities of detoxification enzymes could be significantly detected (Seixas et al., 2017) as the level of these enzyme activities is too low. Moreover, universal substrates are utilized in the biochemical assays which could or could not be sensitively identified by all enzyme members of the enzyme families (Morou et al., 2010).

By using molecular tools, genes and their biochemical products involved in the resistance mechanisms could be characterized and the significant roles of these biochemical products in overpowering the toxic effects of insecticides could be determined. Recently, more updated methods like gene expression microarrays and next-generation sequencing technology are being applied in order to further identify and understand the genes that encode enzymes involved in the interaction with insecticides

molecules (David et al., 2010). However, these advanced approaches are costly whereby not many entomology laboratories could afford to have these kind of facilities for now.

5.8 The Utilization of Insecticides in Malaysian Vector Control Strategies

Both DDT and dieldrin had been previously applied in vector control activities in Malaysia for long times (Thomas, 1962). The use of these organochlorines had been forbidden in Malaysia since 1994 (Low et al., 2015) and other countries earlier than in Malaysia. Thus, in several countries such as Trinidad and Tobago as well as Malaysia, organophosphates like malathion, fenitrothion and fenthion are primarily utilized in either residual spraying or space treatment for the control of adult mosquitoes (Polson et al., 2010; Ong, 2016). Malathion has been employed in fogging activities in Malaysia since early 1970s (Nazni et al., 1998) in which malathion 96% concentrate is the insecticide of choice in ULV (Vythilingam & Wan-Yusoff, 2017). Fenitrothion which has a slow action against insects (Bong et al., 2013) is applied in the space spraying of dengue control activities in Malaysia since 1980s (Loke et al., 2015) while fenthion is utilized in many countries including Malaysia either as a larvicide or as an adulticide (Stone & Brown, 1969; Polson et al., 2012; Ong, 2016).

Meanwhile, temephos is usually applied in larviciding of mosquito larval breeding grounds (Raghavendra et al., 2011). Temephos is one of four larvicides that have been approved by WHO to be applied in potable water (Polson et al., 2001). Temephos is the only larvicide of choice for many countries such as Andaman and Nicobar Islands, Colombia, Trinidad and Tobago, India as well as Brazil (Polson et al., 2010; Maestre-Serrano et al., 2014; Sivan et al., 2015; Bharati & Saha, 2018; Carvalho et al., 2018). In Malaysia, the Ministry of Health Malaysia has recommended temephos as a larvicide in potable water for the control of container-breeders which has been practised since 1973

(Lee, 1991; Chen et al., 2005a). For *Aedes* larval control in Malaysia, temephos is employed at operational dosage of 1 mg/L (Chen et al., 2005b). Since the operational dosage is about 84 times higher than the WHO recommended diagnostic dosage of 0.012 mg/L, the application of temephos in the field is expected to remain effective for now (Vythilingam & Wan-Yusoff, 2017).

Other than that, chlorpyrifos is being applied in the control of Coleoptera, Diptera, Homoptera and Lepidoptera insects (Chai et al., 2009b). Nevertheless, chlorpyrifos has so far never been formally utilized either as a larvicide or an adulticide in the vector control programmes in Malaysia. There is also no previous record on the use of bromophos in vector control strategies in Malaysia. However, in other countries, bromophos had formerly been employed as a larvicide and an adulticide to control *Ae. aegypti*, *Cx. pipiens fatigans* and anopheline mosquitoes as well as for crop pest and residual spraying of fly control (Brown, 1967; Schoof, 1967; Hill, 1971; Wightman & Whitford, 1982; Rozendaal, 1997). In Thailand, the use of bromophos has been banned due to its high risk to users' health (Overgaard, 2006).

Oil-based malathion was the adulticide of choice in dengue control operations in Malaysia before being replaced by water-based pyrethroids in 1996 due to nonacceptance of communities on the foul odour malathion and its oily residues left on the floors and walls of premises upon fogging activities (Teng and Singh, 2001). Pyrethroids have been utilized in the control of adult mosquitoes in Malaysia replacing the use of DDT since 1999 (Rosilawati et al., 2017). Pyrethroids are preferred insecticides when exposure to humans is expected such as during vector control and households spraying activities (Hardstone et al., 2015). This is because pyrethroids possess fast action, great repellency and irritation effects on mosquitoes and are less toxic to humans (Darriet & Chandre, 2013). Pyrethroids are also widely utilized as repellents to avoid the mosquito bites without killing them or exposing the mosquito

populations to selection pressure in which repellency effects will stimulate physiological resistance only at a very minimal level (Kawada et al., 2010).

Pertaining to the use of insecticides for vector control activities in the study areas, only three of them which were clustered as dengue prone residential areas had regularly been exposed to the public health insecticides. Vector control strategies such as larviciding and space spraying like fogging and ultra low volume (ULV) are carried out in all dengue areas in Malaysia (Vythilingam & Wan-Yusoff, 2017), including the three dengue prone residential areas of this study. As outlined by the Ministry of Health Malaysia, when a dengue case is reported, house-to-house fogging is performed in the case house and also the area within 200 meters radius from the case house (Omar et al., 2011). ULV is only conducted when there is a dengue epidemic to cover a larger area of space spraying (Vythilingam & Wan-Yusoff, 2017). Temephos is the only larvicide employed for local larviciding (Chen et al., 2005a), including in those three dengue prone residential areas of this study. On the other hand, for adult mosquito control in all dengue prone residential areas of this work as well as other dengue areas in Malaysia, pyrethroids are mainly used in fogging while malathion is often utilized in ULV (Vythilingam & Wan-Yusoff, 2017). Hence, resistance development among *Ae. albopictus* populations from all three dengue prone residential areas of this study against both temephos and malathion either at larval or adult stage was expected. Nevertheless, despite constant exposures of pyrethroids that are being used in the vector control programmes and as commercial household insecticides, high susceptibility against pyrethroids demonstrated among *Ae. albopictus* populations from dengue prone residential areas are a great relief for now.

Meanwhile, other study sites of this work that were grouped into different types of agricultural areas and fogging-free residential areas were free from any vector control programmes conducted by either the Ministry of Health Malaysia or the local

authorities as there was no report on dengue cases or occurrence of any other mosquito-borne diseases within these areas. The only exposure of vector control insecticides within these agricultural areas and fogging-free residential areas came from the use of household insecticides by residents in which pyrethroids were the main active ingredients of these commercial products. However, high susceptibility against pyrethroids was exhibited among *Ae. albopictus* larvae and adults from these agricultural areas and fogging-free residential areas which indicate that the use of pyrethroids in these areas are still relevant. In contrast, the utilization of organochlorines, organophosphates and carbamates for vector control programmes or pest crop management in these areas should be cautiously considered since the resistance development against these insecticides were already detected in some populations of *Ae. albopictus* from these areas.

Above all, incessant use and excessive reliance on insecticides have led to the occurrence of resistance among mosquito populations and consequently failures in vector control strategies. Higher cost and volumes of chemicals are demanded to ensure the effectiveness of vector control activities due to the development of insecticide resistance (Reid et al., 2014; Agramonte et al., 2017). Besides, insecticide resistance in mosquitoes has indirectly preceded the increase of human morbidity and mortality due to mosquito-borne infections (Paul et al., 2006). In Batticaloa district of Sri Lanka, heavy reliance on DDT and malathion selection for the control of malaria had triggered high resistance development against both insecticides among *Ae. aegypti* and *Ae. albopictus* adults (Dharshini et al., 2011). The occurrence of DDT resistance among mosquito vectors in South Andaman was also due to the persistent use of DDT in the vector control activities undertaken since 1958 (Sivan et al., 2015). Additionally, temephos resistance exhibited among *Ae. albopictus* larvae collected from several countries such as Greece, Italy and Malaysia was due to extensive utilization of

temephos in the mosquito control operations for years (Vontas et al., 2012). The susceptibility status of *Ae. albopictus* against insecticides worldwide including Malaysia is poorly documented. Development of insecticide resistance among *Ae. aegypti* has been reported throughout many regions but very little information is available regarding the insecticide resistance among *Ae. albopictus* (Ranson et al., 2010; Ngoagouni et al., 2016).

5.9 The Use of Pesticides in Agricultural Areas: The Malaysian Scenario

Agriculture is still among the main sectors of many developing countries. Agriculture and plantation are also named as primary sectors in Malaysia which received a total investment of RM 44.9 million and RM 672 million, respectively in 2017 [Malaysian Investment Development Authority (MIDA), 2018]. Oil palm, rubber and paddy are the top most widely planted industrial crops in Malaysia (Department of Agriculture Peninsular Malaysia, 2016). A total of 5,811,145 hectares, 1,077,870 hectares and 681,559 hectares of areas in Malaysia were planted with oil palm, rubber and paddy, respectively (Department of Agriculture Peninsular Malaysia, 2016; Department of Agriculture Malaysia, 2017). Oil palm and rubber are planted in large scale throughout Malaysia to fulfil the economic demand (Sharip et al., 2017). Oil palm is extensively planted in the southern region of Peninsular Malaysia particularly in Johor (Maznah et al., 2015). Paddies are cultivated in more localised but largely developed areas in Selangor, Perak, Kedah, Negeri Sembilan and southern Pahang (Sharip et al., 2017). The Muda Irrigation Scheme along the Muda River in Kedah is the widest rice cultivation area in Malaysia (Sapari & Ismail, 2012).

The prevalence of mosquito populations is governed by agricultural activities, urbanization and habitations by humans which provide various breeding habitats for mosquitoes. *Ae. albopictus* breeds around and close to premises as well as in the forests

and plantations (Lim et al., 2010). Paddy cultivation areas offer ideal breeding grounds for several important mosquito vectors as paddy plants are cultured under flooded conditions especially during early phases of the plant growth (Lytra & Emmanouel, 2014). In Thailand, rice fields had been identified as one of the most significant breeding areas for *Aedes* mosquitoes (Sarfraz et al., 2012). Lytra and Emmanouel (2014) showed that the occurrence of mosquito larvae was closely associated with the rice cultivation cycle in Greece. Mosquito survey performed in Tamil Nadu, India by Suganthi et al. (2014) also demonstrated rice fields as one of the main mosquito breeding habitats. Moreover, paddy cultivation areas, agricultural ditches, irrigation canals and drains were also among the commonly preferred breeding grounds of *Cx. quinquefasciatus* (Bhattacharya & Basu, 2016). Rice fields had been recognized as the most productive habitats for *Cx. quinquefasciatus* immatures in Mwea, Kenya (Mwangangi et al., 2008). In Nagasaki, Japan, Chaves et al. (2015) demonstrated that the abundance of *Ar. subalbatus* at Mount Konpira became greater when the ground area was filled with leaf litter. Since rice cultivation areas in Malaysia are more likely open areas as compared to other types of agricultural areas, stronger and less barrier wind movement is expected which could instigate an increased volume of leaf litter within these areas. This leaf litter could also be one of the potential breeding habitats of *Ar. subalbatus* in Malaysian rice cultivation areas. Other than that, in Calicut, India which is comprised of coconut, rubber, areca and cocoa plantations, breeding of *Ae. albopictus* had been demonstrated in many types of natural and artificial breeding habitats especially in coconut shells that are employed for rubber sap collection (Rao & George, 2010). In Laos, higher numbers of *Ae. albopictus* were also collected from the mature rubber plantations as compared to villages and immature rubber plantations whereby 37% of these samples were discovered from latex collection cups (Tangena et al., 2018).

The use of pesticides in agriculture is one of the principal methods in controlling insect pests, crop diseases and weeds (Department of Agriculture Malaysia, 2012). Pesticides are also extensively applied in the agricultural sector for plant protection to maintain the yield and quality of crop production (Hamsan et al., 2017). Pyrethroids, organophosphates and carbamates are regularly applied in the agricultural practices (Nordin et al., 2002). As such, with about twenty species of pest insects that are known to infest the rice crop in the tropical region, different insecticides are applied in paddy cultivation areas to eliminate each of these insect species (How et al., 2015). Numerous guidelines on the management of pesticides have been documented by many countries in order to reduce the potential risks of pesticide exposures to human and also the environment (World Health Organization, 2012a).

The utilization of pesticides in Malaysian agriculture became popular especially in rubber estates, oil palm plantations, rice fields, cocoa plantations and vegetable farms since 1960s due to agricultural extension programs (Triantafillou, 2001). The Muda River Irrigation Scheme in Kedah which is the widest paddy cultivation area in Malaysia possesses the history of pesticides application since 1980 (Ismail et al., 2015). Weed management in Malaysian paddy cultivation areas is heavily dependent on herbicide application (Anwar et al., 2013).

Dieldrin had been widely employed as a pest poison in tea, vegetable and cotton cultivation areas (Leong et al., 2007). In Malaysia, since 1990, the use of organochlorines in the agricultural sector had either been prohibited or confined to only oil palm and coconut plantations (Leong et al., 2007). However, high level of dieldrin had still been significantly detected in water samples collected from paddy cultivation areas of Muda Scheme, Kedah, Malaysia (Zakaria et al., 2003). Organophosphates like malathion, fenthion and fenitrothion are massively used in the agricultural sector and gardening activity (Cui et al., 2006b). Organophosphates are regularly applied in the

rice cultivation areas in Malaysia to eradicate the proliferation of crop pests (How et al., 2014). Malathion and methyl parathion are commonly used in the vegetable farms, fruit orchards, paddy fields and oil palm plantations in Sabah, Malaysia (Hossain et al., 2010). Chlorpyrifos is widely used for the control of insect pests in the agricultural areas such as in rice fields (Zhang et al., 2012; Vijaya Bhaskar Reddy et al., 2016). In Malaysia, chlorpyrifos is regularly applied at large volumes to control insect pests in soils and crops of oil palm plantations, rice fields as well as vegetable and fruit farms (Leong et al., 2007; Ismail & Ngan, 2005; Chai et al., 2013; Halimah et al., 2016). Other organophosphates like dimethoate as well as dichlorvos and diazinon are also widely utilized in the Malaysian and Australian agricultural sector, respectively (Ahmad et al., 2008; Ismail et al., 2018b). The employment of synthetic pyrethroids like cyfluthrin in agriculture is also rising due to their high efficacy even at low dosages, impermanence in the ecology, fast biodegradation, selective insecticidal action and low toxicity against humans and animals (Lee-Yin et al., 2013). Various organochlorines, organophosphates and pyrethroids had been detected in vegetables cultivated in Cameron Highlands, Malaysia (Farina et al., 2018) which proved the utilization of these pesticides in the local agricultural activities. In Kelantan, Malaysia, alphacypermethrin of pyrethroids, methamidophos of organophosphates, chlorothalonil of organochlorines and mancozeb of dithiocarbamates were the primary pesticides utilized in tobacco plantation areas (Kimura et al., 2005). The use of cypermethrin and chlorpyrifos in citrus orchards in Sarawak and other localities within Malaysia as well as in other tropical countries is also not possible to be avoided due to the high humidity that provides favourable conditions for crop pests such as the citrus psyllid *Diaphorina citri* Kuwayama (Leong et al., 2012).

Despite numerous guidelines published by Department of Agriculture Malaysia on registered pesticides, banned pesticides and working procedures for pesticide operators,

the main pitfall in agriculture is the lack of information and proper records of the pesticides used in each agricultural plantation owned by corporate companies and also in open agricultural areas cultivated by personnel like farmers. Based on the informal interview with farmers and officer-in-charge for the agricultural plantations selected for this study, it can be concluded that the decision on which pesticides to be utilized in each plantation or agricultural area relies more on the decisions made by the plantation management members or supervisors-in-charge as well as the farmers themselves. The affordability of farmers to purchase the pesticides or the budget allocated by the top management of the corporate companies for crop pest control influence the selection of pesticides to be used. Consequently, every agricultural plantation and open agricultural area is currently being exposed to similar or different pesticides at various volumes and application frequencies which would worsen the insecticide resistance development among mosquito populations in these agricultural areas. Furthermore, more adequate training and knowledge need to be provided to pesticide operators and farmers to increase their understanding on the correct doses as well as the handling and applying procedures of pesticides for their agricultural plantations or sites.

From the casual conversation with supervisors-in-charge and farmers of the agricultural areas selected for the present study, common pesticides that are being applied in all types of agricultural areas selected have been listed out. Some of these pesticides are widely utilized in more than one type of agricultural areas and overlap with their application in public health. Glyphosate of organophosphates and paraquat are employed as weed herbicides in all oil palm plantations and rubber estates selected in this study but their application dosages and regularity varied between one another. On the other hand, pyrethroids namely alphacypermethrin, cypermethrin and lambdacyhalothrin as well as organophosphates like malathion, chlorpyrifos and propoxur are recurrently applied as insecticides in all oil palm plantations, paddy

cultivation areas and rubber estates of the present study to control insect pests such as the cotton leafworm (*Spodoptera litura*), the bagworm (*Metisa plana*, *Pteroma pendula* and *Mahasena corbetti*), the brown planthopper (*Nilaparvata lugens*) and the red cotton stainer (*Dysdercus cingulatus*). However, these pyrethroids and organophosphates are also employed at different dosages and consistency in each study locality.

Excessive use of pesticides in agriculture is one of the primary causes of environmental contamination in Malaysia (Sutris et al., 2016b). The selection pressure of agrochemicals in agricultural areas could promote resistance development in mosquitoes in several ways. Agricultural pesticides are dispersed into nature through the contamination in water and soil as well as by spraying activities (Hamsan et al., 2017). Direct pesticide spraying on the aquatic breeding grounds created inadvertently by agricultural practices or irrigation systems will expose the mosquito larvae in these breeding spots to insecticide resistance development. Furthermore, pesticides in soils or sprayed on crops could also be washed out and run off into water bodies, streams, drains and even rivers that could consist of mosquito larvae. Heavy rainfall and tropical soil types in Malaysia stimulate soil erosion in agricultural areas which will then cause the agricultural pesticide-contaminated soil to run off from plantations to water ways and water bodies (Sharip et al., 2017). For instance, although the use of both DDT and dieldrin in agriculture were previously restricted before being discontinued and deregistered in the Malaysian Pesticide Board by 1998, the DDT and dieldrin residues were still detected in water bodies and soils in local agricultural areas particularly rice fields and vegetable farms (Ramachandran & Mourin, 2006). In 2007, Leong et al. reported on several pesticides including dieldrin and chlorpyrifos that were detected in water samples collected from Selangor River whereby multiple agricultural activities like oil palm and rubber plantations as well as vegetable farms were available along the river. Fenitrothion residue was also detected in water, soil and crops from local

agricultural areas while chlorpyrifos was discovered in river water samples including from Langat River in Selangor, Malaysia (Taib et al., 2014; Vijaya Bhaskar Reddy et al., 2016; Wee & Aris, 2017). These findings indirectly showed the utilization of pesticides in the agricultural farms and also the possibility of pesticide contamination in natural habitats of mosquito larvae presented within and around these agricultural areas.

5.10 Crop Pest Management using Pesticides in Agricultural Areas: A Contribution Factor of Insecticide Resistance Development among Mosquito Vectors of Public Health

Adult mosquitoes in agricultural areas are subjected to insecticide selection through the agricultural pesticide spraying activities on crops whereby these pesticide particles are carried and spread by wind to the surrounding areas. According to Sutris et al. (2016b), pesticide residues could waft from agricultural fields to immediate human dwellings by air, water, carriage and even workers' attire. In fact, less than 0.1% of pesticides applied in the agricultural practices that actually reached the pest while the rest are dispersing out into the ecosystem including the air, soil and water (Baharuddin et al., 2011). In Thailand, the use of permethrin-treated materials by workers in rubber estates and fruit orchards to protect them from outdoor biting mosquitoes including *Ae. albopictus* was also believed to be one of the factors in the permethrin resistance development among this mosquito species (Chuaycharoensuk et al., 2011). Other than that, the use of household insecticides by residents staying within agricultural areas in order to combat mosquito attacks indoors would aggravate the insecticide resistance development among mosquito vectors. As described by Etang et al. (2016), discrepancies of resistance levels are closely influenced by the mosquito dynamics based on weather-related events as well as human actions in agriculture and household protection against mosquito attacks.

Physicochemical factors of the water bodies such as temperature, turbidity, alkalinity, salinity, conductivity, dissolved oxygen as well as dissolved organic and inorganic substances significantly influence the abundance of mosquito larvae in these breeding habitats (Nikookar et al., 2017). In fact, these physicochemical characteristics of potential breeding habitats could also influence the oviposition of adult mosquitoes as well as the survival and spatial dispersal of the mosquito population as a whole (Emidi et al., 2017). Therefore, in agricultural areas, the use of pesticides for crop pest management is also expected to affect the physicochemical parameters of aquatic breeding habitats presented within these areas which will eventually influence the density of mosquito larvae within these breeding grounds and also the distribution of adult mosquitoes in the environment of these agricultural areas.

However, the more important issue is the selection of resistant mosquito populations steered by the use of pesticides in agriculture which are from similar classes of insecticides employed in vector control activities that have been accumulating in water bodies within cultivation areas and causing indirect pesticide exposure to mosquito immatures (Djegbe et al., 2011). Interestingly, contamination of pesticides in agricultural areas could also affect the susceptibility of adult mosquitoes against vector control insecticides since the resistance level of adult mosquitoes are influenced by the nature of their breeding habitats (Djouaka et al., 2008). *Aedes albopictus* is exophilic which increases the chance of its exposure to agricultural pesticides (Ponlawat et al., 2005). Moreover, adult mosquitoes have also been observed to rest on pesticide-treated rice which indirectly exposed them to insecticide resistance development (Yoo et al., 2013).

Pesticides, insecticides, herbicides, fungicides and fertilizers used in the agricultural activities and have been detected in mosquito breeding sites within the agricultural areas affect the mosquito susceptibility against vector control insecticides through the

modifications of detoxification pathways (Nkya et al., 2013). Very few literature references have highlighted the insecticide resistance development among mosquito vectors captured from agricultural areas. In Pakistan, *Ae. albopictus* larvae collected from cotton cultivated fields were found to be resistant to chlorpyrifos, deltamethrin and lambdacyhalothrin at 157-266 fold, 15 to 53 fold and 21 to 58 fold, respectively, as compared to the laboratory strain (Khan et al., 2011). These findings proved the resistance development in mosquito larvae tested that was due to the selection pressure of agrochemicals used to eliminate cotton pests. On the other hand, *Ae. albopictus* adults captured from different types including the mature and immature rubber plantations in Laos exhibited resistance to both DDT and malathion with mortality percentages ranging from 27% to 90% and 20% to 86%, respectively (Tangena et al., 2018). However, all these *Ae. albopictus* populations were susceptible to both permethrin and deltamethrin. In Malaysia, *Ae. albopictus* adults collected from an animal farm which is located within an oil palm plantation in Tanjung Sepat, Selangor, Malaysia had been reported with high resistance against public health insecticides namely malathion 5.0%, fenitrothion 1.0%, propoxur 0.1% and bendiocarb 0.1% with mortality percentages of 20% and below (Chen et al., 2013b). Other than that, Noor Afizah et al. (2015b) had described the complete absence of *Ae. aegypti* populations in Carey Island, Selangor, Malaysia discovered during their ovitrap surveillance study which could be due to the intolerance of this mosquito species against the agricultural pesticides used in the study area which was an oil palm plantation.

In addition, glyphosate or commercially known as Roundup is an herbicide that is widely used in crops management in agricultural areas including in Malaysia. Glyphosate is one of the organophosphorus compound that is commonly used as an herbicide in the paddy growing areas such as in Kuala Selangor, Selangor, Malaysia (Nahi et al., 2016). Glyphosate has possible indirect effects on the insect susceptibility

against public health insecticides (Riaz et al., 2009). As demonstrated in a study by Riaz et al., 2009, *Ae. aegypti* larvae that were pre-exposed to glyphosate showed significant increase in their tolerance to permethrin and propoxur. Not only that, the activities of mixed function oxidases, glutathione-S-transferases and esterases in these *Ae. aegypti* immatures were also moderately induced upon the exposure to glyphosate.

Apart from that, pyrethroids were previously not applied as larvicides due to their high toxicity to aquatic living organisms. Nevertheless, with the advancement of technology, current commercialized pyrethroids have been broadly utilized in watery environments including the paddy cultivation areas (Kawada et al., 2009). Hence, the selection of pyrethroids in water bodies present in agricultural areas should also be expected to cause significant resistance development among mosquito immatures in these agricultural areas against public health insecticides in the near future.

Among three types of agricultural areas selected for the present study, distribution and susceptibility of various mosquito species especially *Ae. albopictus* captured from rice cultivation areas were the most frequently reported. To the best of my knowledge, no such studies have been exclusively performed in any oil palm plantations and rubber estates including in Malaysia. Hence, this study has provided elementary information on the dispersal and insecticide susceptibility of mosquitoes from these agricultural areas which could facilitate the local authorities on the most appropriate chemical control method for mosquitoes and other crop pests within these agricultural areas.

5.11 The Use of Piperonyl Butoxide (PBO): An Alternative Control Measure

By considering the fact that the use of insecticides in both public health and agricultural sector is indeed inevitable for now, there is a crucial need to design and implement sensible methods that could prevent or at least delay the resistance

development among mosquito vectors due to selection pressure. The development and application of resistance-delaying or resistance-escaping approaches such as the use of a synergist in combination with an appropriate insecticide could minimize the volume of insecticides to be utilized (Kumar et al., 2002). Furthermore, the synergist could diminish the mosquito's capability to break down the insecticide and thus, enhance the efficacy of the insecticide (LeClair et al., 2017). The enhancement of insecticide activities by the synergist is also beneficial for susceptible mosquitoes in localities with no resistance occurrence in mosquito populations (Gunasekaran et al., 2016) as less amount of insecticides are required to be applied in these areas with shorter knockdown time in these populations. Other than that, the utilization of synergists like piperonyl butoxide (PBO) in bioassays conducted in the laboratory offers rapid and simple baseline data to detect the presence and development of resistance in mosquito populations using alive mosquito samples and inexpensive materials (Chouaibou et al., 2014).

Only PBO was employed as a synergist in the synergism study conducted which was combined with either organochlorines or pyrethroids. Since PBO is an oxidase inhibitor, it is always being used in combination with pyrethroids as well as DDT as cross resistance could also occur between these two classes of insecticides due to similar mode of action. However, there were also previous studies in which PBO was tested in combination with organophosphates and carbamates but no significant reduction of resistance to these insecticides was obtained with the use of PBO. For example, malathion resistance was detected in *Cx. tarsalis* but no synergism effect was observed with the pre-exposure to PBO (Whyard et al., 1994). The use of PBO in combination with organophosphates and carbamates as well as the enzyme microassays of non-specific esterases (EST), glutathione-S-transferases (GST) and insensitive acetylcholinesterase (AChE) on PBO-exposed mosquito populations could also be

undertaken in the future research so that any association between the changes of the susceptibility status after the use of PBO in combination with each insecticide class with the activity level of these metabolic enzymes could be clarified. In fact, the combination of other synergists such as triphenyl phosphate (TPP) and diethyl maleate (DEM) with all classes of insecticides could also be assessed in order to verify the most appropriate synergist to be used to complement each insecticide utilized in the vector control approaches.

In this research work, only adult mosquito populations were subjected to the synergism study which was in line with the fact that pyrethroids alone and PBO + pyrethroids are presently available as adulticides in the market such as in the form of aerosols, mat, coil, repellent and impregnated bednets, but not as larvicides. However, the use of PBO had actually demonstrated the diverse involvement of mixed function oxidases at both larval and adult stages (Paul et al., 2006). The combination of PBO with different larvicides was evaluated on *Ae. aegypti* larvae previously but results obtained were not significantly promising for larval control so far. For instance, despite the use of PBO in combination with deltamethrin, the resistance development against deltamethrin among field strains of *Ae. aegypti* larvae from India failed to be stopped and the resistance ratios of these strains kept rising throughout successive generations (Kumar et al., 2002). Meanwhile, in Singapore, the combination of PBO with permethrin and etofenprox displayed significant increase of mortalities in few field strains of *Ae. aegypti* larvae, whereas the pre-exposure to PBO prior to temephos had reduced the mortality rates instead of increasing the effectiveness of temephos among several strains of the same mosquito populations (Koou et al., 2014a). Thus, synergism study using mosquito larvae could also be taken into account in the forthcoming research so that the differences between the effectiveness of the synergist in

combination with insecticides on larval and adult stages of mosquitoes could be evaluated.

Besides the synergist assays to confirm the involvement of detoxification enzymes in the resistance development in mosquito vectors, more modernized but economical techniques should also be put under consideration to be performed in order to validate the resistance mechanisms discovered using current procedures. For instance, dieldrin resistance in *An. gambiae* from villages within the rice fields in Burkina Faso was shown to be associated with *Rdl* mutation due to massive use of agrochemicals (Kwiatkowska et al., 2013). On the other hand, high frequencies of *kdr* and $ace\text{-}1^R$ mutations were detected in *An. gambiae* originating from the cotton growing site and paddy fields in Cote d'Ivoire, respectively (Camara et al., 2018). The underlying mechanisms involved in the resistance among these mosquitoes could not be confirmed by the synergist bioassays alone but need to be supported with other assays like the molecular tools (Prasad et al., 2017). Hence, in addition to WHO larval and adult bioassays, enzyme microassays and synergist assays, advanced molecular methods should also be undertaken in the future to replenish the current shortcomings in detecting and verifying the presence of alterations in the voltage gated sodium channel, acetylcholinesterase (AChE) and/or γ-aminobutyric acid (GABA) genes that could confer the modified target site resistance among Malaysian mosquito vectors.

5.12 The Way Towards Integrated Vector Management (IVM)

Source reduction remains the best method to be applied in the vector control strategies. In Mayotte, although temephos has been used as a larvicide since 1973 until 2012, both *Ae. aegypti* and *Ae. albopictus* larvae were still susceptible to temephos (Pocquet et al., 2014). This is because before the chikungunya outbreak in 2005-2006, both species were not the target of vector control activities. Thus, the insecticide

selection pressure was rarely performed and elimination of breeding habitats was the main tool of mosquito control in Mayotte at that time (Pocquet et al., 2014). In fact, without the exposure of insecticides in the environment, the susceptible mosquitoes will produce more progeny than the resistant individuals which will eventually increase the proportion of susceptible individuals in the population (Lee et al., 1997). Whenever source removal is not feasible, larviciding could become the next choice of control tool instead of adulticiding. This is because larviciding involves the eradication of mosquito vectors at their breeding source with less insecticide exposure to humans and minimal killing of nontarget organisms as compared to adulticiding in which the adulticides may not reach the adult mosquitoes as they tend to rest in sheltered spots and surfaces (Koou et al., 2014a, Koou et al., 2014b).

Due to the fact that source reduction is a labour-intensive and time-consuming approach, the chemical control using insecticides has become the priority in the vector control strategies of many countries worldwide including Malaysia. The susceptibility of *Ae. albopictus* from all types of area selected for the present study against all pyrethroids tested had positively indicated that the application of pyrethroids in vector control strategies in these areas is the most appropriate and acceptable. However, the rotation of different classes of insecticides should be continuously undertaken as an early prevention action from resistance development against these pyrethroids that may later lead to *kdr* resistance which is irreversible. Continuous selection of insecticide reduces the susceptible individuals in a population and increases the proportion of resistant insects (Brown, 1986). Previous laboratory investigations had demonstrated that constant exposure of insecticides could rapidly trigger the development of insecticide resistance among mosquito vectors. As such, Hamdan et al. (2005) demonstrated temephos tolerance with resistance ratio of 4.49 fold among *Ae. albopictus* larvae after the selection pressure of temephos for 20 generations while Selvi

et al. (2010) showed that persistent selection of malathion in the laboratory had prompted the increase of LC_{50} value from 0.0472 mg/L at first generation to 1.233 mg/L at sixth generation of *Ae. albopictus* larvae. The rotation of insecticides applied in Mexico had significantly delayed the development of high resistance to chlorpyrifos among *Ae. aegypti* populations (Lopez et al., 2014). On the contrary, rotational and regular use of permethrin and fenitrothion in dengue control operations conducted by the Kampar district health division and the local municipal council in Kampar, Perak, Malaysia has exerted high fenitrothion resistance among *Ae. albopictus* adults of two residential areas (Ho et al., 2014). Hence, it is important to determine the insecticide resistance among mosquitoes in the target area and the resistance mechanisms involved in these populations before selecting the insecticides to be employed in the insecticide rotational programme of vector control. Also, only insecticides that are safe, effective and inexpensive should be utilized in these vector control activities (Aizoun et al., 2013).

Other than the rotational use of insecticides, temporary discontinuation of insecticide application in vector control activities for certain duration could also facilitate the prevention or delay of the insecticide resistance development. According to Nazni et al. (2005), resistance could sometimes be reversed when mosquitoes are kept insecticide-free for a long time.

Furthermore, since *Ae. albopictus* shares environmental conditions with *Ae. aegypti* as the main vector of important mosquito-borne diseases, it is likely to experience similar insecticide exposure and resistance development. Nevertheless, this scenario is not always true all the time. A local study by Rong et al. (2012) in a dengue endemic area in Shah Alam, Selangor, Malaysia which is quite near to one of the dengue prone areas selected in the present study; Shah Alam DEN showed resistance among *Ae. aegypti* against DDT, permethrin, propoxur and bendiocarb with mortality percentages

of less than 80% as well as the development of moderate cyfluthrin resistance in these mosquitoes. However, the same mosquito populations were susceptible to malathion and fenitrothion. The contradiction in the susceptibility status of *Ae. aegypti* and *Ae. albopictus* from the same or nearby localities against common public health insecticides such as malathion and permethrin could jeopardise the efficacy of mosquito control strategies conducted. Thus, once again, the meticulous selection of insecticides that are effective in controlling all target mosquitoes within an area is crucial. Moreover, the mixtures of insecticides with different targets and action mechanisms are also a practical short- to medium-span mosquito control method (Darriet & Chandre, 2011). Aside from that, the utilization of plant-based larvicides which typically are biodegradable and have low toxicity to animals and water creatures could also become one of the alternative control tools of mosquitoes (Araujo et al., 2016).

The efficacy of vector control programmes could only be recuperated by comprehending the mosquito breeding grounds and patterns of insecticide resistance development in mosquitoes (Etang et al., 2016). Mosquito surveillance using ovitraps or any other trapping devices, mosquito larval survey and mosquito breeding habitats survey should be carried out in order to identify the mosquito vectors present in the target area and their geographical dispersal in determining the cost-effectiveness of vector control strategies to be conducted. On the other hand, insecticide monitoring surveillance is essential to prevent the underestimation of insecticide resistance development in local mosquito vectors due to insufficient surveillance and under-reporting. Consistent monitoring should be performed since early detection of insecticide resistance emergence in order to prevent advanced development of the resistance which eventually will lead to complete failure of vector control interventions. Even though temephos and pyrethroids are currently the most common larvicide and adulticides recommended by the Ministry of Health Malaysia, respectively, it is

important to include all public health insecticides in the susceptibility testings conducted since breeding habitats of mosquito vectors could also still being contaminated by these insecticides when they are applied as adulticides such as via space spraying instead of only through larviciding activities. Besides, many more insecticide monitoring testings focusing on *Ae. albopictus* and other mosquito species as well should be performed instead of similar testings that are being carried out on *Ae. aegypti* alone.

For agricultural sector, environmentally friendly farming strategies with better yields should be fostered and rotation farming approaches should be practised and incorporated with proper agricultural practice. The employment of potential biological predators such as in oil palm plantations is also one of the alternative methods in eradicating the infestation of crop pests (Jamian et al., 2016). In terms of the application of agricultural pesticides, despite the initiation of Integrated Pest Management (IPM) strategies in agriculture that had been initiated in Malaysia since early 1980s to encourage planters to use pesticides in the most cost-effective ways (Triantafillou, 2001), more efforts are still needed so that these cultivators could be educated and trained on the appropriate use of pesticides as their control tools. A strong collaboration between the agricultural and public health sectors is also demanded in order to prevent, minimize or delay the resistance development among mosquito vectors due to double exposure of insecticides in agricultural areas.

CHAPTER 6: CONCLUSION

1. The dengue vector *Aedes albopictus* was the predominant container-breeder mosquito species collected in ovitraps placed in each study area.

2. The ecological plasticity of *Ae. albopictus* larvae was proven by its co-breeding with other mosquito species in the same ovitraps in which the mixed infestation of *Ae. albopictus* and *Ar. subalbatus* as well as co-infestation of *Ae. albopictus* and *Uranotaenia* sp. were reported for the first time in Malaysia via this research.

3. Most *Ae. albopictus* larvae from different types of area exhibited moderate to high resistance level against organochlorines and organophosphates tested at WHO recommended diagnostic dosages.

4. Inconsistent trends of susceptibility were presented among *Ae. albopictus* larval populations upon selection to all classes of larvicides at independent discriminated diagnostic dosages established from the local reference strain of *Ae. albopictus* larvae.

5. Significant differences in the susceptibility levels of *Ae. albopictus* larvae from dengue prone residential areas as compared to agricultural areas were observed against fenitrothion, fenthion, temephos, propoxur and permethrin.

6. With the exception of fenthion of organophosphates, the WHO recommended diagnostic dosages of organophosphate and organochlorine larvicides were much

lower than the independent discriminated diagnostic dosages of all classes of larvicides established from the local reference strain of *Ae. albopictus* larvae.

7. Larvicides for mosquito control that should be utilized in each type of area are diversified since different *Ae. albopictus* population possessed various susceptibility levels.

8. *Aedes albopictus* adults from different types of area displayed a wide range of knockdown time at 50% (KT_{50}) values but with resistance ratios (RR) of less than 3.00.

9. Although most *Ae. albopictus* adult populations from different types of area exhibited high resistance at indicated exposure time of adulticides, several populations had recovered to moderate resistance or even fully susceptible at 24 hours post-treatment due to increased mortality percentage indicating that certain adulticides required some time to achieve their full efficacy.

10. Significant differences in the susceptibility levels of *Ae. albopictus* adults from residential areas with or without insecticide exposure as compared to *Ae. albopictus* adults from agricultural areas had been observed only in the selection of fenitrothion, propoxur and bendiocarb.

11. Pyrethroids are the best adulticides to be applied for *Ae. albopictus* control in all study areas.

12. The cross resistance involving insecticides within the same classes were observed at larval stage alone (DDT and dieldrin; Fenitrothion and fenthion; Fenitrothion and temephos; Fenthion and temephos; Fenthion and chlorpyrifos; Temephos and chlorpyrifos), at adult stage only (between all pyrethroids) and also at both developmental stages of *Ae. albopictus* (propoxur and bendiocarb).

13. The cross resistance between different classes of insecticides (Organophosphates and carbamates; carbamates and pyrethroids) were demonstrated at both larval and adult stages of *Ae. albopictus* but involving different insecticides of these classes.

14. The activities of non-specific esterases (EST) comprising both α-EST and β-EST engaged significantly in the metabolic resistance in *Ae. albopictus* adults from all types of area but only significant role of α-EST activity was observed in *Ae. albopictus* larvae from non-agricultural areas.

15. Mixed function oxidases (MFO) activity was not significantly involved in the resistance development among *Ae. albopictus* larvae and adults from all types of area.

16. The glutathione-S-transferases (GST) was significantly involved in the metabolic resistance detected in *Ae. albopictus* larvae from almost all types of area. The significant role of GST in the resistance mechanism at adult stage of *Ae. albopictus* was only noticeable in the population from paddy cultivation areas.

17. The enzyme microassay of insensitive acetylcholinesterase (AChE) revealed the effectiveness of propoxur for the control of *Ae. albopictus* adults in all types of area but this insecticide was only useful as a larvicide in oil palm plantations and fogging-free residential areas.

18. The significant elevated activities of different detoxification enzymes at larval and adult stages of *Ae. albopictus* as well as very few associations demonstrated between these enzymes indicated that the role of each detoxification enzyme was self-determining, self-reliant and varied at each developmental stage of mosquitoes.

19. The pre-exposure to the synergist, piperonyl butoxide (PBO) prior to the exposure of organochlorines and pyrethroids had caused rapid knockdown in *Ae. albopictus* adult populations by 1.10 to 1.87 fold.

20. Rapid knockdown due to the utilization of PBO in combination with organochlorines and pyrethroids verified the significant role of PBO in delaying the insecticide resistance development among *Ae. albopictus* adult populations against organochlorines and pyrethroids which will enhance the efficacy and prolong the usage validity of these insecticides.

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4. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2018). Breeding habitats of mosquito larvae in a paddy growing area in Kuala Pilah, Negeri Sembilan, Malaysia. *Asian Journal of Microbiology, Biotechnology & Environmental Sciences*, *20*(2), 404-408.

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1. **Wan-Norafikah, O.**, Chen, C.D., & Sofian-Azirun, M. (2019). *Larvicidal effects of organochlorines and organophosphates against Aedes albopictus from agricultural areas of West Malaysia*. Paper presented at the School of Biological Sciences Postgraduate Conference 2019 (SBS-PGC 2019), 20-21st August 2019, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

2. **Wan-Norafikah, O.**, Chen, C.D., & Sofian-Azirun, M. (2019). *Larvicidal activities of organochlorines and organophosphates against Aedes albopictus from residential sites of Peninsular Malaysia*. Paper presented at the 55th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) 2019, 13-14th March 2019, InterContinental Hotel Kuala Lumpur, Kuala Lumpur, Malaysia.

3. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2018). *Sole and co-infestation of several mosquito species in selected residential areas in Peninsular Malaysia*. Paper presented at the 3rd International Symposium on Insects (ISoI 2018), 19-21st March 2018, Bayview Hotel, Langkawi Island, Kedah, Malaysia.

4. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2018). *Solitary and shared breeding of mosquito larvae in various agricultural areas in Peninsular Malaysia*. Paper presented at the 54th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) 2018, 14-15th March 2018, Connexion Conference & Event Centre, Bangsar South City, Kuala Lumpur, Malaysia.

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6. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2017). *Distribution of Aedes immatures in fogging-free and dengue prone residential areas in Peninsular Malaysia*. Paper presented at the 3rd Entomology Postgraduate Symposium (ENTOPOST) 2017, 13-14th December 2017, Malaysia Genome Institute (MGI), Kajang, Selangor, Malaysia.

7. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2016). ***Diversity in mosquito larval habitats in a rice cultivation area in Padang Serai, Kedah, Malaysia***. Paper presented at the 3rd International Conference on Science & Social Research (CSSR2016), 6-7th December 2016, The Everly Putrajaya Hotel, Putrajaya, Malaysia.

8. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2016). ***Breeding habitats of mosquito larvae in a paddy growing area in Kuala Pilah, Negeri Sembilan, Malaysia***. Paper presented at the Malaysia International Biology Symposium 2016 (*i*-SIMBIOMAS 2016), 26-27th October 2016, Putrajaya International Convention Centre (PICC), Putrajaya, Malaysia.

9. **Wan-Norafikah, O.**, Chen, C.D., Mohd-Amir, M.H., Azahari, A.H., Zainal-Abidin, A.H., Nazni, W.A., Mariam, M., Mohd-Shahizan, J., & Sofian-Azirun, M. (2016). ***Mosquito larval surveillance in a rice field in Tanjung Karang, Selangor, Malaysia***. Paper presented at the 2016 International Conference on Science and Technology Applications in Climate Change (STACLIM 2016), 11-12th August 2016, The Pacific Sutera Hotel, Sutera Harbour Resort, Kota Kinabalu, Sabah, Malaysia.