CHAPTER 7

GENERAL DISCUSSION

Insecticide resistance is an increasing problem faced by those who need insecticides to efficiently control medical, veterinary and agricultural insect pest. In many insects, the problem extends to all four major groups of insecticides. Effective resistance management depends on early detection of the problem and rapid assimilation of information on resistant insect population so that rational pesticide choices can be made (WHO, 1998). In many cases insecticidal pressure on the larval stage is more effective than on adults. This may be due to the fact that larval exposure operates over a longer period. The effect of selection pressure in terms of mortality in each generation is again dependent on the size, growth and relative isolation of the population. The larger the population and the higher the rate of growth, the higher the selection pressure that can be tolerated without loss of the necessary genetic material (WHO, 1976).

Cx. quinquefascistus has developed resistance to various insecticides including OP in California, Okinawa and France (Pennington, 1968; Georghiou, 1969; Georghiou *et al*, 1975; Georghiou and Pasteur, 1978; Tang and Wood 1986), carbamate in California and France (Georghiou *et al*.,1975; Tang and Wood 1986) and pyrethroid in France (Malcom and Wood, 1982; Tang and Wood, 1986). The 10th report of the WHO Expert Committee also stated that *Cx. quinquefasciatus* has developed resistance to carbamate and pyrethroid (WHO, 1986).

The existence of of malathion resistance in Malaysian adult and larval *Cx. quinquefasciatus* has been confirmed by biochemical test (Lee, 1990; Lee *et al*., 1992).

The larval bioassay test against *Cx. quinquefasciatus* from the present study revealed malathion and permethrin selected strains of larvae exhibited a significant reduction in resistance towards LC_{50} values after few generations. It was not clear why such variations on the LC_{50} value was found. However the reason for this can be explained as follows, insects can have various degrees of resistance and these are usually expressed as the ratio of the mean susceptibility (LD_{50}) of the R-population to that of the S-population. This value can depend greatly on the materials and methods used in gathering the data for estimating the LD_{50} and it should therefore be emphasized that there is no such thing as an absolute resistance factor. If different treatment methods are employed, the resistance mechanism will have a different opportunity to affect the final toxicity, which is determined by the interaction of factors such as penetration, transport and detoxification and intoxication reactions (Oppenoorth & Welling, 1976). A good example of this was provided by Busvine (1951), who showed that a certain strain of houseflies was 300-fold resistant to DDT applied topically in acetone, but only 16-fold when mineral oil was used as solvent. Higher resistance factors are often obtained when dosing is slow, such as in contact method where the toxicant is picked up gradually. At the same time, it does not imply that different methods provide results that are all equally meaningful. The penetration rate is generally only proportional to dose until a certain upper limit is reached. Above this limit, the effect of increasing the dose gradually becomes smaller. Therefore, R-strain can appear to be so resistant that they cannot be killed at all or only with very high doses. In such cases, a different method of treatment (larger amount of solvent, use of certain adjuvants) may give more satisfactory results (Oppenoorth & Welling, 1976).

LC_{50} for both malathion (F61 – F70) and permethrin (F54 – F63) resistant *Culex quinquefasciatus* increased steadily to the subsequent 10 generations indicating a marked development of resistance. A strain with a resistance factor of 5 and above is considered as resistant while that between 2-4 as tolerant (Brown, 1958). Variation of the LC_{50} values could be due to the differences among breeding areas, the larval density prior to tests, the volume, surface area and the depth of the solution (Das and Needham, 1961; Thomas 1970).

Generally, *Ae. aegypti* selected strain were more resistant than *Ae. albopictus* strains. One explanation for the higher resistance level in *Ae. aegypti* is the fact that this species naturally prefers to breed and rest indoors. Therefore, it is likely to be exposed to household insecticides and organized indoor spray treatments by public health workers more often than *Ae. albopictus* which is more exophilic. In turn, *Ae. albopictus* is likely to be exposed more often to agricultural insecticides (Ponlawat, 2005).

Malathion selected *Ae. aegypti* showed a modest increase in resistance compared to temephos. The gradual and steady movement of the dosage mortality line without change in slope also suggest an anology with vigor tolerance or at least a polyfactorial genetic origin for the malathion-tolerance (Matsumura & Brown, 1969).

When selection is done in laboratory population, the environment in the laboratory is different from the field (being usually more uniform and constant) and hence natural selection is different. In the laboratory, the selection pressure with the insecticide (e.g., constant mortality or constant dosage) may also be quite different from what happens in the field. If laboratory selection leads to the development of resistance (positive result), it can be concluded that such a resistance is likely to occur in the field.

Larviciding is the first step in chemical mosquito control, since the mosquitoes are killed at the breeding site, prior to dispersing and infesting a community (Chen *et al*., 2005). Today the early detection and monitoring of resistance is recognized as a vital part of resistance management. Resistance management is a relatively new area of research that is directed at developing insecticide use strategies that minimize the rate of evolution of resistance (Ferrari, 1996).

The most widely used organophosphates have been temephos, used as a larvicide in focal treatments and fenthion, fenitrothion and malathion used as perifocal spraying, indoor residual treatments or space treatments. At present, the World Health Organization (WHO, 2006) recommends treating drinking water with temephos at dosage not exceeding 1 mg of a.i. (1 ppm). Although spray programs with malathion have been active in some Latin American countries where *Ae. aegypti* and *Cx. quinquefasciatus* Say (Diptera: Culicidae) cohabitate, the latter species has developed higher resistance than the former (Rodriguez *et al*., 2000; Hamdan *et al*., 2005). There are some recent report showing that *Ae. aegypti* is still susceptible to malathion (Sames *et al*., 1996; Sharma *et. al*., 2004; Ponlawat *et al*., 2005).

The regular application of temephos as 1% (w/w) sand granules to domestic stored water in Malaysia is one of the major methods of controlling both the *Ae aegypti* and *Ae. albopictus* (Cheong, 1978). As a result of this, and other methods of control, the *Ae. aegypti* index has been brought down from 72% in 1973 (Wallace *et al*., 1980) to 14% in 1980 (Cheong, unpublished data). In Malaysia, *Ae. aegypti* has already developed resistance to DDT, fenthion and malathion (Thomas, 1976). Study conducted by Lee et al., (1984) revealed LC50 values of field-collected *Ae. aegypti* were about 2 to 3 times

that of laboratory strain., showing some degree of tolerance to Abate®, however no resistance to Abate® was detected (Lee, *et al*., 1884).

WHO has developed susceptibility bioassay tests (available kit form for purchase from WHO) for mosquitoes, lice, bedbugs, reduviid bugs, cockroaches, blackflies, houseflies, ticks and fleas (WHO, 1992). A study conducted in Centers for Disease Control and Prevention, Atlanta, Georgia, USA revealed that time-mortality bioassays were more sensitive than dose-mortality bioassays in detecting changes in susceptibility and had better correlation with microplate-based biochemical assays for resistance mechanisms (Brogdon, 1998, Brogdon *et al*., 1988a & Brogdon *et al*., 1988b).

In adult bioassay for *Cx. quinquefasciatus,* malathion at 5.0% concentration caused the least mortality rate in malathion resistant adults with 11.7% to 48.3 % of compared to permethrin 0.75% concentration at 98.3%-100% in *Culex quinquefasciatus.* This showed permethrin is the most potent insecticide to produce high level of mortality rate in adults. Cross-resistance in malathion resistant strain of adults with propoxur showed an increase in a high level from 0.6 folds of resistance to 1.3 folds of resistance however in 24 hours post- exposure treatment indicated 75% - 100% of mortality and permethrin resistant strain showed 100% of mortality.

Although larviciding induces more larval resistance than adult resistance and adulticiding may produce more adult resistance than larvae, resistance s not restricted to one or the other stage. The larval test by its nature is more sensitive than the adult test in detecting change in susceptibility level; roughly, a 2 fold increase in adult LC_{50} is accompanied by a 10 fold increase in larval LC_{50} and a 4 fold adult by a 100 fold increase in larval LC_{50} (Brown, 1986). Again roughly, a population may be termed resistant when

its larval LC_{50} has increased by 10 times (Knipling, 1950). In a study comparing the susceptibility of different instar of a laboratory strain of *Culex pipiens quinquefasciatus*, Mulla (1961) reported varying results, depending on the insecticide used. In general, early instar susceptibility are more susceptible to the compounds tested than were late instars; resistance was higher in fourth instars than in second instars of an OP-resistant strain (Wilder, 1972).

Presently, synthetic pyrethroids are preferred chemicals used widely in vector control operations in many parts of the world. These chemicals are assuming increasing importance by virtue of their exceptional high potency against vector insects, quick knock down effects, biodegradability and low mammalian toxicity (Lee, 1993). In vector control operations, permethrin is generally used as a contact adulticide such as for the impregnation of bednet. Relatively little known about the larvicidal activity of permethrin against mosquito larvae, so it is toxic to fish and some non-target microorganisms. The results obtained from bioassay indicated permethrin resistance was higher compared to malathion and temephos,

Early research conducted by (Lee & Winita, 1993), found to be contradicting with present study, where it was concluded that permethrin was equally effective as temephos and a promising chemical larvicidal agent in the control of container breeding *Aedes* larvae. The tolerance of *Aedes* larvae against other insecticides such as temephos in Malaysia have also been reported. Brown (1986) confirmed that the presence of temephos resistance in Malaysian *Ae. aegypti* larvae while Lee *et al*. (1984) reported that two Malaysian larvae populations of *Ae. aegypti* exhibited some degree of tolerance to temephos. Pyrethroids are not recommended for use as larvicides, because they have

broad-spectrum impact on non-target arthrpods and therefore may potentiate larval selection for pyrethroids resistance (Zaed, 2006; Chavasse & Yap 1997). Pyrethroids also break down very easily and are not suitable for use as larvicides.

Cross-resistance against propoxur was not obvious in all the selected strains from this study. High levels of carbamate resistance are generally not found in *Cx. quinquefasciatus* strains which contain only elevated esterase-based resistance mechanisms. Resistance levels to the carbamate propoxur were less than 4-fold in *Cx. quinquefasciatus* from Saudi Arabia and Sri Lanka after 20 generations of selection (Peiris & Hemingway, 1990).

There are two ways in which insecticide selection an affect the activity of an enzyme that can lead to resistance: by selecting for an aberrant structural gene producing an enzyme with different properties due to an alteration of its amino acid sequence or by selecting regulatory factors that determine the amount of the normal enzyme produced. The use of α-naphthyl acetate as a substrate combined with a sensitive method for the estimation of α-naphthol by coupling with diazo compound opens many possibilities for the study of esterases. It was introduced by Gomori and it seems to especially suitable for the study of esterases in insects. The use of of α-naphthyl acetate could be used to determine the non-specific esterases particularly the carboxyl-esterase (CarE) and cholinesterase (ChE) (Asperen, 1962). The use of biochemical resistance detection at a mechanistic level can provide a powerful tool for analyzing field and laboratory populations and clearly the biochemical assays provide more information about the insect population being analyzed (WHO, 1998).

The vast majority of commercial insecticides act at one of two targets in the nervous system. Organophosphate (OP) and carbamates interact with acetylcholinesterase (AChE) and changes in the sensitivity of this enzyme to inhibition have been well characterized biochemically. The changes are clearly due to mutant forms of the enzyme, multiple allelic forms of which have been identified in some species. The other major insecticide class, the synthetic pyrethroids, affect the function of voltage-dependent sodium channels by binding to a site distinct from those of o ther neurotoxins acting on the same protein. Electrophysiological and pharmacological studies have revealed a decreased sensitivity of this target to the toxic effects of pyrethroids.

Three broad enzyme classes are involved in insecticide detoxification, the glutathione transferases, monooxygenases and hydrolases. However, all three classes exist in multiple forms within each species and it is often not known whether increased activity arises from qualitative or quantitative changes in these enzyme complexes. This can often be determined by the use of model, surrogate substrates, especially for esterases and glutathione transferases but it then becomes important to demonstrate that the activity measured plays a role in the breakdown of insecticide. Resistance to insecticides is mediated by qualitative and quantitative changes in proteins that can often be difficult to define precisely at the biochemical level. The main changes involve a limited range of detoxifying enzymes and the few insecticide target side proteins and to a lesser extent completely unknown factors that can delay penetration of pesticide into insects.

The primary routes of insecticides resistance in all insects are alterations in the insecticide target sites (acetylcholinesterase) or changes in the rate at which the insecticide is detoxified. Three major enzyme systems, gluthathione S- transferase,

esterases and monooxygenases are involved in the detoxification of the four major insecticide classes. These enzymes act rapidly metabolizing the insecticide to non-toxic products or b y rapidly binding and very slowly turning over the insecticide (sequestration) (Nazni, *et al*., 2004 & Hemingway, *et al*., 1998). Organophosphorus and carbamate resistance due to detoxifying enzymes can be detected when decrceased inhibition of target enzyme by an organophosphate or carbamate may be overcome by an inhibitor of a specific detoxifying mechanism. (Brown, 1987).

Insecticide detoxification by esterases has been best characterized at the molecular level in mosquitoes and aphids, where increased synthesis of enzyme results from amplification of structural genes. In aphids the massive overproduction of esterase protein (60-fold) results in detoxification of insecticidal esters by both sequestration and hydrolysis when the inhibited esterase reactivates (Devonshire and Moores, 1982) and this could also be the case for *Culex* mosquitoes where there can be up to 500 fold increase in esterase equivalent to 15% of the insects' total proteins (Mouches et al., 1987).

The increased detoxifying ability and malathion resistance was associated with an abnormally low content of aliesterase and these characters were proved to be genetically inseparable (Oppenoorth, 1959). It was concluded that the malathion-resistant alleles was simultaneously responsible for increased phosphatase and decreased aliesterase (Oppenoorth & van Asperen, 1960).

In many insect species, such ad *Cx. quinquefasciatus* (Say), *Culex pipiens* (L.) and *Myzus persicae* (Sulzer), increase in esterase activity has been associated with insecticide resistance (Devonshire & Sawicki, 1979). Mixed mixed function oxidases

involved in insecticide detoxication by insects are comparable to the drug metabolizing enzymes of mammals (Gillette, 1966) and numerous investigations have shown that the latter vary in activity with species, sex, age and nutritional status of the animal. It is clear that the relative activity of these detoxication enzymes is of great significance in determining the concentration of drug ultimately available at the target site of action (Shawky, 1969).

Enhanced synthesis of these esterases were reported as to the mosquito's ability to detoxify insecticides into their non-toxic derivatives. Reports by Brogdon (1984) and Wu, *et al*., (1992) confirmed that mechanism of resistance in organochlorines and pyrethroids, e.g. DDT and permethrin were due to insecticide less efficiently transported to reach its target site action (Rohani, 2001). Brogdon (1984) reported that changes in the membrane phospholipids may have played a role in pyrethroid (kdr) resistances. Biochemical aspects of malathion resistance were first studied in the house fly (Musca doomestica L.). Insecticides resistance due to detoxifying enzymes can be determined if decreased inhibition of target enzyme is offset by an inhibitor of specific detoxifying mechanism. In addition, other factor such as migration may also influence the population of susceptible individual in the field population (Lee, *et al*., 1998). Resistance to pyrethroid in *Culex* mosquitoes occurs due to detoxification by cytochrome P450 monooxygenases (Kasai, 1998), as well as target site insensitivity (i.e. kdr) (Martinez-Torres, 1999).

In *Culex*, broad spectrum organophosphate resistance has generally been associated with quantitative changes in one or more phosphotriesterase enzymes (Curtis & Pasteur, 1981; Georghiou & Pasteur 1978; Pasteur and Sinerge, 1975). Recently

altered acetylcholinesterase (AChE) resistance mechanisms have been reported in two species, *C. pipiens* L. and *C. tritaeniorhynchus* Giles (Hemingway, 1986). Selection of this type of mechanism should be avoided, if possible as it produces a broad range of cross-resistance which drastically limits the choice of future replacement insecticides (Hemingway, 1986).

Present study clearly illustrated that non-specific esterase did not play a role in conferring OP's, pyrethroid and carbamate resistance in *Aedes aegypti* and *Aedes albopictus*. Contradicting to this finding, Paeporn (2003) reported that esterases play a significant role in temephos resistance. An earlier study conducted by Pethuan (2007) revealed that non-specific esterase could play a role in pyrethroid resistance. Esterase metabolism contributed to pyrethroid resistance in *An. Gambiae* (Brogdon & Barber 1990; Vulule *et al*., 1999) and elevation of a-esterase is correlated to permethrin tolerance in *Ae. aegypti* (Adriana *et al*., 2005). There was a clear association between OP resistance in *Culex* larvae and high level of esterase activity. Previous studies using biochemical assays have shown that the rise in GST or EST activity may result in resistance to OP insecticides (Brogdon, 1989; Hemingway and Karunaratne, 1998). Insensitive AChE is an indicator of resistance to organophosphates and carbamate insecticides whereas elevated non-specific ESTs often account for resistance to organophosphates, carbamates and pyrethroids (Terriere, 1984; Brogdon, 1989; Rosales *et al*., 1990; Hemingway & Karunaratne, 1998). Pyrethroid resistance is often a result of elvated non-specific EST activity.

Butz (1965) reported that alkaline phosphate activity rose sharply during the first few days of adult life in the flour beetle, *Tribolium confusum* with very little detectable

age-related changes in males. They reported that the difference of enzyme levels in females and males could be due to females having to undergo the process of egg production. As reported by Nazni *et. al*., (1999) aging is a biological phenomenon which has undergone evolution and the possession of a finite life span is an essential feature of survival. Only through more research in areas of biological and physical sciences will a better perception of age in relation to aging and resistance development can be ascertained.

Multiple molecular forms of enzymes or isozymes are commonly found in most organisms. Their study is providing notably promising in answering many basic questions pertaining to evolutionary, genetic and developmental research (Simon, 1991).Early work on the development of resistance to organophosphates in larval *Ae. aegypti* suggested that in the case of malathion, detoxification by esterases was of little importance and that physical mechanisms such as decreased absorption were prominent (Ziv & Brown, 1969). Later, Field (1984) showed a clear relationship between the level of tolerance to the organophosphorus insecticide malathion and the intensity of the esterase bands that are the product of the locus previously described as esterase-6 (Field & Hitchen, 1981).

Temephos-resistant strain of *Cx. quinquefasciatus* from California demonstrate upon electrophoresis a strongly staining esterase, which we designated as Esterase-B and a weakly straining esterase (Esterase-A). OP-susceptible adults display only weakly straining Esterase-A and Esterase-B (Georghiou and Pasteur, 1978). A similar association between OP resistance and the presence of a strongly staining esterase (named Esterase-3) has been described in *C. pipiens pipiens* L. from Southern France showed that the gene coding this esterase (Est-3) and gene coding resistance to chlorpyrifos are closely linked

(Georghiou, 1980). However, Esterase-3 of French mosquitoes and Esterase-B of California mosquitoes have very different biochemical properties indicating that they are coded by different genes. Additionally, OP resistance in each country displays different cross resistance patterns within this chemical group, thus supporting further the hypothesis that the genes for resistance may be different (Georghiou and Pasteur, 1978).

Insecticide resistance genes have developed in a wide variety of insects in response to heavy chemical application. At the gene level, two genetic mechanisms are involved in esterase overproduction, namely gene amplification and gene regulation (Raymond, 1998).

The polyacrylamide gel electrophoresis made it possible to observe a well-stained band with a relative mobility value of 0.779; this band was called A4 it was not observed in the reference strain of *Ae. aegypti* and may be associated to organophosphate resistance which remains to be proved in future research (Rodriguez, 1999). Adult bioassay tests conducted on selected strains showed no correlation against larval bioassay to pemethrin and temephos selected strains exceptional for *Cx. quinquefasciatus* malathion strain. This finding corroborated with poly-acrylamide gel electrophoresis on *Cx. quinquefascistus* malathion strain which showed over-expressed or elevated levels esterase levels based on staining with α- and β- naphthyl acetate. A handful of overproduced alleles have occurred in *Culex* mosquitoes in response to OP selection throughout the world ((Raymond, 1998).

To determine the influence of the non-specific enzyme esterase in conferring resistance, genetic analysis can be done as a further analysis. Further analysis is needed for a complete understanding of the genetics of ESTs in the selected strains.