Cytochrome P450s and Their Roles in Insecticide Resistance in the Mosquito, *Culex quinquefasciatus*

by

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Abstract

The southern house mosquito, *Culex quinquefasciatus*, is an important disease vector of West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), and filariasis. Insecticides are the most important component in the vector-control effort. Pyrethroids are currently the most widely used insecticides for indoor sprays of mosquitoes worldwide. However, mosquitoes have developed resistance to insecticides. An improved understanding of the mechanisms governing insecticide resistance is necessary and would be helpful to develop novel strategies for preventing resistance development, and controlling resistant mosquitoes.

A mosquito strain of *Cx. quinquefasciatus*, $HAmCq^{G0}$, was collected from Huntsville, Alabama, and established in the laboratory in 2002. The level of resistance to permethrin in the $HAmCq^{G0}$ strain is 10-fold, compared with an insecticide susceptible mosquito strain S-Lab, which has served as reference strain. The strain, $HAmCq^{G8}$, was the 8^{th} generation of permethrin-selected offspring of $HAmCq^{G0}$, with a resistance level to permethrin at 2700-fold compared with S-Lab strain. There are multiple mechanisms reported to be involved in pyrethroid insecticide resistance, including two major ones, cytochrome P450-mediated detoxification and voltage-gated sodium channel insensitivity. In order to gain insight in understanding the mechanisms conferring pyrethroid resistance in the *Culex* mosquito, I investigated the distribution of the resistance levels in the field strain $HAmCq^{G0}$ to permethrin by isolating 104 single-egg-raft colonies. I further characterized both the frequency of the L-to-F *kdr* mutation allelic expression of sodium channels and the expression levels of multiple up-regulated cytochrome P450 genes in single-egg-raft colonies with different levels of resistance to

determine their possible roles in resistance. To better understand the mechanism of cytochrome P450-mediated detoxification involved in insecticide resistance, I demonstrated the expression profiles for the total 204 P450 genes that are reported in the genome of *Cx. quinquefasciatus* from different strains bearing different pyrethroid resistance phenotypes (susceptible, intermediate and highly resistant). My objectives were to pinpoint the key P450 genes involved in insecticide resistance. With those key P450 genes identified, I then examined their functions in conferring resistance using double-stranded RNA-mediated gene interference (RNAi) techniques, and revealed that silencing the expression of up-regulated genes in mosquitoes resulted in a reduction of resistance. Furthermore, characterization of transgenic lines of *Drosophila melanogaster* with the mosquito P450 genes showed an induction of the levels of tolerance to permethrin in these transgenic lines. Taken together, my research indicated that multiple up-regulated P450 genes are co-responsible for detoxification of insecticides and insecticide selection, and also that multiple mechanisms co-work on the development of insecticide resistance in mosquitoes.

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List of Abbreviations

AChE	acetylcholinesterase
ANOVA	analysis of variance
bp	base pair
cDNA	complementary DNA
CYP	cytochrome P450
DDT	dichloro-diphenyl-trichloroethane
DEF	S,S,S-tributylphosphorotrithioate
DNA	deoxyribose nucleic acid
dsRNA	double stranded RNA
EMSA	Electrophoretic mobility shift assay
GABA	gamma aminobutyric acid
GST	glutathione S-transferase
$HAmCq^{G0}$	a field collected pyrethroid resistant mosquito strain
$HAmCq^{G8}$	a highly resistant mosquito strain following permethrin selection
JH	juvenile hormone
kdr	knockdown resistance
LC_{50}	lethal concentration necessary to kill 50% of a test population
LD_{50}	lethal dosage necessary to kill 50% of a test population
MFO	mixed function oxidases

mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
OP	organophosphate
P450	cytochrome P450 monooxygenase
PBO	piperonyl butoxide
PCR	polymerase chain reaction
PSMO	polysubstrate monooxygenases
qRT-PCR	quantitative real-time polymerase chain reaction
RISC	RNA induced silencing complex
RNA	ribose nucleic acid
RR	resistance ratio
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SiRNA	small interfering RNA
S-Lab	an insecticide susceptible laboratory mosquito strain
SNP	single nucleotide polymorphism
SSC	sodium chloride and sodium citrate
TAE	tris-acetate/EDTA

Chapter 1

Literature Review

1.1 Insecticide resistance

During the past few decades, insecticides have been widely and frequently used to control agricultural pests and disease vectors. However, the severe and repeated application of insecticide has resulted in insecticide resistance, which now has become a serious global problem. The definition of resistance given by the World Health Organization (WHO) is "the development of an ability in a strain of some organisms to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). In addition, the Insecticide Resistance Action Committee (IRAC) defines insecticide resistance as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". In this case, it is known that no individual itself has been changed by insecticides or any other toxicants, but the structure of the population as a whole has been modified by the insecticides selection process, during which more tolerant individuals bearing resistance factors (i.e. genes) have survived under such selection pressure (Brown and Pal, 1971). Consequently, the portion of the resistant individuals in a population increased, who are then able to pass the resistance factors to their offspring, causing the resistant level of the whole population to further increase. Therefore, some scientists consider the insecticide resistance as a pre-adaptive phenomenon due to the existence of resistant individuals prior to insecticide exposure.

In 1908, Melander first observed insecticide resistance, reporting about San Jose scale becoming resistant to lime sulfur in 1914 (Melander, 1914). However, the development of resistance to insecticides takes place rapidly. In 1939, the insecticidal effect of dichloro-diphenyl-trichloroethane (DDT) was discovered by Paul Muller, but unfortunately, not long after the discovery, it was reported that house flies developed resistance to DDT (Georghiou, 1986). By the 1960s, mosquitoes were also found to be resistant to DDT, which prevented the worldwide eradication of malaria (Desowitz, 1991). As more new types of insecticides have been applied to control agricultural pests and disease vectors, insecticide resistance has been continuously increasing. There were 1797 cases of resistance in arthropods (including all species and all insecticides) in 1984, and by 1991, over 500 species of insects were shown to be resistant to at least one insecticide at some level (Georghiou & Lagunes-Tejada, 1991). Moreover, as of 1984 at least 17 insect species were found to be resistant to all major classes of insecticides (Georghiou 1986). So far, resistance has developed to all five major classes of insecticides: cyclodienes, carbamates, dichloro-diphenyl-trichloroethane (DDT) and its analogues, organophosphates (OPs), and pyrethroids. Recently, resistance to some relatively new insecticides has also been reported, such as imidacloprid in Neonicotinoids (Liu and Yue, 2000), *Bacillus thuringiensis* (*Bt*) (Gerghiou et al., 1983; Goldman et al., 1986; Tabashnik et al., 1990), Abamectin (Scott, 1989), fipronil (Liu and Yue, 2000), and spinosad in spinosyns (Moulton et al., 2000).

Due to the development of insecticide resistance, more pests can survive under a high concentration of insecticide application. As a result, to control pests effectively, more frequent and heavier uses of insecticides are needed, which, in turn, can cause incredibly rapid development of insecticide resistance, serious environmental contamination (Feyereisen, 1995),

and damaging economic losses. Furthermore, resistance will cause resurgence of agriculturally important pests and outbreaks of human and animal diseases when the insect vectors cannot be controlled (Hemingway and Ranson, 2002). Finally, repeated use of the same class of insecticides to control a pest can also cause undesirable changes in the pest gene pool and possibly result in cross or multiple resistance to different insecticides(Huang and Han, 2007).

In sum, to slow down the development of insecticide resistance, operate the pest management wisely, and use the insecticides reasonably in order to protect the humans, animals, and environment, it is very important to deeply understand the mechanisms of insecticide resistance and control its development.

1.2 Mechanisms of insecticide resistance

According to the different characteristics of insecticide resistance, several mechanisms of insecticide resistance have been documented, such as increased metabolic detoxification, target site insensitivity, physiological modification, and behavioral change. Some insects with behavioral resistance may detect or recognize a danger and avoid exposure to the toxicants, while others may modify their physiological factors to decrease penetration/absorption of insecticides through their cuticles, increase the ability for sequestration/storage of toxicants, or accelerate excretion of poisonous compounds/metabolites. However, the most important and significant mechanisms involved in insecticide resistance are increased metabolic detoxification and target site insensitivity (Hemingway and Ranson, 2000), which have been widely studied and reported for many years. There are three enzymes mainly functioning in metabolic detoxification by elevating the amount or activities, which are cytochrome P450 monooxygenases (P450s), hydrolases, and glutathione-s transferases (GSTs). Target site

insensitivity results from alterations of amino acids that are responsible for the binding of insecticide molecules, including three biochemical sites: sodium channels, acetylcholinesterases (AChE) and gamma-aminobutyric acid (GABA) receptors (Liu et al., 2006). Besides these mechanisms of insecticide resistance, an additional one based on thermal stress response has been proposed (Patil et al., 1996), but its importance has not presently been assessed.

1.2.1 Increased metabolic detoxification

Metabolic detoxification is one of the most pivotal and common mechanisms of insecticide resistance (Scott, 1991; Hemingway et al., 2004). Insects use their internal enzyme systems to break down insecticides. Resistant strains may possess higher levels or more efficient forms of these enzymes. In addition to being more efficient, these enzyme systems also may have a broad spectrum of activity. There are three major types of enzymes involved in resistance: cytochrome P450-dependent monooxygenases, hydrolases (esterases), and glutathione S-transferases (Feyereisen, 1995). These enzymes can protect insects from xenobiotics and metabolize these lipophilic compounds into more water-soluble metabolites that can be excreted easily.

1.2.1.1 Cytochrome P450 monooxygenase-mediated detoxification

Monooxygenations of xenobiotics are catalyzed either by the cytochrome P450-dependent monooxygenase system or by the FAD-containing monooxygenase which has not been identified in insects yet (Scott, 1991).

The cytochrome P450 superfamily is a large and diverse group of hemoproteins of the b cytochrome type which is the carbon monoxide-binding pigments of microsomes, named for the unique absorption peak at 450nm when their reduced forms are treated with carbon monoxide.

The P450s are known in the literature by various names, such as cytochrome P450 monooxygenases, mixed function oxidases (MFO), polysubstrate monooxygenases (PSMO), microsomal oxidases, and heme thiolate proteins (Feyereisen, 1999; 2005). The role of cytochrome P450s (P450s) as the terminal oxidase is extremely essential, not only because of their role in regulating the titers of endogenous compounds such as hormones, fatty acids and steroids which are very important for insect growth, development, and reproduction (Feyereisen, 1999), but also in the catabolism and anabolism of xenobiotics such as drugs, pesticides and plant toxins (Scott, 2008), accounting for approximately 75% of the total metabolism.

P450s are found in almost all aerobic organisms, from plants, bacteria, and fungi to insects, birds, fishes, and mammals. The majority of cytochrome P450s in eukaryotes is embedded in the endoplasmic reticulum. The three most important components of the P450 monooxygenase system are cytochrome P450, which acts as the substrate binding protein; NADPH-cytochrome P450 reductase (P450 reductase), which transfers electrons from NADPH to cytochrome P450; and cytochrome b5, which transfers electrons from NADH to cytochrome P450 in some P450 monooxygenase systems as an additional potential electron donor (Scott, 1993; Feyereisen, 1999; 2005).

1.2.1.1.1 Nomenclature and discovery

Cytochrome P450 monooxygenases (P450s) derives its name from the discovery of a liver microsomal pigment (P) fifty years ago. This group of enzymes has an absorption peak at 450nm, when the reduced form is bond and saturated with carbon monoxide (CO). The nomenclature of P450s is introduced by Nebert et al, and now universally accepted, designates all gene members of theP450 superfamily with a CYP prefix, followed by a numeral for the family, a letter for the

subfamily, and a numeral for the individual gene, for instance CYP6A1. If it refers to a gene or cDNA sequence, it is shown in italics (*CYP6A1*), whereas the gene product, mRNA, and enzyme are shown in capitals (Feyereisen, 1999). For example, *CYP9M10* refers to a cytochrome P450 gene, family 9, subfamily M and gene number 10. Alleles are designated *v1*, *v2*, etc and pseudogenes are designated *p1*, *p2*, etc. P450s with >40% of the amino acids identical are usually grouped into the same family and members with >55% of the amino acids identical are generally grouped into the same sub-family. This nomenclature system is based on the overall amino acid identity, but no information regarding the function of a P450 should be assumed (Scott & Wen, 2001).

Currently, around 11,600 CYP genes in total have been identified and named. Among those, nearly one third (~3,282) genes are animal P450s and more than one third (~4,479) are from plants. The last part of P450 genes cover bacterial, protist and fungal P450s. In animal P450s, about half of the genes, more than 1675 genes belong to insects. Up to August 2009, there are 977 P450 families and 2519 subfamilies had been reported (Alzahrani, 2009), including 120 families in animals, 126 in plants, 731 in other groups (i.e. bacteria, protist, fungi, archaea, and viruses). Dr. David Nelson on behalf of the P450 Nomenclature committee is in charge of a website at the University of Tennessee, Memphis (http://drnelson.utmem.edu/nelsonhomepage.html). Most recent P450 sequence information can be found at this website.

1.2.1.1.2 P450 catalytic cycle

P450 systems are similar in mammals and insects. Outlines of the catalytic cycle are as follows (Ortiz de Monetellano, 1995; Figure 1.1): The heme protein in the oxidized form, P450

(Fe III), binds the substrate (RH). The P450-substrate complex receives a single electron from a redox partner, and P450 (Fe II) then binds oxygen. A second one-electron reduction step precedes the rather remarkable chemistry where molecular oxygen is split and a reactive oxygen complex inserts an atom of oxygen into the substrate (ROH) in a radical reaction with the other atom being reduced to water. The classical stoichiometry of a P450 monooxygenase, RH + $NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$, is only achieved in tightly coupled reactions, whereas the reactions are uncoupled to varying degrees and superoxide and hydrogen peroxide are generated by the collapse of one- or two- electron-reduced P450-dioxygen complexes (Feyereisen, 1999). After a serial of reactions during one P450 catalytic cycle, the substrates can be metabolized into more water soluble form of metabolites.

1.2.1.1.3 Insect cytochrome P450s

According to the sequences of insect P450 genes, insect CYP genes fall into four major clans, as the CYP2, CYP3, CYP4 and mitochondrial clans (Feyereisen, 2006; Nelson, 1998). CYP2 clan includes P450s that are responsible for essential physiological functions. CYP3 clan includes the most numerous P450 genes that play roles in xenobiotic metabolism and also insecticide resistance. Multiple P450 genes in this clan are insect-specific. Some genes in CYP4 clan are considered to be inducible by xenobiotics; others are reported to play a part in odorant or pheromone metabolism. In addition, mitochondrial P450 seems to be found only in animals. The P450 genes in this clan are involved in essential physiological functions and xenobiotic metabolism, but except insect ecdysteroids (Feyereisen, 2006). Insects have evolved a large variety of P450 genes because of their shorter generation time, wider ecological dispersal, and biological phenomenon of metamorphosis (Feyereisen, 1999). The first inset cytochrome P450

was first reported in houseflies in 1967, two years after the detection of P450 in mammalian liver (Scott, 2008). However, the first insect P450 (CYP6D1) was purified to a high specific content suitable for production of highly specific antisera (Wheelock and Scott, 1989). In 1989, the first insect P450 (*CYP6A1*) was cloned by screening a cDNA expression library from a housefly strain (Feyereisen et al., 1989). Subsequently, many researchers found multiple P450s in several insect species. There are more than 1600 P450s known and identified thus far. Two hundred four putative P450 (CYP) genes in *Cx. quinquefasciatus* have put them in the largest P450 repertoire for any insect genome that has been reported so far (Arensburger et al., 2010; Yang and Liu, 2011). There are 111 P450s in *Anopheles gambiae* (Ranson et al., 2002), 160 P450s in *Aedes aegypti* (Strode et al., 2008), 90 P450s in *Drosophila melanogaster* (Tijet et al., 2011), 92 P450s in jewel wasp *Nasonia vitripennis* (Oakeshott et al., 2010), 86 P450s in silk moth *Bombyx mori* (Li et al., 2005), 46 P450s in honeybee *Apis mellifera* (Claudianos et al., 2006), 134 P450s in red flour beetle *Tribolium castaneum* (Richards et al., 2008), 83putative/ 58 complete P450s in pea aphid *Acyrthosiphon pisum*, 115 P450s in green peach aphid *Myzus persicae* (Ramsey et al., 2010), 37 P450s in human body louse *Pediculus humanus* (Lee et al., 2010).

Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides and plant toxins by their transcriptional up-regulation, resulting in increased P450 protein levels and P450 activities, which, in turn, cause enhanced metabolic detoxification of insecticides and plant toxins in insects, and also known to be an important part of the biosynthesis and degradation pathways of endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH) and thus play important roles in insect growth, development, and production.

1.2.1.1.3.1 Functions in metabolism of endogenous compounds

Cytochrome P450s are reported to be an important metabolizer to juvenile hormones, which are synthesized from endocrine glands, the corpora allata (CA) and critical in insect development, metamorphosis, reproduction, and many other physiological processes (Helvig et al., 2004). Methyl farnesoate epoxidation to juvenile hormone III (JH III) was catalyzed by a P450–like enzyme in corpora allata homogenates of giant cave roach, *Blaberus giganteus* (Hammock, 1975). Moreover, a study on CYP4C7 of cockroach demonstrated that this P450 is either working on the decline in JH titer, mopping up the glands of potential JH precursors, or its expression stimulates CA changing its function to produce a new JH-related molecule (Sutherland et al., 1998). P450s are also involved in metabolism of a variety of terpenoids (Andersen et al., 1997).

The role of P450s in the metabolism of ecdysteroid is well established. Several P450 enzymes are identified to catalyze the desaturation of hydrocarbons in a metabolism (Feyereisen, 2011). The conversion of ecdysone to 20-hydroxyecdysone (20E) is catalyzed by a P450 enzyme, ecdysone 20-monoxygenase, initially localized in microsomes or in mitochondria (Feyereisen and Durst, 1978; Johnson and Rees, 1977). *CYP6H1* that was cloned from malpighian tubules of locust was studied to be involved in 20E biosynthesis (Winter et al., 1999). Another P450 *CYP302A1* was also reported to be take part in the metabolism of ecdysone in *Drosophila melanogaster* (Alzahrani, 2009). The conversion of cholesterol to 7-dehydrocholestrol is also mediated by P450s (Grieneisen et al., 1993).

In addition, insect P450s are also considered to catalyze the synthesis of insect pheromones and metabolize fatty acid and eicosanoid (Ahmed et al., 1979; Cuany et al., 1990). Bradfield et al. (1991) demonstrated that CYP4C1 cloned from the fat body of cockroaches associated with lipid

metabolism. Moreover, Reed et al. (1994) referred that the biosynthesis of hydrocarbons, abundant molecules in the cuticle of insects is also related to a P450 with specific properties. Several P450s, such as *CYP9A13*, *CYP4G20* in male moth *Mamestra brassicae*, were investigated predominantly expressed in sensory organs, (Maibeche-Coisne et al., 2005), and *CYP341A2* in the swallowtail butterfly, *Papilio xuthus* L. (Ono et al., 2005), suggesting their functions in olfactory systems.

1.2.1.1.3.2 Functions in metabolism of xenobiotics

It is best-known that P450 enzymes are involved in the metabolism of xenobiotics, such as insecticide and plant toxins, converting the foreign compounds to less toxic metabolites (Feyereisen, 1999). Metabolism of insecticides by cytochrome P450s leads insects in becoming resistant to insecticides, which is due to increased detoxification or decreased activation, resulting from change of enzyme catalytic activity or protein expression level (Oppenoorth, 1984; Schuler, 2012). There are several reactions involved in P450-mediated detoxification of xenobiotics on the basis of a variety of substrates, such as epoxidation, aromatic hydroxylation, thiophosphate ester oxidation, ester bond cleavage and aliphatic hydroxylation. However, when cytochrome P450s act on organophosphate or cyclodiene insecticides, the substrates can become more active toxicants after catalysis by P450s. The activation of OP insecticides is implemented by the substitution of sulphur on the double bond with phosphate to oxygen (P=S to P=O). For instance, the activation of malathion to more toxic metabolite, malaoxon. For another example referring to cyclodiene insecticides, aldrin and heptachlor can be metabolized into their epoxides, which also have more insecticidal effectiveness (Feyereisen, 2011).

Cytochrome P450s have a fundamental role on plant-insect interactions as well, due to their detoxification of certain plant secondary chemicals, leading to the behavioral changes of

herbivore insects (Schuler, 1996). Some research clarified that certain P450s and monooxygenases can be induced by elevating their gene expressions and enzyme activities after exposure to particular plant toxins (Feyereisen, 2011). For instance, a linear furanocoumarins, xanthotoxin, is toxic to most organisms due to their abilities to cross-link DNA strands, affecting transcription and replication (Berenbaum et al., 1990). However, larvae of black swallowtail butterflies, *Papillo polyxenes*, are able to digest this toxin and metabolize it to harmless form of metabolite by P450s in the midgut (Berenbaum et al., 1990). In addition, when plants get attacked and damaged by herbivorous insects, the plants produce volatile secondary chemicals, jasmonate and salicylate, to prevent them from insect damage by attracting natural enemies. In turn, in response to plant defense, insect enhance P450 enzymes to detoxify allelochemicals. The corn earworm, *Helicoverpa zea*, uses jasmonate and salicylate to activate four cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals (Li et al. 2002).

1.2.1.2 Hydrolase-mediated detoxification

Hydrolases or esterases, including carboxylesterases and phosphorotriester hydrolases are a large group of enzymes that are involved in Phase I detoxification, present in most organisms (Scott, 1991). Although hydrolases can metabolize all three major types of insecticides, which are the substrates organophosphate (OP), carbamate, and pyrethroids, hydrolase-mediated insecticide resistance has mainly been found in OPs and carbamates (Hemingway et al., 2004), especially in OPs. There are at least two mechanisms established so far in terms of the ability of the enzymes involved in insecticide resistance: 1) quantitative changes of esterases and 2) qualitative changes in enzyme structure.

1.2.1.2.1 Quantitative changes

Quantitatively changed esterase-base mechanism, also known as gene amplification (Small and Hemingway, 2000), is based on the over-production of the enzyme, resulting in the elevation of the hydrolytic enzymes for sequestering penetrated insecticides through hydrolysis of the enzyme catalytic center (Devonshire and Field, 1991). Gene amplification of esterases resulting in the production of abundant proteins in resistant insects has been identified as the primary mechanism of hydrolase-mediated resistance (Hemingway and Karunaratne, 1998; Small and Hemingway, 2000), protecting insects by rapidly binding and sequestering insecticides before the insecticides reach the target site. The mechanism of overexpression of these esterase enzymes involved in resistance to OPs or carbamates is most widely documented in certain aphid species and in *Culex* mosquitoes (Hemingway and Karunaratn, 1998; Devonshire and Field, 1991). The peach-potato aphid, *Myzus persicae* has developed resistance to OPs worldwide in both the field and greenhouse, resulting from the synthesis of high levels of esterase E4 (Devonshire and Field, 1992). Devonshire (1977) also found that, comparing the total carboxylesterase activity in resistant aphids to that in susceptible aphids, there was 60-fold increased activity observed and also a positive correlation between resistance level and carboxylesterase activity. Nevertheless, after purification and characterization, the esterase E4 was recognized up to 3% of the total protein present in resistant aphids, but exhibited weak enzyme activity, indicating that the resistance of the aphids to OPs was caused by the elevation of esterase E4 for sequestration of the insecticide (Devonshire and Moores, 1982). The greenbug, *Schizaphis graminum*, was also reported to have developed resistance to OPs by elevating esterase activity (Ono et al., 1994; Shufran et al., 1996). In *Culex* mosquitoes, resistance to OPs was also correlated with highly

active carboxylesterases (Soderlund, 1997). Two loci (esterase B and esterase A) were found to code for carboxylesterases that were over-expressed in resistant strains and resulted in the detoxification of OPs by sequestration and hydrolysis. Approximately 250-fold amplification of the esterase B1locus was detected in a resistant strain of *Culex quinquefasciatus* from California (Mouches et al., 1986).

1.2.1.2.2 Qualitative changes

Qualitatively changed esterase-base mechanism, also called non-elevated esterase mechanism, depends on the change of enzyme function (Hemingway et al., 2004), which confers resistance by increased metabolism caused by single point mutations in the structural genes. Consequently, the alteration to the amino acids of the enzyme can dramatically result in the change of the enzyme-substrate specificities, although few have been characterized and reported at the nucleotide level. Such a mechanism has been frequently in relation to resistance to the organophosphate insecticide malathion (Hemingway, 2000). Several insect species have been documented with an increased ability to degrade insecticides through alterations to the structure of an esterase, such as *Musca domestica* (Van Asperen and Oppenoorth, 1959), *Lucilia cuprina* (Hughes and Raftos, 1985), *Chrysomya putoria* (Townsend and Busvine, 1969), *Culex tarsalis* (Whyard et al., 1995) and *Plodia interpunctella* (Beeman and Schmidt, 1982). These structural changes resulted in the loss of activity for model substrates and led to the "mutant ali-esterase hypothesis" (Oppenoorth and Van Asperen, 1960), which elucidated that a carboxylesterase was modified to gain the ability for OP hydrolysis at the expense of activity for artificial substrates (Parker et al., 1996). Purification of malathion carboxylesterase (MCE) in both *Cx. tarsalis* and *L. cuprina* indicated that the enzymes with mutations existed in resistant strains but were absent in

susceptible ones (Parker et al., 1991; Whyard and Walker, 1994; Whyard et al., 1995). The resistant gene *LcαE7* encodes the major ali-esterase of *L. cuprina*, also known as the esterase isozyme E3. An amino acid substitution (Gly137→Asp) in the enzyme active site appeared to increase the ability for dephosphorylation of OPs and reduce ali-esterase activity (Newcomb et al., 1997). The same amino acid substitution was found in the OP-resistant house fly *Musca domestica* (Claudiano et al., 1999). A second mutation (Trp251→Leu) in the *LcαE7* was reported in the esterase E3 of *L. cuprina* associated with malathion resistance, indicating that mutations of amino acids in carboxylesterase enhance the hydrolase activity of the enzyme (Campbell et al., 1998).

1.2.1.3 Glutathione S-transferase-based detoxification

The glutathione S-transferases (GSTs), which currently have been recognized in most organisms, belong to a multi-functional superfamily (Snyder and Maddison, 1997), involved in Phase II detoxification. The first GST was reported to catalyze the conjugation of 1,2-dichloro-4-nitrobenzene (DCNB) with glutathione by Booth et al.(1961). The function of GSTs is catalyzing detoxification of xenobiotics, including insecticides, by conjugating endogenous substrates, such as reduced glutathione (GSH), to foreign substrates or their primary toxic metabolic products via a sulfhydryl group. This results in the conjugates becoming more water-soluble and more easily excreted (Hemingway, 2000; Salinas and Wong, 1999; Douglas, 1987). In addition to this primary role of GSTs, these enzymes also can bind and transport a variety of compounds as intracellular carrier proteins, which causes inactivation and immobilization by covalent binding. This is considered as an additional protective role of GSTs (Mannervik and Danielson, 1988). The GST mechanism often acts as a secondary resistance mechanism in linkage disequilibrium

with a monooxygenase or esterase-based resistance mechanism (Hemingway et al., 1991). There are at least two ubiquitously distributed and distantly related groups of GSTs, classified according to their location within the cell: microsomal and cytosolic GSTs (Enayati et al, 2005). In insects, there have been six different classes of GSTs (i.e. Delta, Epsilon, Omega, Theta, Sigma and Zeta) identified so far (Enayati et al., 2005). Among these six classes, the two largest classes, Delta GSTs and Epsilon GSTs, are unique to insects (Ranson et al., 2001).

In insecticide resistance mechanisms, elevated GST activity has been associated with resistance to all the major classes of insecticides, especially organophosphorus and carbamate, in many insects (Prapanthadara et al., 1993; Huang et al., 1998; Vontas et al., 2001). GST enzymes have been considered to be involved in resistance by association only, rather than by any individual GST. In addition, based on detailed molecular study, the elevation of the amount of GST(s), which can result from gene amplification, an increase in transcriptional rate or enhanced stability of mRNA, mainly confers the resistance rather than the qualitative change in individual enzymes (Grant and Hammock, 1992; Ranson et al., 2001). Two GST genes in *Drosophila melanogaster*, *DmGSTD1* and *DmGSTD21*, were expressed and detected as functionally active with different substrates. Insecticide-induced *GSTD1* was expressed more at the level of transcription initiation, while *GSTD21* was activated due to the increased stability of mRNA (Tang and Tu, 1995). The resistant housefly, *Musca domestica*, has been found to be able to metabolize OP insecticides higher than the susceptible strain with enhanced GST activity (Yang et al., 1971; Lewis and Sawicki, 1971). Four different GST genes from a resistant Cornell strain of housefly, *MdGST1, 2, 3,* and *4*, expressed in recombinant protein in *Escherichia coli*, which exhibited conjugating activity (Wang et al., 1991; Syvanen et al., 1994). Compared with the susceptible strain, all four of these GSTs were over-produced in the Cornell-R strain. However,

only *MdGST3* and *MdGST4* were subsequently reported to be responsible for the OP resistance (Syvanen et al., 1994; 1996). In the diamondback moth (DBM), *Plutella xylostella*, glutathione conjugation was reported as a major detoxifying reaction for parathion and methyl parathion, and the degradation of both insecticides was exhibited more highly in the resistant strain than in the susceptible one (Kao and Sun, 1991). Huang (1998) also confirmed the correlation of GST overexpression with high level of insecticide resistance.

Some other GSTs are also able to dehydrochlorinate insecticides, such as DDT, in a reaction where GSH acts as a co-factor rather than a conjugate (Clark and Shamaan, 1984), thus contributing to DDT resistance in insects. This has been studied in many insect species, such as the housefly *Musca domestica*, and the mosquitoes *Aedes aegypti* (Grant et al., 1991), *Anopheles gambiae* (Prapanthadara et al., 1993) and *Anopheles dirus* (Prapanthadara et al., 1996).For instance, in the African malaria mosquito, *Anopheles gambiae*, DDT resistance is attributed to an enhanced level of DDT-dehydrochlorination by GSTs (Hemingway et al., 1985). DDTase activity of all seven GSTs was detected in a resistant strain (Proapanthadara et al., 1995).

1.2.2 Target site insensitivity

Most insecticides, such as organophosphates, carbamates, organochlorine, and pyrethroid insecticides, are neurotoxic and aim for the nervous system. Three major targets, involved in insecticide resistance that is associate with point mutations, have been identified: 1) the sodium channels, responsible for raising the action potential in the neurons during the nerve impulses; 2) acetylcholinesterases, which have a role in the hydrolysis of the neurotransmitter acetylcholine; and 3) γ-aminobutyric acid (GABA) receptors, which are related to chloride-ion neurotransmission channels in the insect nervous system.

1.2.2.1 Insensitivity of voltage-gated sodium channels

Sodium channels are integral membrane proteins and play a significant role in the generation and propagation of action potentials in electrically excitable cells in the neurons of both vertebrates and invertebrates (Zlotkin, 1999), conducting sodium ions (Na^+) through the plasma membrane of a neuron. The sodium channel, while in the resting phase (deactivated) with no stimulus present, has sodium ions outside the cell and chloride and potassium ions inside. When the stimulus arrives and becomes strong enough, sodium channels open (activated) and sodium ions flush into the cell, resulting in depolarization of the membrane. When the action potential reaches its peak in milliseconds, the sodium channels close (inactivated) and potassium ions flow out, resulting in repolarization of the cell membrane occurring. Extra potassium ions continuously flowing out of the cell then causes temporary hyperpolarization of the cell membrane. After this stage, the cell membrane returns to the resting potential when sodium channels are then ready for the next stimulus (Dong, 2007).

Insect voltage-gated sodium channels are different from those in mammals because there is only a 241kDa α subunit, which folds into 4 hydrophobic repeat domains (I-IV) separated by hydrophilic linkers, but no auxiliary β subunits, whereas there are one or more 33-36kDa auxiliary β subunits in mammals.(Zlotkin, 1999; Hemingway and Ranson, 2000; Yu and Catterall, 2003). The α subunit is considered to conduct the main function of the entire channel. In each domain, there are six putative transmembrane helical segments (S1-S6) connected by small extra/intracellular loops (Yu and Catterall, 2003). Among these six segments, S4 is the most conserved segment, present in each repeated domain, and thought to be the voltage sensor.

Insect sodium channels have been widely studied as target sites for several insecticides, including DDT, pyrethroids, N-alkylamides and dihydropyrazoles, especially for the first two types (Soderlund and Kniple, 1995). Insecticides, such as pyrethroids, act on sodium channels to slow their activation and inactivation, causing the channels to open constantly and the eventual death of the affected insects (Narahashi, 2000). However, after repeated use of insecticides, insects have developed resistance to the insecticides due to reduced neuronal sensitivity of voltage-gated sodium channels to insecticides, also known as knock down resistance (*kdr*), conferred by one or more point mutations in the sodium channels. The point mutations, leading to resistant sodium channels by affecting gating, make the activation of the channels more difficult or inactivation much easier.

Knockdown resistance to DDT was first identified in the house fly *Musca domestica* (Busvine, 1951). The subsequent studies on *kdr* traits demonstrated that *kdr* was also found to confer cross-resistance to the entire class of pyrethroid compounds (Fine, 1961). An allelic form of *kdr* termed *super-kdr* was found to confer much higher resistance, up to 500-fold, to delta-methrin (Sawicki, 1978). The first two point mutations reported in house fly strains associated with the insecticide resistance are L1014F and M918T, conferring *kdr* and *super-kdr* respectively. L1014F is a substitution of phenylalanine for leucine at amino acid residue 1014 in domain IIS6, which is also called L-to-F mutation. Meanwhile, M918T is a mutation of methionine to threonine at residue 918 in the domain IIS4-S5 linker (Williamson et al., 1996; Soderlund and Knipple, 1999). To further study the function of the mutated sodium channels, Vais et al. (2000) expressed the wild-type and mutated sodium channels in *Xenopus laevis* oocytes and elucidated that *kdr* or *super-kdr* mutations reduced the fraction of available channels and the pyrethroids affinity for the sodium channels.

To date, at least 20 resistance-associated mutations on the sodium channels of 15 insect species in a range of important agricultural pests and disease vectors have been identified (Soderlund and Knipple, 2003). The majority of these mutations are detected on the S4/S5 linker and S5 helices of domain II, or the S6 helices of domain I, II and III (Wang and Wang, 2003). The mutation of L1014F has been found and studied mostly in other pest species, such as *Blattella germanica* (Dong, 1997), *Haematobia irritans* (Guerrero et al., 1997), *Plutella xylostella* (Schuler et al., 1998), *Anopheles gambiae* (Martinez-Torres et al., 1998), *Leptinotarsa decemlineata* (Lee et al., 1999), *Myzus persicae* (Martinez-Torres et al., 1999b; Criniti et al., 2008), *Frankliniella occidentalis* (2002), *Myzus persicae* (Eleftherianos et al., 2002), *Anopheles stephensi* (Enayati et al., 2003), and *Anopheles subpictus* (Karunaratne et al., 2007). Not only is this mutation reported to be associated with DDT and pyrethroids resistance, but several other mutations on sodium channels have been investigated in relation to knockdown resistance. A mutation of leucine 1014 to serine (L1014S) has been found in several mosquito species, *Culex pipiens* (Martinez-Torres et al., 1999), *Anopheles gambiae* (Ranson et al., 2000), and *Anopheles arabiensis* (Stump et al., 2004). In *Pediculosis capitis*, two mutations, threonine 929 to isoleucine (T929I) and leucine 932 to phenylalanine (L932F), in the IIS5 transmembrane segment were identified to be associated with permethrin resistance (Lee et al., 2000). Liu and Pridgeon (2002) demonstrated the leucine 1014 to histidine (L1014H) mutation in the house fly, *Musca domestica*. In the IIS5 segment, a mutation threonine 929 to valine (T929V) was also identified in *Bemisia tabaci* (Roditakis et al., 2006).In addition, in the IIS6 segment of *Aedes aegypti*, two mutations (I1011V and V1016I) were found (Saavedra-Rodriguez et al., 2007). Recently, the amino acid substitution F979S within the *kdr* gene was also documented in *Myzus persicae* (Criniti et al., 2008). Morgan et al. (2009) discovered a mutation (L925I) in the domain

IIS4-5 linker of the sodium channel gene in the cattle tick, *Boophilus microplus*, that is associated with cypermethrin resistance.

1.2.2.2 Insensitivity of acetylcholinesterase

Acetylcholinesterase (AChE) is a very important enzyme in the central nervous system of almost all animals and is responsible for hydrolyzing the neurotransmitter acetylcholine at the nerve synapse. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to terminate synaptic transmission and nerve impulses. In this case, once the AChE is inhibited, build-up of acetylcholine takes place, resulting in repeated stimulation of neurons and eventually death by exhaustion. In vertebrate species, two enzymes, acetylcholinesterase and butyrylcholinesterase exist according to their different substrates, whereas only one enzyme exists in arthropods, which is generally considered to be AChE with the characteristics of both (Toutant, 1989). There are two AChEs in insects encoded by two genes (Fournier, 2005).

In insects, the predominant molecular form of AChE is a globular amphiphilic dimer which is membrane-bound via a glycolipid anchor (Hemingway and Ranson, 2000). The key role of AChE is to break down acetylcholine into acetate and choline by hydrolysis (Kozaki et al., 2002). Acetylcholine is a chemical excitatory transmitter in the nervous system in humans and many other organisms. It is released at the synaptic cleft (the space between two nerve cells) so that the next nerve impulse can be transmitted across the synaptic gap. In the insect nervous system, AChE is the primary target of organophosphate (OP) and carbamate insecticides, which can inhibit AchE activity by phosphorylating or carbamoylating the serine residue within the active site of the enzyme (Corbett, 1974). Consequently, the inhibition of AchE prevents the

hydrolysis of acetycholine, causing continuous stimulation to synapses, and finally the death of the insects (Hemingway and Ranson, 2000; Nabeshima et al., 2003). To date, insects have developed the resistance to organophosphate and carbamate insecticides by alterations in AChE, resulting from multiple point mutations in theri structural genes which lead to a decreased sensitivity to inhibition of the enzyme by these insecticides (Ayad and Georghiou, 1975; Hemingway, 2004). The first case of an AChE exhibiting insensitivity to an organophosphate insecticide was reported in a paraoxon-resistant strain of red spider mite, *Tetranychus urticae* in 1964 (Smissaert, 1964). A few decades later, Fournier et al. (1989) succeeded in identifying a single gene *Ace*, coding for AChE in the central nervous system of *Drosophila melanogaster*, by cloning, sequencing and characterizing the gene. Five point mutations associated with insecticide resistance, F115S, I199V, I199T, G303A and F368Y, were identified in the resistant *Drosophila* strains by comparison to susceptible strains, indicating that recombination between resistant alleles preexists in the natural populations and confers the development of insecticide resistance (Feyereisen, 1995; Mutero et al., 1994). However, in *Drosophila* there is only one gene *Ace* encoding AChE, whereas there are two genes, *Ace1* and *Ace2* (or designated as *o-Ace* and *p-Ace*), in some other insect species (Holt et al., 2002).

According to the numeration of the *Torpedo californica* mature enzyme, many positions were found to possess mutations in different insect species, which are related to insecticide resistance (Fournier, 2005). In *Position 78*, in addition to the mutation F115S in *Drosophila*, mutation F139L has been detected in *Aphis gossypii*, which has proved to be resistant also to insecticides (Li and Han, 2004). In *Position 227*, the mutation G342A/V has been found in *Musca domestica* resistant strains (Kozaki et al., 2001; Walsh et al., 2001), and G303A mutation, which is the most frequent mutation providing resistance, has been identified in *Drosophila*

populations (Menozzi et al., 2004; Shi et al., 2004). *Position 331* provides another example where mutations found at this position in several species conferred resistance, such as the mutation S431F in *p-Ace* in resistant strains of *Aphis gossypii* (Toda et al., 2004; Andrews et al., 2004) and *Myzus persicae* (Nabeshima et al., 2003), the mutation F445W in *Culex tritaeniorhynchus p-Ace* (Nabeshima et al., 2004), and the mutation F439C in *Tetranychus urticae* (Anazawa et al., 2003).

1.2.2.3 Insensitivity of gamma-aminobutyric acid (GABA) receptors

The GABA receptors are a class of receptors including the nicotinic acetylcholine receptors, which respond to the neurotransmitter gamma-aminobutyric acid (GABA) (Hemingway, 2000). Ionotropic GABA receptors are widely distributed within both vertebrate and invertebrate central nervous systems and exhibit a differential topographical distribution, where they mediate rapid, mostly inhibitory synaptic transmission. Vertebrate GABA receptors are grouped into three types, $GABA_a$, $GABA_b$, and $GABA_c$, whereas GABA receptors in insect do not fall into the same categories (Rauh et al., 1990; Hosie et al., 1997). These receptors are thought to be comprised of five subunits (i.e. α, β, γ, δ and ρ subunits). However, so far only three subunits have been identified in insects, RDL (resistance to dieldrin), GRD (GABA and glycine-like receptor of *Drosophila melanogaster*), and LCCH3 (ligand-gated chloride channel homologue 3) (Wolff and Wingate, 1998; Knipple et al., 1995). GABA receptors binding with GABA form the chloride ion selective channels that contain five protein molecules of various subunits surrounding a central pore. Each subunit has four trans-membrane regions (M1-M4). The second one (M2) provides many of the residues that line the integral chloride channel through which chloride ions can pass (Buckingham et al., 2005).

GABA receptors can be activated by a number of compounds, GABA being a major one (Martin and Dunn, 2002; Buckingham et al, 2005). GABA is an amino acid transmitter in the central nervous system and neuromuscular junctions, mostly inhibitory in the adult vertebrate. When activated by GABA, the receptors allow the flow of chloride ions across the membrane of the cell, hyperpolarizing the cell and making it less likely to generate an action potential, in order to take part in the regulation of neuronal excitability throughout the nervous system. However, in insects, GABA also plays excitatory actions, mediating muscle activation at synapses between nerves and muscle cells and also the stimulation of certain glands (ffrench-Constant et al., 1993). Widely distributed in the insect nervous system, GABA receptors are targets of both natural compounds (i.e. picrotoxinin, short for PTX) and synthetic insecticides and convulsants (i.e. cyclodienes, trioxabicycloctanes, lindane, phenyl pyrazoles and fipronil) (Hosie et al, 1995). However, due to frequent use of such insecticides, recently a large number of insect species have developed insecticide resistance due to the reduction/loss of sensitivity of GABA receptors, caused by point mutations in the receptors.

A GABA receptor subunit gene, *Rdl* (Resistance to dieldrin), was indentified in field-collected strains of *Drosophila melanogaster* found to be resistant to dieldrin, a cyclodiene insecticide. A mutation at a single codon in the *Rdl* gene, causing an alternation of alanine with either a serine or a glycine at position 302, conferred the dieldrin resistance in these highly-resistant strains (ffrench-Constant et al., 1993). In the peach aphid, *Myzus persicae*, an Ala3Gly mutation was detected in a cyclodiene-resistant strain (Anthony et al., 1998). The laboratory selected resistant strain of *Drosophila simulans* displayed high resistance to the insecticide fipronil due to two mutations, A301G present in the RDL GABA receptor subunit, and an additional mutation T350M present in the third transmembrane domain (Le Goff et al., 2005). In

the M2 transmembrane domain, two amino acid substitutions have been associated with dieldrin resistance in various insect species. In the mosquito, *Anopheles gambiae*, Du et al. (2005) demonstrated the A296G substitution, while the A296S substitution was associated with dieldrin resistance in *Anopheles arabiensis*, *Anopheles stephensi* and *Aedes aegypti* (Ffrench-Constant et al., 2000; Du et al., 2005). Wondji et al. (2011) also observed two substitutions, A296S and V327I, in the M2 transmembrane domain of the mosquito *Anopheles funestus* in Africa.

1.2.3 Physiological modification

In addition to metabolic detoxification and target site insensitivity, physiological modification is another important mechanism involved in insecticide resistance. This mechanism includes decreased penetration/absorption of the insecticide through the cuticles or intestines, increased sequestration/storage of the insecticide in fat depots or other inner organs, and accelerated excretion of the active ingredients of the insecticide.

1.2.3.1 Decreased penetration/absorption

When the insecticides, either stomach insecticides or contact insecticides, act on the target insects, they must first pass through the cuticles or digestive tract lining in order to reach the site of action. Modifications of these tissues prevent or decrease the absorption or penetration of the chemicals, which also confers insects with certain levels of resistance. The insects are able to detoxify the insecticides more rapidly and easily due to smaller amounts of the insecticides passing through the body to the target sites. This resistance mechanism can widely affect most insecticides and frequently cooperate with other types of resistance mechanisms as a contributing factor rather than as an important mechanism of resistance on its own (Scott, 1991). It is

documented that resistant insects may absorb the toxin more slowly than susceptible insects. In the house fly, *Musca domestica*, a chromosome III gene *pen*, conferring insecticide resistance, was isolated and determined to reduce rates of insecticide absorption in the resistant strain (Plapp and Hoyer, 1968). A later study showed that ^{14}C-labelled fipronil penetrated more slowly in resistant house fly strains than in susceptible ones (Wen and Scott, 1999). Ahmad et al. (2006) demonstrated that the penetration of deltamethrin into resistant *Helicoverpa armigera* was much slower than into susceptible one. Some mosquitoes have also evolved thicker or altered cuticles, reducing penetration of the insecticide (Stone and Brown, 1969; Apperson and Georghiou, 1975; Wood et al., 2010). However, the molecular basis for this mechanism has not been clearly identified. Recently, a study on the laccase 2 gene (*CpLac2*) encoding for Laccase enzymes related to the cuticular tanning in the mosquito, *Culex pipiens pallens*, revealed that the *CpLac2* was expressed significantly higher in the resistant strain than in the susceptible strain, suggesting that resistance could derive from reinforcement of the cuticle, resulting in decreased penetration of insecticide through the cuticles (Pan et al., 2009).

1.2.3.2 Increased sequestration/storage

Another mechanism of insecticide resistance, sequestration involves storage of the toxicant at some other sites than the target site. Therefore, the insecticides inside the body can be kept away from vulnerable tissues and organs by sequestration and storage (Yu, 2008). In the elevated esterase-based mechanism, the overproduced esterase quickly sequesters OP insecticide with low hydrolysis activity (Claudianos et al., 1999). For example, resistant peach potato aphid, *Myzus persicae* (Sulzerz), produces very large amounts of carboxylesterase E4, about 3% of the total protein in resistant aphids. Carboxylesterase E4 catalyzes the hydrolysis of organophosphates

and carbamates in the aphids. However, its catalytic activity is low. It has been suggested that the resistant effect caused by E4 is not mediated by hydrolysis but by storage/sequestration (Devonshire and Moores, 1982). Moreover, most esterases that function by sequestration are elevated by gene amplification (Hemingway, 2000).

1.2.3.3 Accelerated excretion

Accelerated excretion is also a mechanism often involved in insecticide resistance, which prevents the concentration of the toxicant at the target site from reaching a threshold needed to act on the insects. For example, the excretion rate is much faster in malathion-resistant strains than that in susceptible strains of grain borers, *Rhizopertha dominica* (Fabricius) (Matthews, 1980). It has been found in diazinon-resistant thrips, *Frankliniella occidentalis* (Pergande), that excretion was faster than in the susceptible ones (Zhao et al., 1994). The Colorado potato beetle, *Leptinotarsa decemlineta*, may develop resistance to azinphosmethyl by fast excretion of this insecticide (Argentine et al., 1995). Scharf et al. (1999) also observed an increased excretion rate in the resistant Western corn rootworm, *Diabrotica virgifera.* In the beet armyworm, *Spodoptera exigua*, the rate of excretion was about twice as high in the greenhouse-selected strain than in the susceptible strain, resulting in a more rapid clearance of insecticide amounts from the insect body (Smagghe et al., 2003). However, so far there have not been conclusive findings on the molecular basis of increased rates of excretion.

1.2.4 Behavioral resistance

Insects may also acquire the ability to detect and recognize a harmful toxicant, which can lead them to escape these types of chemicals behaviorally. The first documented case of

"behaviouristic resistance" was reported by Trapido (1954) on *Anopheles albimanus* in Panama. Behavioral resistance is defined as the development of behaviors (i.e. escaping from harmful chemicals, repelling insecticides, or avoiding contact with insecticides) that reduce an insect's exposure to a toxin or that allow an insect to survive in an environment that is harmful and/or fatal to the majority of other insects under such selection pressures (Sparks et al., 1989).The behavior is categorized based on the causes underlying their expression, into either stimulus-dependent behavioral resistance or stimulus-independent behavioral resistance (Georghiou, 1972). Stimulus-dependent behavioral resistance, including increased irritability and enhanced repellency, requires sensory stimulation of the insect at the time when the behavior takes place. In irritability, an insect is stimulated to leave the immediate toxic environment upon contact with a treated surface, whereas in repellency, an insect is stimulated to leave the immediate toxic environment before contact with a treated surface. After ten generations of selection, a laboratory strain of the mosquito, *Anopheles atroparvus*, was observed to be more irritable to DDT-treated paper than the original strain (Gerold and Laarman, 1964). Another example of the house fly, *Musca domestica*, elucidated that after two-year use of malathion-sugar baits, the flies increased their repellency since the majority of them only approached the bait in flight instead of alighting on it, a reaction in contrast to their previous response (Kilpatrick and Schoof, 1958). Stimulus-independent behavioral resistance does not require sensory stimulation of the insect in order for avoidance to occur, an example being exophily, in which an insect behaves in a manner that avoids exposure to a toxicant by chronic occupation of nontoxic habitats. Thus, the measure of success for stimulus-independent behavioral resistance is the ability to occupy habitats preferentially or modify other behaviors selectively, thereby preventing contact with a toxicant (Lockwood et al., 1984). The mosquito, *Anopheles gambiae* was abundant in human habitations,

but became nonexistent after eight years of BHC treatments, and this remained the case even after the treatments were discontinued (Georghiou, 1972). Although behavioral resistance is mostly reported in mosquitoes, it has also been observed in some other insect species, such as the German cockroach, *Blattodea: Blattellidae* (Ross, 1992; Silverman and Bieman, 1993; Wang et al., 2004), the horn fly, *Haematobia irritans* (Lockwoodet al., 1985), and the maize weevil, *Sitophilus zeamais* Motschulsky (Guedes et al., 2009).

1.3 Mosquitoes as an insect pest

1.3.1 Introduction of mosquitoes and their medical importance

Mosquitoes are little flies and belong to the family Culicidae (Diptera), with two wings and a long piercing-sucking mouthpart. So far, over 3,500 species of mosquitoes have been described from various parts of the world, more than 200 species in the United States alone. Most species are nuisances because of the bloodsucking behavior of the females on living vertebrates, including humans. Mosquitoes are holometabolous insects and have four distinct developmental stages in their life cycles, including egg, larva, pupa, and adult. Female mosquitoes lay their eggs on the surface of the water or somewhere nearby since the hatching of the eggs requires water. The shape of the eggs is commonly oval or cigar-shaped measuring approximately 0.6mm in length (Clements, 1992). The modes of oviposition vary among genera but are mostly found in common among species in one genus. Some mosquitoes drop their eggs singly, such as *Anopheles* and *Aedes* mosquitoes, while others lay their eggs in arrays called “rafts” such as *Mansonia*, *Coquillettidia*, *Culex*, *Culiseta*, and *Uranotaenia* mosquitoes. Most eggs hatch into larvae within 48 hours. The larvae of mosquitoes are aquatic and often called wigglers because they “wiggle” up and down in the water. Most larvae have siphon tubes for breathing and hang

from the water surface, and they dive below only when disturbed. Larvae have four developmental stages, called instars, ranging from 4 to 14 days, during which they feed on microorganisms in the water. After the fourth molt, larvae change into pupae. As with the larvae, the pupae are also aquatic and are called tumblers, usually looking comma-shaped. Mosquitoes in this stage also need to come to the surface for breathing but do not feed. It takes approximately two days before the pupae emerge into adults (Foster and Walker, 2002). Male adults usually emerge before females. The newly-emerged adults rest on the surface of the water for a short time in order to dry and harden themselves. Adult mosquitoes mate within a few days after emergence. Typically, males live shorter lives than females, only about one week, feeding on the nectar of flowers or other sources of sugar, while females who also feed on the nectar live longer than a month, but also need a blood meal after mating. The blood meal provides the females with the necessary nutrition to develop their eggs. Female mosquitoes are able to seek out hosts for blood meals by trace chemicals (i.e. carbon dioxide, amino acids and octenol) emitted from the hosts (Clements, 1999). Most mosquitoes attack and bite animals at dusk or after dark, but some species can attack during daylight hours or in the shade during the day, such as *Aedes* and *Culiseta*, which are more aggressive biters. The lifespan of mosquitoes varies depending on temperature mostly, humidity, and the acquirement of a blood meal. Mosquitoes are active for the entire year in the humid tropical regions, but exhibit diapause over the winter in cold regions.

As blood-feeding biters, female mosquitoes are considered vectors for many diseases via viruses and parasites. Different mosquitoes can transmit different diseases. For instance, *Aedes* mosquitoes (i.e. *Aedes albopictus*, *Aedes aegypti* and *Aedes cinereus*) transmit yellow fever, dengue fever and Chikungunya. Severe dengue can be fatal and spread through the bites of

infected mosquitoes. *Anopheles* mosquitoes (i.e. *Anopheles gamniae*, *Anopheles funestus*, and *Anopheles stephensi*) are the only ones which transmit malaria to humans, and they also can transmit canine heartworm and filariasis. Another important vector is the *Culex* mosquitoes (i.e. *Culex quinquefasciatus*, *Culex pipiens*, and *Culex territans*), which transmit West Nile virus (WNV), filariasis, Japanese encephalitis, St. Louis encephalitis, avian malaria and tularemia (Sardelis et al., 2001; Granwehr et al., 2004; Liu et al., 2006). These diseases can be transmitted by various mosquitoes to more than 700 million people annually, causing at least two million deaths in the areas of Africa, South America, Central America, Russia and much of Asia (Morel et al., 2002; Muslu et al. 2011).

1.3.2 Insecticide resistance in mosquitoes

Since mosquitoes are annoying insects bothersome to people around homes and public places and important vectors capable of transmitting fatal diseases to both humans and other animals, control of the mosquitoes has become a very vital strategy. So far, chemical means mostly administered as insecticide applications have played a principal role in mosquito control due to their efficiency and easy application. However, development of insecticide resistance has proven to be a significant problem in mosquito control as it has been with other insects. To control malaria, DDT was first used in 1946, but unfortunately, only one year later, the mosquitoes, *Aedes tritaeniorhynchus* and *Aedes solicitanswas*, were found to display resistance to DDT (Brown, 1986). In an effort to combat the problem, more and more insecticides were produced for mosquito management, yet the resistance problems continued with the newer insecticides, such as the organophosphates, carbamates and pyrethroids. Currently, pyrethroids are most widely used as indoor residual house sprays for bednets and curtains due to the low

toxicity to humans and high effectiveness against pests (Zaim et al., 2000; Hougard et al., 2002). So far, more than 125 mosquito species have been reported as resistant to one or more insecticides (Hemingway and Ranson, 2000). Most resistance mechanisms involved in mosquito resistance to insecticides can be grouped into two categories, metabolic detoxification (alterations in the levels or activities of detoxification enzymes), and target site insensitivity (mutations in the sodium channel, acetylcholinesterase and GABA receptor genes) (Hemingway et al., 2004).

Increased metabolic resistance, which involves the sequestration, metabolism, and/or detoxification of the insecticides, is considered to decrease the effective dose acting on the target sites. There are three groups of enzymes involved in insecticide resistance: cytochrome P450s, hydrolases, and GSTs. Enhanced P450 monooxygenase activities have been detected in the resistant mosquitoes *Anopheles gambiae* (Vulule et al., 1999) and *Anopheles albimanus* (Brogdon et al., 1999). Subsequent research has proved that overexpression of P450 genes is mainly responsible for elevated P450 enzyme activities and, in turn, insecticide resistance. Since the whole genome sequences of *Anopheles gambiae,* Aedes *aegypti*, and *Culex quinquefasciatus* have been released (Holt et al., 2002; Nene et al., 2007; Arensburger et al., 2010), a comprehensive study of total P450 genes in these three genomes can be conducted. Prior to this, Kasai et al. (2000) reported a ~2.5-fold elevated level of a P450 gene, *CYP6F1,* in a strain of *Culex pipiens quinquefasciatus* with permethrin resistance. Another adult-specific P450 gene, *CYP6Z1,* has been shown in increased transcript levels in a pyrethroid-resistant strain of *An. gambiae* from East Africa (NiKou et al., 2003). In the same year, up-regulation of *CYP4* family members was found in a relative species, *Culex pipiens pallens* with deltamethrin resistance (Shen et al., 2003). In pyrethroid-resistant strains of *Anopheles funestus,* the CYP6P4 and

CYP6P9 genes are overexpressed 51- and 25-fold compared with the susceptible strain (Wondji et al., 2009). In the mosquito, *Culex quinquefasciatus,* analysis of the total P450 genes in the whole genome revealed that up- and down-regulation of multiple P450 genes co-occur following permethrin selection (Yang and Liu, 2011). The *Culex* mosquitoes, *Culex pipens, Culex quinquefaciatus*, and *Culex tritaeniorhynchus*, are also widely studied and documented to have a resistant mechanism of over-production of carboxylesterases, also called quantitative changes in response to OPs and carbamate insecticides (Hemingway and Karunaratne, 1998). There are some reports of enhanced esterase activities in the permethrin-resistant *Anopheles gambiae* (Vulule et al., 1999), *Anopheles albimanus* (Brogdon and Barber, 1990), and *Aedes aegypti* (Mourya et al., 1993). However, qualitative changes of carboxylesterase, resulting in rapid hydrolysis instead of increased sequestration, have also been observed in several mosquito species, *Anopheles culicifacies, Anopheles stephensi* and *Anopheles arabiensis* (Herath et al., 1987; Hemingway, 1982; 1983). Other detoxification genes, such as the glutathione transferases *GSTE2* and *GSTE3* were also documented as over expressed in an *Aedes aegypti* permethrin resistant strain (Strode et al., 2008). Recently, members of the Epsilon class of *GST*s have been identified by microarray analysis as up-regulated in the DDT-resistant populations of *Aedes aegypti* (Marcombe et al., 2009).

Target site insensitivity is another mechanism conferring insecticide resistance in mosquito species due to point mutations on the target sites. Knock-down resistance (*kdr*) is attributed to severe use of DDT and pyrethroids and linked to point mutations in the *para*-type voltage-gated sodium channel genes. The most common *kdr* mutation associated with pyrethroid resistance, occurs in the S6 segment of domain II in the sodium channel gene and involves a substitution of leucine to phenylalanine (L1014F) (Williamson et al., 1996). It has been investigated in several

mosquito species, such as *Anopheles gambiae* (Martinez-Torres et al., 1998), *Anopheles stephensi* (Enayati et al., 2003), *Aedes aegypti* (Brengues et al., 2003), *Culex quinquefasciatus* (Xu et al., 2005), and *Anopheles subpictus* (Karunaratne et al., 2007). However, another substitution (Leucine to serine, L1014S) at the same position has also been reported in *Culex pipiens* (Martinez-Torres et al., 1999), *Anopheles gambiae* (Ranson et al., 2000), *Anopheles sacharovi* (Luleyap et al., 2002), and *Anopheles arabiensis* (Stump et al., 2004). In addition to mutations in sodium channel genes, mutations in acetylcholinesterase (AChE), which is the target site of OPs and carbamate insecticides, are also found to contribute to insecticide resistance in mosquitoes. A single amino acid substitution, glycine to serine (G119S), has been found in the *ace-1* coding region associated with insecticide resistance in the mosquitoes *Anopheles gambiae* (Weill et al., 2003), *Culex pipiens* (Weill et al., 2003), and *Culex quinquefasciatus* (Liu et al., 2005). The mutation in gene *ρ-ace*, phenylalanine to tryptophan (F445W) has also been reported in high-level OP resistant *Culex tritaeniorhynchus* (Nabeshima et al., 2004). Insensitivity of GABA receptors is also considered to confer resistance to cyclodiene and fipronil insecticides in mosquitoes. In *Anopheles gambiae*, the A296G substitution was found to confer dieldrin resistance (Du et al., 2005), while the A296S substitution was found to confer resistance in *Anopheles arabiensis*, *Anopheles stephensi* and *Aedes aegypti* (Ffrench-Constant et al., 2000; Du et al., 2005). Another two substitutions, A296S and V327I in the M2 transmembrane domain of the GABA receptor, have also been demonstrated to confer dieldrin resistance to the mosquito *Anopheles funestus* in Africa. (Wondji et al., 2011).

1.4 Techniques for gene functional study on mosquitoes

As insecticide resistant mechanisms (metabolic caused by changes in the levels or activities of detoxification proteins and target site resulting from mutations in the sodium channel, acetylcholinesterase and GABA receptor genes) have been described in mosquitoes, more and more research have concentrated on the molecular and genetic basis of those mechanisms to elucidate development of insecticide resistance (i.e. how genes and the proteins they encode function in the organism), and amazing progress has been made using molecular methods and technology.

1.4.1 RNA interference technology

RNA interference (RNAi) is a posttranscriptional gene silencing technique accomplished by knocking down or silencing the activity of specific target genes, an efficient way to study gene function in living organisms. RNAi was first noticed in petunias (Napoli et al., 1990), but first reported in the nematode *Caenorhabditis elegans* in 1998 (Fire et al., 1998). In 2006, Andrew Fire and Craig Mello were awarded the Nobel Prize in physiology or medicine for this discovery.

RNAi works by destroying the messenger RNAs (mRNAs), which include information encoding proteins, with the double-stranded RNA (dsRNA) trigger (Bernstein et al., 2001; Figure 1.2). Upon entering a cell, the dsRNAs are recognized and cut into ~23 bp small fragments by an enzyme, named Dicer that belongs to the RNase III family of nucleases (Hamilton and Baulcombe, 1999). The small fragments of double-stranded RNA molecules, termed short interfering RNAs (siRNAs), are unwound to single-stranded by the endonuclease Argonaute 2 (Ago 2), and incorporated into additional components to form the RNA-induced Silencing Complex (RISC). The RISC uses the unwound siRNA to find and base pair with the target mRNA that has a complementary sequence, thus guiding the RNAi machinery to the target

mRNA resulting in the cleavage and subsequent degradation of the mRNA (Hammond et al., 2000; Zamore et al., 2000; Pham et al., 2004; Fuchs et al., 2004). In this case, the degraded mRNA is successfully deprived of its function and prevented from being used as a template for protein translation (Ahlquist, 2002).

In insects, the fruit fly, *Drosophila melanogaseter* was chosen as the first model to conduct gene functional studies using RNAi. In 1998, the genes, frizzled and frizzled 2, were studied in *D. melanogaster* using RNAi (Kennerdell and Carthew, 1998). Since then, RNAi has been applied as a powerful tool in studying gene function in many insect species, such as studies of *Hox* genes in the red flour beetle, *Tribolium castaneum* (Brown et al., 1999), and the milkweed bug, *Oncopeltus fasciatus* (Hughes and Kaufman, 2000). RNAi has also allowed the disruption of vitellogenin expression in the honey bee, *Apis mellifera* (Guidugli et al., 2005), and the cockroach, *Blattella germanica* (Martin et al., 2006). RNAi has also played a very critical role in gene functional studies of mosquitoes. Silence of the apyrase gene *AgApy* in the salivary glands of the malaria mosquito, *Anopheles gambiae*, showed that this gene is important in the host probing behavior of the mosquitoes (Boisson et al., 2006). Injection of dsRNA of the gene *defensin* into the mosquito, *An. gambiae* knocked down the gene, which revealed that *defensin* is important for protecting the mosquito against infections of Gram-positive bacteria (Blandin et al., 2002). In the study of insecticide resistance in the same mosquito, *An. gambiae*, knockdown of the gene encoding for NADPH cytochrome P450 reductase by RNAi increased the sensitivity of mosquitoes to pyrethroids (Lycett et al., 2006).

1.4.2 Transgenic technology

To understand the function of a gene, not only can we knock down the gene in the organism to investigate the response, but also we can transform the target exogenous gene into the genome of otherwise organisms to exploit the changes of the host. The latter process is known as transgenic technology, an effective tool to elucidate insect gene function and genetically modify insects for pest control. Transgenic technology, using transposable P-element transposition system, was first applied in the fruit fly, *Drosophila melanogaster*, due to its short generation time, low maintenance requirements, and relatively simple genome (Rubin and Spradling, 1982; Figure 1.3). The use of transposable elements, especially P-elements, in *D.melanogaster* has led to many valuable applications (Ryder and Russell, 2003).Since the completion of the fly genome sequence in 2000 (Adams et al., 2000), the precise genomic location of transposon insertions can be determined. P-elements are Class II transposons, segments of DNA capable of being moved directly to different position in the genome of a single cell, making them valuable tools to generate transgenic flies for gene functional study. The gene of interest is placed between P-element ends, usually within a plasmid vector, and injected into embryos. The P-element, as a cargo, carries the target gene and transposes it from the plasmid to a random chromosomal site. Another plasmid vector, named helper-plasmid that includes a transposase gene to inhibit autonomous movement, is used along with the P-element-vector (Karess and Rubin, 1984; Laski et al., 1986). To identify transformants, a visible marker gene is used to insert the inverted terminal repeats (Rubin and Spradling, 1983), such as *white*, *rosy*, *vermilion*, or *yellow* (Klemenz et al., 1987; Pirotta, 1988; Fridell and Searles, 1991; Roseman et al., 1995), which are consequently expressed as eye colors. However, P-element was determined not to work outside the genus *Drosophila* after several attempts (Atkinson and James, 2002). Several other transposable elements have been widely used in other insects though for genetic transformation,

such as *mariner*, *Hermes*, *Minos* and *piggyBac* (Robinson et al., 2004). The transgenic technology has also been broadly applied to implement pest management programs against both agricultural pests and disease vector (Benedict and Robinson, 2008), by either population reduction, relying on Sterile Insect Technique, or population replacement, introducing a resistance mechanism to prevent disease transmission (Scott et al., 2005; James, 2005). This powerful approach has been reported to have been employed successfully in several pest insects, such as the pink bollworm *Pectinophora gossypiella* in California (Henneberry, 2007), the tsetse fly *Glossina austeni* in Zanzibar (Vreysen, 2001), the new world screwworm Cochliomyia hominivorax in North and Central America (Wyss, 2000), the yellow fever mosqutito *Aedes aegypti* (Kokoza et al., 2000), the malaria mosquitoes *Anopheles stephensi* (Ito et al., 2002), *Anopheles gambiae* (Marshall and Taylor, 2009), and various tephritid fruit fly species in various regions of the world (Klassen and Curtis, 2005).

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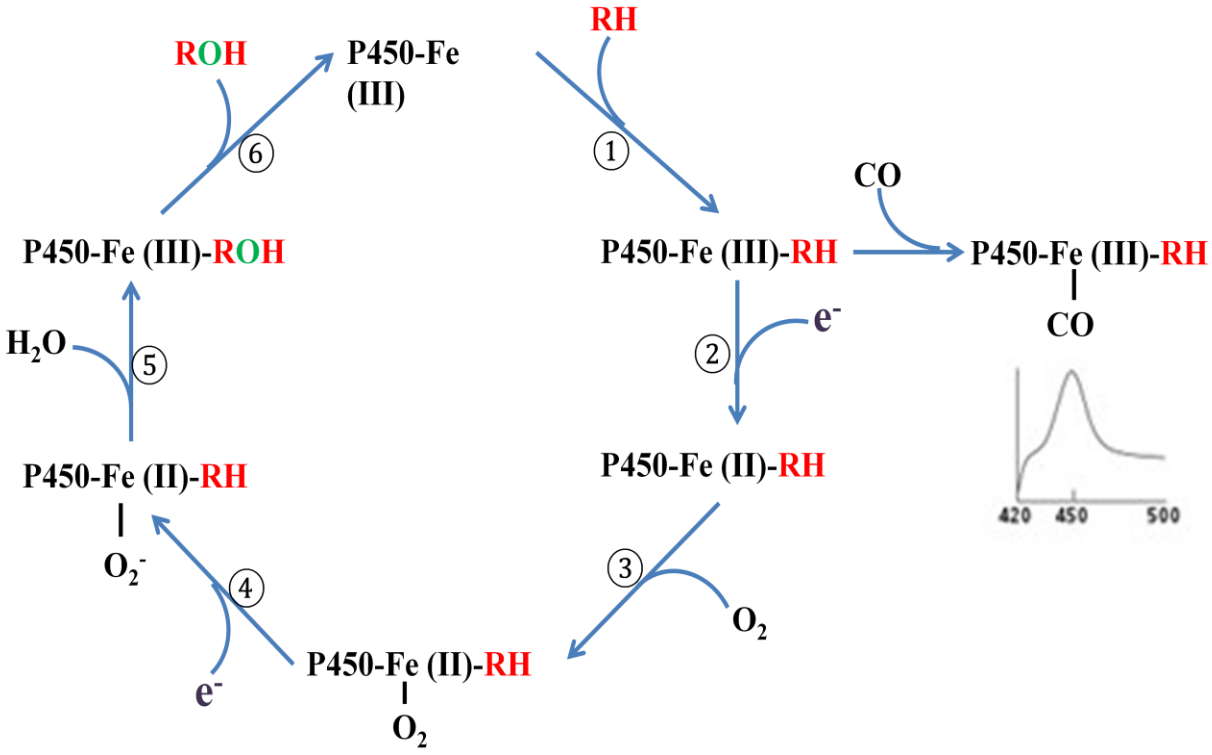

Figure 1.1 Cytochrome P450 catalytic cycle

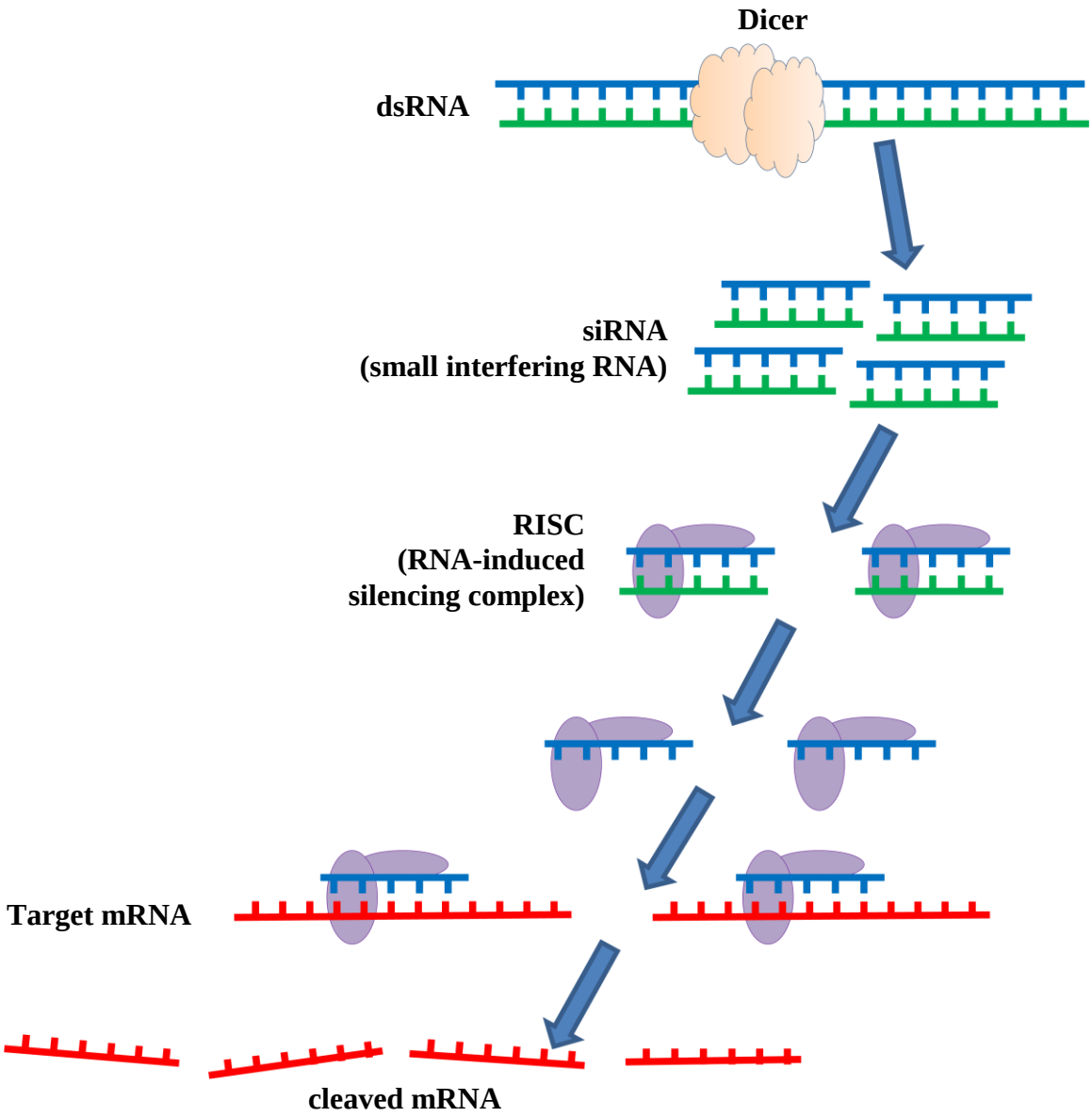

Figure 1.2 The scheme of RNA interference

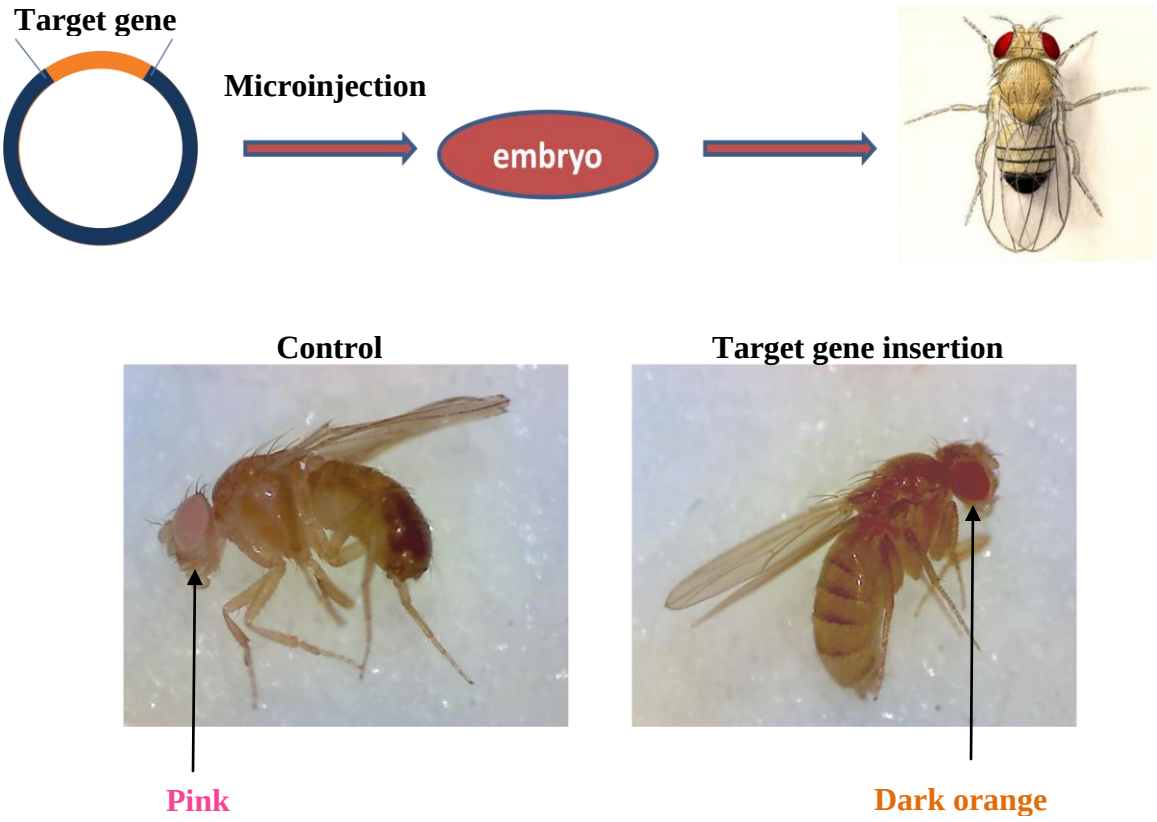

Figure 1.3 The scheme of *Drosophila melanogaster* transgenic technique

Chapter 2

Research Goal and Specific Objectives

2.1 Introduction

The mosquito, *Culex quinquefasciatus* is a cosmopolitan nuisance biting pest. In the Southeastern USA, this species is moderately competent as an important vector of viruses such as West Nile virus (WNV) and St. Louis encephalitis virus (SLEV), and nematodes that cause lymphatic filariasis (Sardelis et al., 2001; Jones et al., 2002; Foster and Walker, 2002). The approach used to control transmission of these pathogens and other mosquito-associated diseases has primarily relied on the application of insecticides, especially pyrethroids (McCarroll and Hemingway, 2002). Pyrethroid insecticides are currently the most widely used insecticides for the indoor control of mosquitoes and are the only chemical group recommended for the treatment of mosquito nets, the main tool for preventing malaria in Africa (Hemingway et al., 2004).However, mosquito-borne diseases are now resurgent, mainly resulting from the difficulty in controlling vectors that have developed resistance to insecticides.

The most significant mechanisms involved in pyrethroid resistance are increased metabolic detoxification by cytochrome P450s and decreased target site sensitivity of sodium channels. Cytochrome P450s are known to be involved in the metabolism of insecticides and plant toxins by their transcriptional up-regulation, which result in increased P450 protein levels and P450 activities, causing the development of resistance to insecticides and facilitating the adaptation of insects to their plant hosts (Scott, 1999). Insensitivity of the insect sodium channel resulting from point mutations in its amino acid sequence has been also demonstrated to contribute resistance to pyrethroids, termed knockdown resistance (*kdr*). It has been suggested that due to genetic

variability, rare individuals with an altered genome (mutations) conferring resistance exist in a population even before it has been exposed to insecticides. Those individuals can thus survive the selection pressure of insecticides, leading to the development of insecticide resistance (Liu and Yue, 2000).

2.2 Research goal and specific objectives

In order to better understand the current status and development of pyrethroid resistance, the long term goal of my project is to characterize the molecular mechanism of P450-mediated detoxification involved in pyrethroid resistance in the mosquito, *Culex quiquefasciatus*. To achieve the long term goal, the following five objectives will be addressed: 1) To investigate permethrin resistance profiles in a field population of the mosquito, *Cx. quiquefasciatus*; 2) To identify *kdr* allelic variation and frequency in the *Culex* mosquitoes from the same field population in response to different resistant levels; 3) To characterize the expression tendency of P450 genes following different resistant levels of the mosquitoes bearing different *kdr* alleles; 4) To compare the expression profiles of total P450 genes among different *Culex* mosquito populations bearing different phenotypes in response to permethrin; 5) To elucidate the functions of P450 genes in response to pyrethroid insecticide resistance in the mosquito, *Cx. quiquefasciatus*.

2.3 Hypotheses and significance of research

Currently, we have two field populations of the Southern house mosquito *Culex quinquefasciatus*, $HAmCq^{G0}$ and $MAmCq^{G0}$ in our lab, collected from Huntsville and Mobile, respectively, in the state of Alabama in 2002. Both populations exhibited 10-fold level of

resistance to pyrethroid insecticides, especially permethrin, compared to an insecticide susceptible laboratory strain S-Lab (Liu et al., 2007). We have further selected $HAmCq^{G0}$ and $MAmCq^{G0}$ strains with permethrin for eight and six generations, respectively, and generated two new offspring strains, $HAmCq^{G8}$ and $MAmCq^{G6}$, which exhibited ~2700- and 570-fold levels of resistance to permethrin (Xu et al., 2005).

For the first objective, investigation of permethrin resistance profiles in a field population of the mosquito, *Cx. quiquefasciatus*, the field-collected population $HAmCq^{G0}$ will be chosen to isolate and build up individual single-egg-raft colonies, in which the mosquitoes share the same genetic background. I hypothesize that the levels of insecticide resistance in the population vary among the single-egg-raft colonies and range from very low to very high resistance. The results of the first objective will reveal the distribution of insecticide resistant levels in a field population and provide an overview of development status of insecticide resistance in the field mosquito, *Cx. quiquefasciatus*.

My second objective focuses on the identification of *kdr* allelic variation and frequency in the *Culex* mosquitoes from the same field population in response to different resistant levels. In this study, based on the results of the first objective, different single-egg-raft mosquito colonies selected based on their resistant levels will be investigate the frequency of allelic (A or T) expression at the L-to-F locus of sodium channels, which are the target sites of pyrethroid insecticide, by SNP determination. I hypothesize that the colony with a lower resistant level will possess a higher frequency of susceptible homozygous A alleles, in contrast, to a colony with higher resistant level which should possess a higher frequency of resistant homozygous T alleles as well as the existence of an intermediate colony with a higher frequency of heterozygous A/T alleles.

The third objective is to characterize the expression tendency of P450 genes following different resistant levels of the mosquitoes bearing different *kdr* alleles. Based on two studies above cited previously, I will select several colonies representing different levels of insecticide resistance to examine the expression of certain P450 genes that are considered to be associated with insecticide resistance. My hypothesis is that overexpression of P450 genes occurs in a colony with a higher insecticide resistant level, while a colony with a lower level of insecticide resistance should exhibit expression level of P450 genes closer to the susceptible strain S-Lab. The results of objectives 2 and 3 will indicate whether these two major mechanisms (P450-mediated detoxification and insensitivity of sodium channels) involved in insecticide resistance work in tandem with each other, and which one is predominant in conferring insecticide resistance in one individual colony.

The fourth objective of my research concentrates on comparison of the expression profiles of total P450 genes among different *Culex* mosquito populations bearing different phenotypes in response to permethrin. I will conduct genome analysis of a total of 204 P450 genes identified in the mosquito, *Cx. quiquefasciatus*, and compare expression profiles of P450 genes in both larvae and adults mosquitoes respectively, among the susceptible strain (S-Lab), field parental strains ($HAmCq^{G0}$ and $MAmCq^{G0}$), and resistant offspring strains ($HAmCq^{G8}$ and $MAmCq^{G6}$). I expect that if the over-expression of a Key P450 gene is involved resistance, its expression level will be higher in the highly resistant $HAmCq^{G8}$ and $MAmCq^{G6}$ strains than in the $HAmCq^{G0}$ and $MAmCq^{G0}$ strains. The accomplishment of this objective will provide comprehensive information of total P450 genes, whether contributing or not to insecticide resistance by either up- or down-regulation.

My last objective is to elucidate the functions of P450 genes in response to pyrethroid insecticide resistance in the mosquito, *Cx. quiquefasciatus*. To achieve this objective, key P450 genes identified from the fourth objective will be selected to conduct the functional study by RNA interference (RNAi) technique and P450 transgenic *Drosophila* lines. We predict that silencing the expression of any one of the key P450 genes in mosquitoes by RNAi will result in decreased permethrin resistance, and conversely the expression of any one of the transformed key P450 genes in *Drosophila* will increase the resistant level of *Drosophila* to permethrin. The result of this objective will provide new insight into the function of single or group of P450 genes in conferring insecticide resistance.

Insect cytochrome P450s are critical for detoxification and/or activation of xenobiotics and play a fundamental role in biosynthesis and degradation of endogenous compounds. The accomplishment of the proposed research will provide an overview of insecticide resistant levels and resistance development status in the field population of the mosquito, *Culex quinquefasciatus*, and novel information of mechanisms involved in insecticide resistance, as well as a more comprehensive understanding of the expression profiles of total P450 genes and their role in insecticide resistance. Information from these studies will also provide ideas for controlling the development of insecticide resistance and novel targets for synthesizing new insecticides to control insect pests.

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Chapter 3

Permethrin Resistance Profiles in a Field Population of Mosquitoes, *Culex quinquefasciatus* (Diptera: Culicidae)

3.1 Abstract

Insecticides, especially pyrethroids, are important components in the vector-control effort. In order to better understand the development of resistance, the current study characterized resistance profiles in individual single-egg-raft colonies of a field population of *Culex quinquefasciatus*, $HAmCq^{G0}$. Our study, with 104 colonies derived from each of the single-egg-rafts of $HAmCq^{G0}$, indicated that the levels of resistance to permethrin in 4^{th} instar larvae ranged from 0.4- to 280-fold compared with laboratory susceptible S-Lab larvae. We characterized the distribution of single-egg-raft colonies with different levels of resistance in the $HAmCq^{G0}$ population and found that 65% individual colonies had < 10-fold levels of resistance to permethrin, 16% from 10- to 20- fold, 7% from 20- to 30-fold, and 12% ≥ 30-fold. There is a clear link (strong interaction) between overall levels of pyrethroid resistance in a field *Culex* mosquito population and the distribution within that population of different levels of resistance. We further characterized the frequency of the L-to-F *kdr* allelic expression of sodium channels in the single-egg-raft colonies with different levels of resistance to determine its possible role in resistance. The correlation between allelic expression and levels of resistance clearly showed the importance of L-to-F *kdr* mutation mediated sodium channel insensitivity in resistance development. However, our results also suggested that the sodium channel insensitivity is unlikely to be the sole mechanism and multiple mechanisms may present among the single colonies in response to insecticide resistance.

Key words: permethrin resistance profiles, *Culex quinquefasciatus*, single-egg-raft colony, mutations

3.2 Introduction

The mosquito, *Culex quinquefasciatus* is a cosmopolitan nuisance biting pest with a high reproductive capacity that is common throughout tropical areas and the lower latitudes of temperate regions worldwide. In the Southeastern United States, this species is moderately competent as an important vector of viruses such as West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) (Sardelis et al. 2001, Jones et al. 2002, Reisen et al. 2005). Outside the United States, *Cx. quinquefasciatus* is also thought to be responsible for transmitting the nematodes that cause lymphatic filariasis (Foster and Walker 2002). At present, the control of transmission of these pathogens and other mosquito-associated diseases primarily relies on the application of insecticides, especially pyrethroids such as permethrin (Zaim and Guillet 2002). Pyrethroid insecticides are currently the only insecticides considered suitable for impregnating bed nets for the control of mosquitoes due to their efficient knockdown of target insects and low toxicity to humans (Lengeler et al. 1996, Hemingway et al. 2004). However, mosquitoes have been shown to develop a resistance to pyrethroid insecticides when pyrethroids are broadly and repeatedly applied.

It has been suggested that due to genetic variability, rare individuals with an altered genome that can result in one or more potential resistance mechanisms or factors exist in a population even before it has been exposed to insecticides. Those individuals can thus survive the selection pressure of insecticides, leading to the development of insecticide resistance (Sawicki and Denholm 1984, Brattsten et al 1986, Liu and Yue 2000). The possible mechanisms or factors that

may result in the evolution of insecticide resistance include decreased response of insects to toxicants, decreased exposure, and/or alternate metabolic pathways (Taylor and Feyereisen 1996). One mechanism known to be important in the development of pyrethroid resistance in insects is insensitivity of the voltage-gated sodium channel, which is the primary target of both pyrethroids and DDT. Modifications in the sodium channel structure (specifically, point mutations resulting from single nucleotide polymorphisms [SNPs]), lead to insensitivity in insect sodium channels to pyrethroids and DDT and hence the development of insecticide resistance, known as knockdown resistance (*kdr*) (Soderlund and Bloomquist 1990, Williamson et al. 1996, Soderlund 2005, Dong 2007, Davies et al. 2007, 2008). Among these *kdr* mutations, the substitution of leucine by phenylalanine [L to F], histidine [L to H], or serine [L to S] in the 6th segment of domain II (IIS6) results from a single nucleotide polymorphism, which in the case of mosquitoes is the change from codon TTA to TTT. This has been clearly associated with the development of resistance to pyrethroids and DDT in many insect species, including mosquitoes (Martinez-Torres et al. 1999, Xu et al. 2006a), although other *kdr* mutations appear to be unique to specific species (Soderlund 2005, Davies et al. 2007, Dong 2007).

The mosquito strain *Culex quinquefasciatus* HAmCq^{G0}, collected from Huntsville, Alabama, has been established in our laboratory (Liu et al. 2004b). This *Culex* mosquito strain has an elevated level of resistance to permethrin, a pyrethroid insecticide (Li and Liu 2010). In previous work we have shown that the L-to-F mutation in the sodium channel is present in the HAmCq^{G0} population and this has been linked to the frequency of allelic (A or T) expression at the L-to-F locus of sodium channels of the HAmCq^{G0} population (Xu et al. 2005, 2006a, 2011). This study represents an effort to better understand the genetic variations in insecticide resistance in the HAmCq^{G0} population by generating a susceptible reference line with a similar genetic

background to HAmCq^{G0} in order to examine the correlation, if any, between the L-to-F mutation of sodium channels and levels of resistance, providing valuable new insights into the development of permethrin resistance in field *Culex* mosquitoes. To this end, we isolated 104 single-egg-raft colonies from the HAmCq^{G0} population, examined the levels of susceptibility and resistance of each to permethrin, and then investigated the L-to-F mutation of the sodium channels in each colony.

3.3 Materials and Methods

3.3.1 Mosquito Strains

Three strains of *Culex quinquefasciatus* were used in this study. HAmCq^{G0} was collected from Huntsville, Alabama in 2002 and maintained without further insecticide selection. This strain was used for the construction of the single-egg-raft colonies. HAmCq^{G8} was the 8th generation of permethrin-selected offspring of HAmCq^{G0} (Xu et al. 2006a, Li and Liu 2010). S-Lab was an insecticide susceptible strain obtained from Dr. Laura Harrington (Cornell University) and used as a reference strain for comparing the resistance levels to permethrin among mosquito colonies and strains. All the mosquitoes were reared at 25 ±2^{o}C under a photoperiod of 12:12 (L:D) h (Nayar and Knight 1999), and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

3.3.2 Single-egg-raft Isolation

To isolate single-egg-raft mosquito colonies, in which the mosquitoes shared the same genetic background, more than 300 pupae of HAmCq^{G0} were collected from each of 3 successive generations of the field population, for a total of about 900 pupae. The pupae were placed in the

30 × 30 × 30 cm cages for emergence. In most cases the adult mosquitoes emerged within one or two days, at which point they were provided with access to 10% sucrose. A blood meal was offered to the mosquitoes for two nights after a 4-day mating period. Two to 3 days after the blood meal, each of the female mosquitoes with blood uptake laid an egg-raft on the surface of the water in a container. The egg-rafts were then isolated separately into 100 ml plastic cups for hatching. The resulting colonies, each consisting of the more than 40 larvae hatching from an individual single-egg-raft, were reared in popcorn cups. A total of more than 280 egg-rafts were obtained from $HAmCq^{G0}$ through three sequential single-egg-raft isolations. From those egg-rafts, 106 individual colonies were successfully established and used to produce 2 generations of offspring. These 104 colonies were used for the permethrin toxicity study and SNP determination of the L-to-F mutation in the sodium channel.

3.3.3 Insecticide Bioassays and Data Analysis

Stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) for the insecticide bioassays were prepared in acetone. The bioassay method used for the larvae was as described in previous studies (Liu et al. 2004a, 2004b; Liu et al. 2009, Li et al. 2009, Li and Liu 2010); each bioassay consisted of 4^{th} instar mosquito larvae in regular tap water and 1% insecticide solution in acetone at the required concentration, using four to eight concentrations that resulted in >0 and <100% mortality. Control groups received only 1% acetone. Mortality was assessed after 24 h. At least 3 replications of the bioassay for each generation of the original samples were performed. Bioassay data were pooled and analyzed by standard probit analysis, as described by Liu et al. (2004a, 2004b), utilizing a computerized version of Raymond (1985). Statistical analysis of LC_{10}, $LC_{50,}$ and LC_{90} values was based on non-overlapping 95% confidence

intervals (CI). Resistance ratios (RRs) were calculated by dividing the LC_{50} of the resistant field strains by the LC_{50} of the susceptible S-Lab strain.

3.3.4 Amplification of Sodium Channel Gene Fragments

Ten individual mosquitoes from each single-egg-raft colony had their RNA extracted for each experiment using the acidic guanidine thiocyanate (GIT)-phenol-chloroform method. Three replications were performed, each on a different day, for a total of 30 individual mosquitoes from each colony. The first strand complementary DNA (cDNA) was synthesized using the Transcriptor First Strand cDNA SYN kit (Roche). The L-to-F mutation residues from the sodium channel cDNA fragments were amplified by the primer pair *KDR* S#8 (5'-ATTTCATCATCGTGGCCCTTTCGC-3') and *KDR* AS#1 (5'-TTGTTCGTTTCGTTGTCGGCTGTG-3'), as described previously (Xu et al. 2011). The amplicons generated by this primer pair spanned the intron/exon boundaries without amplifying the genomic DNA to eliminate any influence due to genomic DNA contamination. The PCR solution with JumpStart^{TM} Taq DNA Polymerase (Sigma), cDNA template and the primer pair (*KDR* S#8/*KDR* AS#1) was heated to 95 ℃ for 2 min, followed by 40 cycles of PCR reaction (94 ℃ for 45 s, 65 ℃ for 45 s and 72 ℃ for 2 min) and a final extension of 72 ℃ for 10 min. To confirm that the resulting PCR products were sodium channel gene fragments, the PCR product from the cDNA of each individual was sequenced and in all cases the PCR sequences amplified from cDNA were indeed the sodium channel gene fragments. The PCR products were then subjected to single nucleotide polymorphism (SNP) determination.

3.3.5 SNP Determination for *kdr* Allelic Expression Variation

The frequency and heterozygosity of the allele in mosquitoes were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (Applied Biosystems) (Xu et al. 2006a, 2006b). The primer Cx_SNP2 (5′ GCCACCGTAGTGATAGGAAATTT 3′) used for SNP determination was designed according to the sequence immediately upstream of the *kdr* allele mutation site. Three replications of the SNP determination were carried out using the different preparations of the PCR templates, as described above.

3.4 Results and Discussion

Generally, the level of resistance to permethrin is evaluated for a field population or insect population as a whole, which provides a general picture of the resistance status of a population but fails to take into account details of any variations in the susceptibility of individuals in that population. Our study of characterization of resistance profiles in single-egg-raft mosquito colonies of a field mosquito population enables us to determine the range of resistance present within individuals in the population and hence predict the potential for resistance development under insecticide application and selection.

3.4.1 Permethrin Resistance of Individual Single-egg-raft Mosquito Colonies

The insecticide resistance profiles for the 104 single-egg-raft colonies were characterized to reveal the status of insecticide resistance and the distribution of resistance levels in the natural field population by determining the susceptibility or resistance of each mosquito population to the permethrin insecticide. Insecticide bioassay were conducted on the 4^{th} instar larvae of each of

the 104 single-egg-raft mosquito colonies and strains, as described by Liu et al. (2004a, 2004b), and Li and Liu (2010). The lab susceptible *Culex quinquefasciatus* strain S-Lab was used as a standard susceptible population in order to estimate the resistance ratios in the single-egg-raft mosquito colonies of $HAmCq^{G0}$; the levels of resistance to permethrin in each of the single-egg-raft mosquito colonies were compared with that of the lab susceptible strain, S-Lab. Resistance ratios (RRs) were calculated by dividing the LC_{50} of each of the single-egg-raft mosquito colonies by that for the S-Lab strain (Table 3.1). Of the 104 mosquito colonies tested, most had evolved some degree of resistance to permethrin compared with the S-Lab strain, with resistance ratios at LC_{50} ranging from 0.4 to 290 (Table 3.1). Nevertheless, the majority of the colonies exhibited relatively low levels of resistance to permethrin, with a resistance ratio <10 (Table 3.1, Figure 3.1); 45% of the colonies showed <5-fold levels of resistance and 20% showed 5- to 10-fold levels of resistance. Based on overlapping 95% confidence intervals (Table 1), 16 colonies were observed to share similar susceptibilities with the S-lab strain, and 1 colony, Colony #98, was found to be more susceptible than the S-Lab strain, with the resistance ratios of 0.4. These results strongly indicate a considerable variation of susceptibility and resistance between individuals in a field population in response to insecticides.

Next, we evaluated the resistance in the population $HAmCq^{G0}$ as a whole and compared with it with the levels in individual single-egg-raft colonies in the population. Measurements of the permethrin resistance level in the field population $HAmCq^{G0}$ revealed an overall resistance ratio of ~10 compared with that in S-Lab mosquitoes (current study) (Li and Liu 2010). Looking at the individual single-egg-raft colonies, 66 had resistance levels lower than that of $HAmCq^{G0}$ and 38 colonies were higher. Of the colonies with higher resistance levels than $HAmCq^{G0}$, two individual colonies exhibited relatively high levels of resistance to permethrin, with resistance

ratios of 230 and 280, respectively. This suggests that the resistance level for the whole population may represent only an average level of resistance in most individuals in that population, with apparently elevated levels resulting from the presence of a relatively few resistant individuals. Deciphering the levels of resistance for individual mosquitoes using single-egg-raft colonies has thus enabled us to identify individuals with relatively high levels of resistance within a field population and estimate their effect. The existence of these highly resistant individuals may present a risk for the development of resistance in the population as a whole once environmental conditions favor them.

Our previous studies have reported that after eight generations of selection with permethrin, the level of resistance in $HAmCq^{G8}$, the permethrin selected offspring of $HAmCq^{G0}$, increased 2700-fold compared with that of S-Lab (Table 3.1) (Li and Liu 2010). Although these results appear to indicate that in the field resistant individuals under insecticide pressure would produce a highly resistant strain such as $HAmCq^{G8}$, Li and Liu suggest that the laboratory situation may not accurately reflect the field situation (Li and Liu 2010). They argue that resistance develops more slowly as a large portion of the field insect population may not be directly exposed to insecticide selection, thus providing a pool of susceptible individuals to contribute to the repopulation of insecticide-resistant populations (Georghiou and Taylor 1977, Li and Liu 2010). This hypothesis is supported by the results reported here, where the single-egg-raft colonies had variable levels of resistance and none were as high as those of the laboratory selected offspring, $HAmCq^{G8}$.

3.4.2 L-to-F *kdr* Allelic Expression Variation

As an important mechanism known to be involved in insecticide resistance, target site insensitivity resulting from point mutations in voltage-gated sodium channels and its role in pyrethroid and DDT resistance has been studied in many medical or agricultural pests, including mosquitoes. In earlier studies, a 341 bp PCR fragment at the point where the L-to-F mutation resides was generated from the mosquito sodium channel, revealing a strong correlation between the prevalence of the L-to-F allelic (T) expression at the RNA level and the level of resistance in resistant HAmCq mosquitoes (Xu et al. 2006a, 2011). Xu et al. (2005, 2006a) reported that the L-to-F *kdr* mutation resulting from a single nucleotide polymorphism of adenine to thymine (A to T) in the mosquito sodium channel is an important factor that dramatically reduces the sensitivity of the channel to pyrethroids and, in turn, leads to the development of pyrethroid resistance in *Culex* mosquitoes. In the current study, variations in the expression of A and/or T alleles at the L-to-F *kdr* locus in each of the single-egg-raft colonies was examined to gain valuable insights into the mechanisms involved in permethrin resistance and to determine if there is indeed a correlation between the frequency of *kdr* allelic expression in individual mosquito colonies and their levels of permethrin resistance using the SNP determination method (Xu et al. 2006a, 2006b, 2011; Liu et al. 2009). The sodium channel cDNA fragments were amplified from 10 individuals each from 40 randomly selected single-egg-raft mosquito colonies of the $HamCq^{G0}$ population and all 3 strains of S-Lab, $HamCq^{G0}$ and $HamCq^{G8}$ using a primer pair of *KDR* S#8 and *KDR* AS#1, as previously reported (Xu et al. 2006a, 2011), which generated a ~500 bp sodium channel cDNA fragment for each individual mosquito.

Comparing the frequency of alleles (A and/or T) in the L-to-F *kdr* locus at the RNA level in each of the single-egg-raft mosquito colonies revealed that most colonies showed a clear correlation between the prevalence of the allelic expression of A or T in the L-to-F *kdr* locus at

the RNA level and the level of their susceptibility or resistance to permethrin (Table 3.2). The majority of the single-egg-raft colonies with relatively low or medium levels of resistance showed heterogeneous responses, including individuals who exhibited a combination of allelic expression for both alleles (TTA/T), or for either the A or T allele (TTA or TTT, Table 3.2). Ten mosquito colonies in which all the individual mosquitoes (100%) expressed only the A allele were found to have a susceptibility to permethrin similar to that of the susceptible S-Lab strain, suggesting that expressing the susceptible allele A generates a codon encoding Leu corresponding to susceptibility to insecticides in this strain (Table 3.2). In those colonies with high levels of resistance to permethrin, such as colony #32 (285-fold increase compared to S-Lab), 100% of the individual mosquitoes expressed the T allele, resulting in a change of Leu to Phe (Table 3.2), which is strongly correlated with high levels of resistance in this population. In colonies with relatively low or moderate levels of resistance, their intermediate level of expression for the L-to-F allele corresponded to their resistance levels, ranging from individuals expressing only the susceptible allele A, through those expressing both alleles, to those expressing the *kdr* allele T. Two colonies with relatively low levels of resistance (10- and 8.3-fold, respectively), contained no individuals that expressed the susceptible allele A and a majority or all with the *kdr* allele T (Table 3.2).

These results may suggest that target site insensitivity resulting from the L-to-F substitution is indeed the major, if not the sole, mechanism responsible for the development of permethrin resistance in these mosquito populations. The correlation between allelic expression and levels of resistance in these mosquito colonies confirms the importance of the L-to-F *kdr* mutation in mosquito sodium channels in the evolution of resistance development, although the L-to-F *kdr* mutation is unlikely to be the sole mechanism responsible for pyrethroid resistance in these

colonies or in field populations. Our group has recently isolated the full-length sodium channel cDNAs from S-Lab, HamCq^{G0}, HamCq^{G8} mosquitoes (Xu et al. 2012). Comparing the entire sodium channel cDNA sequences across these 3 strains revealed multiple mutations to be present throughout the *Culex* mosquito sodium channels (Xu et al. 2012). This suggests that multiple mutations may co-exist in the mosquito sodium channels that are involved in pyrethroid resistance. Although the current study focused on evaluating only the L-to-F mutation in resistant field populations of *Culex quinquefasciatus*, the possibility that additional mutations in their sodium channels may also contribute to the development of permethrin resistance cannot be excluded.

Interestingly, two colonies with elevated levels of resistance (24- and 14-fold, respectively) were found to be entirely composed of individuals that expressed the susceptible allele A. These results strongly suggest that target site insensitivity resulting from the L-to-F mutation is not an important mechanism in permethrin resistance for these mosquito colonies. While the possibility that mutations other than L-to-F presented in the sodium channel are responsible for permethrin resistance in these colonies of *Culex* mosquitoes cannot be excluded, it is necessary to consider alternative resistance mechanisms in addition to target site insensitivity. Similar results have also been reported by Liu et al. (2009). Previous studies indicated that the interaction of multiple insecticide resistance mechanisms or genes is likely to be responsible for insecticide resistance (Georghiou and Taylor 1977, Raymond et al. 1989, Liu and Scott 1996, Liu and Yue 2000, 2001; Pridgeon and Liu 2003, Pridgeon et al. 2003, Liu et al. 2005, Xu et al. 2005, 2006a; Liu et al. 2007). In particular, Xu et al. (2005) suggested that multiple mechanisms – in this case P450-mediated detoxification and *kdr*-mediated target site insensitivity – were implicated in the development of resistance in HamCq *Culex quinquefasciatus* mosquitoes from Alabama. A study

comparing the gene expression profiles of resistant and susceptible *Culex* mosquitoes identified multiple genes that were overexpressed in the resistant HamCq mosquitoes, suggesting the importance of these overexpressed genes in resistance (Liu et al. 2007). A recent study by Yang and Liu has identified multiple P450 genes to be up-regulated in HamCq mosquitoes (Yang and Liu 2011). Whether or not metabolic detoxification enzymes, especially P450s, are involved in detoxifying permethrin insecticide and conferring permethrin resistance in these mosquito colonies needs further investigation.

3.5 Conclusions

The current study characterized the resistance profiles in 104 colonies derived from single-egg-rafts of a field population of *Culex quinquefasciatus*, $HAmCq^{G0}$. Insecticide bioassay with permethrin revealed that levels of resistance to permethrin in 4^{th} instar larvae of these colonies ranged from 0.4- to 280-fold compared with a laboratory susceptible S-Lab strain. Of the colonies, 65% had < 10-fold levels of resistance to permethrin, suggesting that the majority of the individuals in the field mosquito population $HAmCq^{G0}$ had low levels of resistance. Seven percent had ≥ 30-fold levels of resistance to permethrin, suggesting that the field population contained a mixture of individuals with different levels of resistance. The frequency of the L-to-F *kdr* allelic expression in the sodium channels examined in individuals from single-egg-rafts colonies with different levels of resistance varied and appeared to be closely correlated with the levels of resistance in those colonies, suggesting the importance of the mutation in resistance of major colonies. However, the results also indicate that multiple mechanisms/different mechanisms could be involved within or among the single colonies in response to insecticide

resistance. This study has enabled us to establish a useful procedure for utilizing isolated susceptible colonies in future studies as reference mosquitoes.

3.6 Acknowledgements

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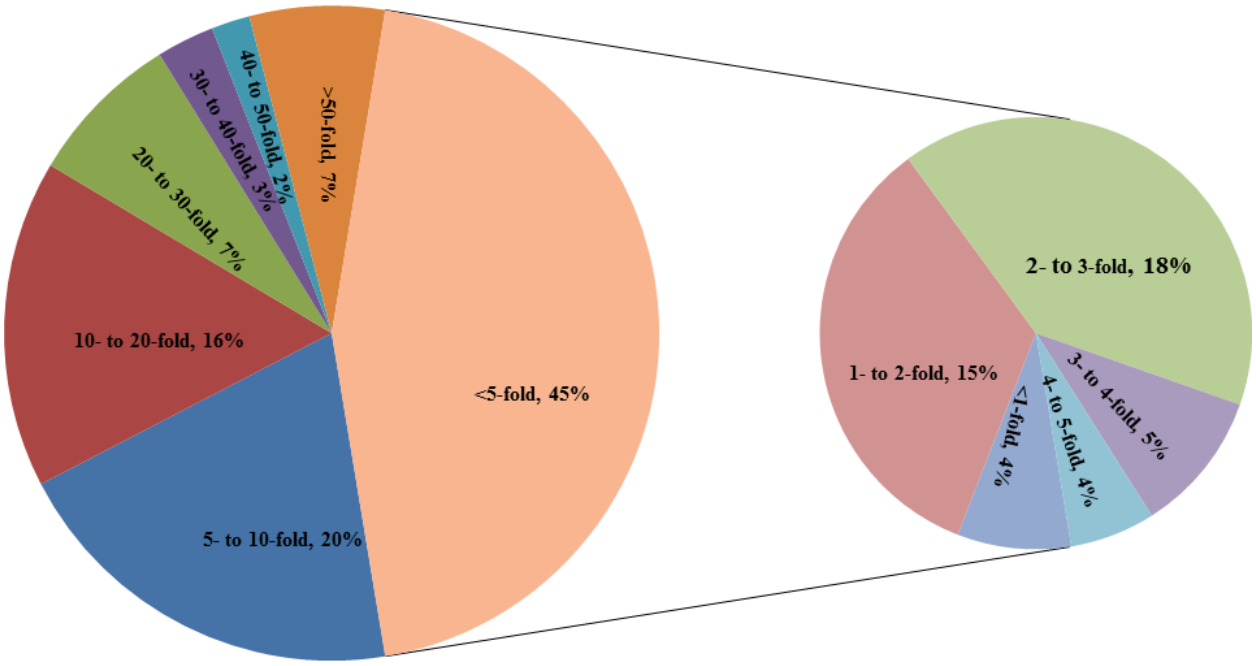

Figure 3.1 Distribution of the levels of insecticide resistance in single-egg-raft colonies of the HAmCq^{G0} population of *Culex quinquefasciatus*. The pie chart represents the percentages of individual single-egg-raft colonies with levels of resistance falling within each range. Resistance ratios (RRs) were calculated by dividing the LC_{50} of each of the single-egg-raft mosquito colonies by that for the S-Lab strain.

Table 3.1 Toxicity of Permethrin to mosquito colonies of *Culex quinquefasciatus*

Colony	df	n[a]	χ^{2}[b]	LC_{50}[c](CI)[d]	Slope(SE)	RR[e]	RR[f]
1	3	147	2.1	0.04 (0.02-0.06)	1.3 (0.2)	5.4	13
2	2	89	0.2	0.007 (0.002-0.01)	1.6 (0.5)	1.0	2.3
3	5	167	3.2	0.01 (0.008-0.02)	3.0 (0.5)	1.6	3.7
4	3	98	0.5	0.2 (0.1-0.5)	1.0 (0.2)	31	73
5	3	85	1.0	0.4 (0.2-2.1)	0.8 (0.2)	58	135
6	5	250	2.6	0.009 (0.008-0.01)	4.3 (0.8)	1.3	3.0
7	3	85	1.7	0.2 (0.1-0.5)	1.0 (0.2)	30	70
8	3	150	1.7	0.02 (0.01-0.02)	2.0 (0.4)	2.3	5.3
9	2	67	0.4	0.06 (0.02-0.1)	1.4 (0.3)	8.1	19
10	2	75	1.9	0.06 (0.02-0.1)	0.8 (0.2)	8.1	19
11	3	128	1.0	0.3 (0.2-0.5)	1.2 (0.2)	36	84
12	3	49	2.6	0.1 (0.05-0.4)	1.0 (0.3)	19	43
13	3	55	1.0	0.3 (0.2-1.7)	1.1 (0.3)	48	111
14	4	57	2.2	0.02 (0.009-0.03)	2.6 (0.7)	2.3	5.3
15	2	54	1.7	0.1 (0.06-0.3)	1.6 (0.4)	16	38
16	6	174	3.5	1.6 (1.0-2.9)	1.2 (0.2)	234	546
17	4	124	3.2	0.1 (0.06-0.2)	1.3 (0.2)	15	35
18	5	230	1.3	0.02 (0.02-0.03)	3.9 (0.5)	3.1	7.3
19	6	81	3.0	0.02 (0.01-0.02)	3.1 (0.7)	2.4	5.7
20	4	158	9.9	0.04 (0.01-0.1)	1.2 (0.2)	5.6	13
21	4	167	9.5	0.09 (0.03-0.3)	1.3 (0.2)	13	30
22	4	74	3.1	0.08 (0.02-0.4)	0.6 (0.2)	11	25
23	3	43	0.8	0.2 (0.07-0.8)	1.4 (0.5)	22	51
24	3	109	2.1	0.2 (0.09-0.3)	1.1 (0.2)	22	52
25	4	216	5.4	0.09 (0.05-0.2)	1.6 (0.2)	13	29
26	7	243	3.3	0.02 (0.02-0.02)	3.9 (0.4)	2.4	5.7
27	6	213	13	0.07 (0.04-0.2)	1.4 (0.2)	10	24
28	6	150	4.3	0.04 (0.03-0.05)	2.5 (0.4)	5.6	13
29	5	187	6.0	0.04 (0.03-0.06)	1.9 (0.3)	5.6	13
30	7	113	2.3	0.01 (0.009-0.01)	3.9 (0.9)	1.6	3.7
31	5	173	5.9	0.5 (0.3-1.0)	1.4 (0.2)	73	170
32	5	74	1.0	2.0 (1.3-4.8)	2.1 (0.7)	285	666
33	6	203	4.6	0.2 (0.1-0.3)	1.2 (0.2)	28	66
34	5	92	3.6	0.5 (0.3-0.7)	2.5 (0.5)	69	161
35	6	226	5.5	0.01 (0.01-0.02)	2.4 (0.3)	2.0	4.7
36	6	218	7.9	0.02 (0.02-0.03)	2.1 (0.3)	3.3	7.7

37	4	190	9.6	0.04 (0.01-0.07)	1.6 (0.2)	5.0	12
38	7	208	14	0.02 (0.01-0.02)	2.2 (0.3)	2.1	5.0
39	8	239	13	0.02 (0.01-0.02)	3.6 (0.5)	2.1	5.0
40	6	100	1.3	0.01 (0.009-0.02)	3.8 (0.8)	1.7	4.0
41	4	380	10	0.08 (0.04-0.2)	1.0 (0.1)	11	27
42	4	53	3.4	0.2 (0.08-0.7)	1.0 (0.3)	27	63
43	2	62	1.1	0.09 (0.03-0.2)	1.0 (0.3)	12	29
44	5	139	3.5	0.005 (0.004-0.006)	3.9 (0.7)	0.7	1.7
45	5	131	9.5	0.06 (0.02-0.2)	1.2 (0.2)	8.4	20
46	2	36	0.3	0.06 (0.02-0.1)	1.6 (0.5)	8.3	19
47	6	158	2.9	0.4 (0.3-0.7)	1.2 (0.2)	60	140
48	4	98	0.9	0.04 (0.03-0.06)	2.1 (0.4)	5.4	13
49	5	74	1.2	0.1 (0.08-0.3)	1.6 (0.3)	19	44
50	5	133	4.5	0.7 (0.3-2.4)	0.8 (0.2)	96	223
51	6	426	29	0.02 (0.01-0.03)	2.2 (0.2)	2.9	6.7
52	4	128	3.1	0.05 (0.03-0.08)	1.3 (0.2)	6.9	16
53	5	349	8.8	0.1 (0.07-0.2)	1.2 (0.1)	17	39
54	5	200	2.0	0.1 (0.08-0.2)	1.1 (0.1)	17	40
55	6	242	6.8	0.3 (0.2-0.4)	2.0 (0.2)	39	92
56	7	226	13	0.04 (0.02-0.06)	2.0 (0.2)	5.4	13
57	4	72	3.0	0.02 (0.01-0.03)	1.7 (0.4)	2.7	6.3
58	3	67	0.1	0.01 (0.004-0.02)	1.7 (0.4)	1.6	3.7
59	5	95	7.2	0.04 (0.02-0.07)	2.3 (0.4)	5.1	12
60	6	152	7.3	0.1 (0.07-0.2)	1.7 (0.3)	17	40
61	6	116	7.3	0.2 (0.09-0.3)	1.6 (0.3)	24	56
62	8	335	4.7	0.08 (0.07-0.1)	2.3 (0.2)	11	27
63	4	72	1.8	0.2 (0.1-0.3)	2.2 (0.4)	26	61
64	4	89	3.4	0.03 (0.02-0.04)	3.7 (0.9)	4.0	9.3
65	6	159	4.8	0.04 (0.03-0.05)	2.8 (0.4)	6.0	14
66	5	150	5.6	0.08 (0.05-0.1)	1.8 (0.2)	11	25
67	5	127	1.8	0.05 (0.04-0.07)	2.1 (0.4)	7.6	18
68	7	165	9.9	0.04 (0.03-0.07)	2.0 (0.3)	6.3	15
69	4	135	1.3	0.02 (0.01-0.02)	5.8 (1.0)	2.3	5.3
70	4	90	3.2	0.3 (0.2-0.6)	1.4 (0.3)	47	109
71	5	140	1.2	0.02 (0.01-0.02)	3.2 (0.5)	2.1	5.0
72	5	97	11	0.07 (0.03-0.3)	2.0 (0.3)	9.7	23
73	5	82	6.6	0.02 (0.01-0.02)	5.0 (1.0)	2.3	5.3
74	5	114	2.0	0.02 (0.01-0.02)	2.7 (0.5)	2.4	5.7
75	3	65	1.9	0.04 (0.03-0.06)	2.2 (0.5)	5.6	13
76	4	101	1.5	0.01 (0.01-0.02)	4.0 (0.7)	2.0	4.7

77	6	175	10	0.01 (0.01-0.02)	3.1 (0.4)	2.0	4.7
78	5	163	12	0.01 (0.002-0.02)	2.4 (0.4)	1.3	3.0
79	4	110	4.2	0.01 (0.009-0.02)	4.5 (0.8)	1.9	4.3
80	4	83	0.9	0.02 (0.01-0.02)	4.1 (0.8)	2.4	5.7
81	3	172	3.3	0.02 (0.01-0.03)	2.1 (0.3)	2.9	6.7
82	4	114	4.8	0.03 (0.02-0.06)	1.7 (0.4)	4.7	11
83	4	63	4.6	0.05 (0.03-0.08)	3.3 (0.8)	6.4	15
84	5	89	2.5	0.1 (0.08-0.1)	3.2 (0.6)	15	34
85	4	110	2.5	0.009 (0.007-0.01)	3.7 (0.7)	1.3	3.0
86	7	227	8.1	0.02 (0.02-0.03)	1.7 (0.2)	3.1	7.3
87	4	175	11	0.01 (0.005-0.02)	2.5 (0.3)	1.4	3.3
88	7	275	8.6	0.03 (0.03-0.05)	1.8 (0.2)	4.9	11
89	6	263	7.8	0.03 (0.02-0.04)	2.2 (0.2)	3.9	9.0
90	3	78	3.0	0.03 (0.02-0.04)	2.5 (0.6)	3.6	8.3
91	3	75	0.7	0.01 (0.005-0.01)	3.2 (0.8)	1.1	2.7
92	4	115	2.0	0.1 (0.06-0.2)	1.4 (0.3)	14	32
93	4	74	0.5	0.006 (0.004-0.007)	3.8 (0.9)	0.9	2.0
94	5	123	2.7	0.008 (0.006-0.01)	3.1 (0.6)	1.1	2.7
95	6	134	3.3	0.01 (0.01-0.02)	4.6 (0.7)	2.0	4.7
96	5	207	10	0.01 (0.006-0.02)	2.6 (0.4)	1.6	3.7
97	4	95	6.7	0.02 (0.01-0.02)	5.4 (0.9)	2.1	5.0
98	5	171	12	0.003 (0.001-0.004)	3.1 (0.4)	0.4	1.0
99	4	73	1.9	0.007 (0.005-0.008)	4.9 (1.3)	1.0	2.3
100	5	136	7.0	0.06 (0.04-0.08)	2.6 (0.4)	8.3	19
101	6	155	5.0	0.07 (0.05-0.1)	1.6 (0.2)	9.9	23
102	4	73	0.9	0.008 (0.006-0.01)	3.6 (0.8)	1.1	2.7
103	5	88	3.8	0.005 (0.002-0.007)	2.6 (0.6)	0.7	1.7
104	5	200	2.6	0.009 (0.008-0.01)	2.9 (0.4)	1.3	3.0
S-Lab	3	150	1.0	0.007 (0.005-0.008)	3.4 (0.6)	1.0	2.3
$HAmCq^{G0}$	3	150	1.3	0.07 (0.04-0.1)	2.4 (0.4)	9.4	22
$HAmCq^{G9}$	3	150	1.4	19 (9.9-30)	3.2 (0.5)	2,700	6,200

[a] Number of mosquito larvae tested.

[b] Pearson chi-square, goodness-of-fit test.

[c] LC_{50} value in ppm.

[d] 95% confidence interval, toxicity of permethrin is considered significantly different when the 95% CI fail to overlap.

[e] Resistance ratio between LC_{50} of each colony/strain and susceptible strain S-Lab.

[f] Resistance ratio between LC_{50} of each colony/strain and most susceptible colony #98.

Table 3.2 Allelic expression at the L-to-F locus of sodium channels in the single colonies of *Cx. quinquefasciatus*

Colony	n[a]	Resistance Phenotype[b]	Resistance Phenotype[c]	Frequency of the specific alleles[d]		
				TTA	TTA/T	TTT
4	30	31-fold	73-fold	0.2	0.2	0.6
13	30	48-fold	111-fold	0	0	1
25	30	13-fold	29-fold	0.7	0	0.3
27	30	10-fold	24-fold	0	0.4	0.6
32	30	285-fold	666-fold	0	0	1
44	30	0.7-fold	1.7-fold	0.4	0	0.6
60	30	17-fold	40-fold	0.22	0.33	0.44
61	30	24-fold	56-fold	1	0	0
63	30	26-fold	61-fold	0.7	0.3	0
71	30	2.1-fold	5.0-fold	0.58	0	0.42
72	30	9.7-fold	23-fold	0.09	0.45	0.45
73	30	2.3-fold	5.3-fold	0.92	0.08	0
74	30	2.4-fold	5.7-fold	1	0	0
75	30	5.6-fold	13-fold	0.42	0.42	0.17
76	30	2.0-fold	4.7-fold	0.33	0.67	0
77	30	2.0-fold	4.7-fold	0.75	0.08	0.17
78	30	1.3-fold	3.0-fold	0.73	0.27	0
79	30	1.9-fold	4.3-fold	1	0	0
80	30	2.4-fold	5.7-fold	0.81	0.19	0
81	30	2.9-fold	6.7-fold	0.5	0.42	0.08
82	30	4.7-fold	11-fold	0.36	0.18	0.45
83	30	6.4-fold	15-fold	0.75	0.17	0.08
84	30	15-fold	34-fold	0.08	0.25	0.67
86	30	3.1-fold	7.3-fold	0.5	0.08	0.42
87	30	1.4-fold	3.3-fold	1	0	0
88	30	4.7-fold	11-fold	1	0	0
89	30	3.9-fold	9.0-fold	0.75	0.25	0
90	30	3.6-fold	8.3-fold	0.58	0.33	0.08
91	30	1.1-fold	2.7-fold	0.92	0	0.08
92	30	14-fold	32-fold	1	0	0
93	30	0.9-fold	2.0-fold	1	0	0
94	30	1.1-fold	2.7-fold	0.97	0	0.03
95	30	2.0-fold	4.7-fold	1	0	0
96	30	1.6-fold	3.7-fold	0.7	0.3	0
97	30	2.1-fold	5.0-fold	0.5	0.25	0.25

98	30	0.4-fold	1.0-fold	1	0	0
99	30	1.0-fold	2.3-fold	0.88	0	0.13
100	30	8.3-fold	19-fold	0	0	1
101	30	9.9-fold	23-fold	0.17	0.08	0.75
103	30	0.7-fold	1.7-fold	1	0	0
S-lab	30	1.0-fold	2.3-fold	1	0	0
$HAmCq^{G0}$	30	9.4-fold	22-fold	0.26	0.46	0.28
$HAmCq^{G9}$	30	2,667-fold	6,223-fold	0	0.04	0.96

[a] The total number of tested flies.

[b] Resistance phenotype is the resistance ratio between LC_{50} of each colony/strain and susceptible strain S-Lab.

[c] Resistance phenotype is the resistance ratio between LC_{50} of each colony/strain and most susceptible colony #98.

[d] The expression frequency of specific alleles at the L-to-F locus in the colonies; the nucleotides at the L-to-F locus changed in the RNA level are underlined.

Chapter 4

Investigation of Main Molecular Mechanisms Involved in Pyrethroid Resistance in a Field Population of the Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae)

4.1 Introduction

Mosquitoes are annoying and important insect pests for both human and animals, not only because the females can repeat their blood feedings on the host causing great discomfort, but they can also act as vectors of parasites and pathogens to transmit a wide variety of diseases, such as malaria, filariasis, dengue, yellow fever and encephalitis, which can result in public health problem, and even eventual death of human. The southern house mosquito, *Culex quinquefasciatus*, is one of the major domestic pests in the urban and semi-urban areas of tropical regions in the whole world. This species is the primary vector of Saint Louis encephalitis virus (SLEV), West Nile Virus (WNV), and the predominant vector of lymphatic filarial nematodes, *Wuchereria bancrofti*, which has caused approximate 120 million people infected (Sardelis et al., 2001; Jones et al., 2002; WHO, 2010).

To avoid the increase of morbidity and mortality of people resulting from the transmission of these pathogens and other mosquito-borne diseases, vector control of mosquitoes becomes one of the important components of the current global strategy. To date, chemical-based control plays a significant role in the management of medically important vectors worldwide. Pyrethroid insecticides, such as permethrin are one of the most widely used classes of insecticides as house sprays for indoor control of mosquitoes. Due to their high insecticidal effectiveness and low mammalian toxicity, pyrethroid insecticides are the only chemical that are considered to treat bed nets for avoiding mosquitoes and preventing malaria in Africa (Najera and Zaim, 2001;

Khambay and Jewess, 2004). However, it has been reported that mosquito vectors have broadly developed their resistance to insecticides, especially to pyrethroids, in turn, which can lead to the emergence of resistant population and resurgence of diseases (Brogdon and McAllister, 1998).

Getting insight into the molecular basis of the mechanisms governing insecticide resistance, such as investigation of genes that are involved into the insecticide resistance, is important for pest control, management of insecticide resistance and novel insecticide development for new targets. It has been known as pre-adaptive phenomenon that some individuals in one population, with one or more potential resistance mechanisms or factors resulting from altered genome, exist prior to insecticide exposure (Sawicki and Denholm 1984, Brattsten et al 1986). As a result, these individuals can contribute to the evolution of insecticide resistance of this population by existing possible mechanisms or factors, which include loss of sensitivity of insects to insecticides, alternate metabolic pathways, and avoidance of exposure to insecticides (Taylor and Feyereisen 1996). There are mainly two significant mechanisms involved in pyrethroid resistance, which are increased cytochrome P450-mediated detoxification and decreased target-site sensitivity of sodium channels (Wondji et al., 2009; Dong, 2007). Cytochrome P450s are found to be the broadest gene super-family in almost all aerobic organisms, responsible for detoxifying xenobiotics such as drugs, pesticides, and plant toxins, and endogenous compounds such as hormones, fatty acids, and steroids (Pavek and Dvorak, 2008). Insect cytochrome P450s play an important role in the detoxification of exogenous compounds such as insecticides and plant toxins, which can help the insects with the development of insecticide resistance and adaption to the plant hosts and environment (Feyereisen, 2011; Schuler, 1996; Terriere, 1984). A significant characteristic of insect P450s is their transcriptional up-regulation, leading to enhanced P450 protein levels and P450 activities, which cause increased metabolic

detoxification of insecticides (Feyereisen, 2011; Liu and Scott, 1998; Zhu et al., 2010; Liu et al., 2011). Another significant mechanism involved in pyrethroid resistance known as knockdown resistance (*kdr*) is insensitivity of voltage-gated sodium channel, which is located at insect central nerve system (CNS), and considered to be the primary target site of both pyrethroid insecticide and DDT. Structural alterations (mutations) of the gene encoding sodium channels, specifically point mutations resulting from single nucleotide polynorphisms (SNPs), confers the conformational changes of sodium channel proteins, which cause the loss of affinity of the proteins to pyrethroids, in turn, leading to the development of insecticide resistance (Dong, 2007; Soderlund, 2005; Davies et al., 2008). The most common *kdr* mutation is the substitution of leucine by phenylalanine [L to F] in the 6th segment of domain II (IIS6) of sodium channel, which in the case of mosquitoes is the change of a single nucleotide from codon TTA to TTT. This has been also reported to be associated with the development of insecticide resistance in many other insect species (Soderlund 2005, Dong 2007; Davies and Williamson, 2009).

The mosquito population *Culex quinquefasciatus* HAmCq^{G0}, collected from Huntsville, Alabama, has been established in our laboratory and demonstrated an elevated level of resistance to permethrin (Liu et al. 2004b; Li and Liu 2010). Moreover, our previous studies have indicated that the L-to-F mutation in the sodium channel is present in the HAmCq^{G0} population and that individuals in this population express their susceptibility/resistance to permethrin at different levels (Xu et al. 2011; Yang and Liu, 2013). Up-regulation of several cytochrome P450 genes have been also found in the population with higher levels of resistance (Yang and Liu, 2010). In an effort to better understand the genetic variation and mechanisms involved in development of permethrin resistance in the HAmCq^{G0} population, we chose 8 individuals of single-egg-raft colonies from the HAmCq^{G0} population, representing different levels of susceptibility/resistance

to permethrin, and investigated the L-to-F mutation of the sodium channels in each colony and the expression profiles of the up-regulated key P450 genes.

4.2 Materials and Methods

4.2.1 Mosquito Strains and Colonies

The strain HAmCq^{G0}of *Culex quinquefasciatus* was originally collected from Huntsville, Alabama in 2002, with a lower level of resistance to permethrin, and maintained without further insecticide selection. This field strain was used for the construction of the single-egg-raft colonies. Eight single-egg-raft colonies were isolated by building up each colony from an egg-raft laid a single female after mating with a single male, as described by Yang and Liu (Yang and Liu, 2013). The HAmCq^{G8} was the 8th generation of permethrin-selected offspring of HAmCq^{G0} (Xu et al. 2006a, Li and Liu 2010). All the mosquitoes were reared at 25±2^{o}C under a photoperiod of 12:12 (L:D) h (Nayar and Knight 1999), and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University). The mosquito was reared strictly under identical rearing conditions for the two mosquito populations and ten single-egg-raft colonies.

4.2.2 Insecticide Bioassays and Data Analysis

Stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) for the insecticide bioassays were prepared in acetone. The bioassay method used for the larvae was as described in previous studies (Liu et al. 2004a, 2004b; Liu et al. 2009, Li et al. 2009, Li and Liu 2010); each bioassay consisted of 4th instar mosquito larvae in regular tap water and 1% insecticide solution in acetone at the required concentration, using four to eight concentrations

that resulted in >0 and <100% mortality. Control groups received only 1% acetone. Mortality was assessed after 24 h. At least 3 replications of the bioassay for each generation of the original samples were performed. Bioassay data were pooled and analyzed by standard probit analysis, as described by Liu et al. (2004a, 2004b), utilizing a computerized version of Raymond (1985). Statistical analysis of LC_{10}, $LC_{50,}$ and LC_{90} values was based on non-overlapping 95% confidence intervals (CI). Resistance ratios (RRs) were calculated by dividing the LC_{50} of the resistant field strains by the LC_{50} of the susceptible S-Lab strain.

4.2.3 Amplification of Sodium Channel Gene Fragments

Ten individual mosquitoes from each single-egg-raft colony had their RNA extracted for each experiment using the acidic guanidine thiocyanate (GIT)-phenol-chloroform method. Three replications were performed, each on a different day, for a total of 30 individual mosquitoes from each colony. The first strand complementary DNA (cDNA) was synthesized using the Transcriptor First Strand cDNA SYN kit (Roche). The L-to-F mutation residues from the sodium channel cDNA fragments were amplified by the primer pair *KDR* S#8 (5'-ATTTCATCATCGTGGCCCTTTCGC-3') and *KDR* AS#1 (5'-TTGTTCGTTTCGTTGTCGGCTGTG-3'), as described previously (Xu et al. 2011). The amplicons generated by this primer pair spanned the intron/exon boundaries without amplifying the genomic DNA to eliminate any influence due to genomic DNA contamination. The PCR solution with JumpStart^{TM} Taq DNA Polymerase (Sigma), cDNA template and the primer pair (*KDR* S#8/*KDR* AS#1) was heated to 95 ℃ for 2 min, followed by 40 cycles of PCR reaction (94 ℃ for 45 s, 65 ℃ for 45 s and 72 ℃ for 2 min) and a final extension of 72 ℃ for 10 min. To confirm that the resulting PCR products were sodium channel gene fragments, the PCR product

from the cDNA of each individual was sequenced and in all cases the PCR sequences amplified from cDNA were indeed the sodium channel gene fragments. The PCR products were then subjected to single nucleotide polymorphism (SNP) determination.

4.2.4 SNP Determination for *kdr* Allelic Expression Variation

The frequency and heterozygosity of the allele in mosquitoes were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (Applied Biosystems) (Xu et al. 2006a, 2006b). The primer Cx_SNP2 (5′ GCCACCGTAGTGATAGGAAATTT 3′) used for SNP determination was designed according to the sequence immediately upstream of the *kdr* allele mutation site. Three replications of the SNP determination were carried out using the different preparations of the PCR templates, as described above.

4.2.5 Quantitative real-time PCR (qRT-PCR)

The 4th instar larvae and 2-3 day-old adults (before blood deeding) of two mosquito population and eight single-egg-raft colonies had their RNA extracted for each experiment using the acidic guanidine thiocyanate-phenol-chloroform method. Total RNA (0.5 μg/sample) from each mosquito sample was reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR, which was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (15 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and a P450 gene specific primer pair designed

according to each of the P450 gene sequences (http://c*quinquefasciatus*.vectorbase.org/, Table 4.1 with accession number for each of P450 genes) at a final concentration of 3-5 μM. All samples, including the A 'no-template' negative control, were performed in triplicate. The reaction cycle consisted of a melting step of 50 ℃ for 2 min then 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min. Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction using Dissociation Curves software (Wittwer et al., 1997). Relative expression levels for the P450 genes were calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software (Livak and Schmittgen, 2001). The 18S ribosome RNA gene, an endogenous control, was used to normalize the expression of target genes (Liu et al., 2007). Preliminary qRT-PCR experiments with the primer pair (Table 4.1) for the 18S ribosome RNA gene designed according to the sequences of the 18S ribosome RNA gene had revealed that the 18S ribosome RNA gene expression remained constant among all 3 mosquito strains, so the 18S ribosome RNA gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated three to four times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

4.3 Results

Generally, according to our previous study, we selected eight single-egg-raft colonies built up from the field population $HAmCq^{G0}$, which had different levels of resistance to permethrin, bearing different variations in the expression of A and/or T alleles at the L-to-F *kdr* locus (Yang and Liu, 2013). Our current study examined the expression profiles of several up-regulated P450

genes in the larvae and adults of these eight single colonies, plus the parental field population of *Culex* mosquitoes, HAmCq^{G0}, and permethrin-selected offspring HAmCq^{G8} respectively. This study enabled us to get insight into the molecular mechanisms involved in pyrethroid resistance in the mosquito, *Culex quinquefasciatus*, and to determine the predominant mechanisms in charge of resistance present within individuals in the population and hence predict the potential for resistance development under insecticide application and selection, and the strategies for control of mosquitoes and mosquito resistance to pyrethroid insecticides.

4.3.1 Bioassays of Individual Single-egg-raft Mosquito Colonies and Strains

In mosquito larvae biassay, eight single-egg-raft *Culex quinquefasciatus* colonies distributed their resistance to permethrin approximately from 1.0- to 23-fold. Of the 8 mosquito colonies tested, Cx_SERC5 and Cx_SERC8 had relatively low resistance level, with resistance ratios less than 2.0-fold; two colonies, Cx_SERC1 and Cx_SERC2 with their resistance levels at around 6.0-fold; colonies Cx_SERC3 and Cx_SERC4 at 11-fold, and the other two, Cx_SERC6 and Cx_SERC7, with higher levels of resistance at around 20-fold. The field population HAmCq^{G0}, from which the single-egg-raft colonies derived, had a resistance level at 22-fold. After 8 generations of permethrin selection, the individuals in the population HAmCq^{G8} showed much higher level of resistance to permethrin (Table 4.2). The levels of resistance to permethrin in each of the single-egg-raft mosquito colonies and strains were compared with that of the most susceptible colonies Cx_SERC5. Resistance ratios (RRs) were calculated by dividing the LC_{50} of each of the single-egg-raft mosquito colonies and strains by that for single colony Cx_SERC5 (Table 4.2).

4.3.2 Frequency of *kdr* Allelic Expression Related to Resistance Levels

Using primers based on genome sequences of *Culex quinquefasciatus* available in Vectorbase, a 341bp PCR fragment of sodium channel gene was amplified by RT-PCR. This fragment covers the L-to-F locus of *kdr* mutation, which confers the resistance of mosquitoes and other medical or agricultural insect pests to pyrethroid and DDT, known as target site insensitivity resulting from point mutations. Our previous studies revealed a correlation between the prevalence of the L-to-F allelic (T) expression at the RNA level and the level of resistance in mosquitoes (Xu et al., 2011; Yang and Liu, 2013). In the current study, variations in the expression of A and /or T alleles at the L-to-F *kdr* locus in each of the eight single-egg-raft colonies was examined using the SNP determination method (Xu et al., 2006, 2011; Liu et al., 2009). Comparing the frequency of alleles (A and/or T) in the L-to-F *kdr* locus at the RNA level in each of the eight single-egg-raft mosquito colonies showed that different genotypes for the L-to-F *kdr* site were exhibited among individuals, including individuals that were heterozygous for both susceptible (A) and resistance (T) alleles or homozygous for either the A or T allele, which is consistent with the results of the earlier study (Xu et al., 2011; Yang and Liu, 2013). Three of eight single-egg-raft colonies, Cx_SERC1, Cx_SERC5 and Cx_SERC8, with relatively low levels of resistance exhibited allelic expression for only A allele within all individual mosquitoes (100%) in those colonies, which is the susceptible allele generating a codon encoding Leu (Table 4.3). With resistance level of the single-egg-raft mosquito colony increased, the individuals in corresponding colony expressed decreased frequency of A allele, but increased frequency of T allele at *kdr* mutation locus resulting in a change of Leu to Phe (Table 4.3). However, there was a colony, Cx_SERC4, with medium level of resistance, in which all individuals expressed only susceptible A allele. The colony or strain with relatively high levels of resistance contained no

individuals that expressed the susceptible allele A and a majority or all with the *kdr* allele T (Table 4.3).

4.3.3 Target P450 Gene Expression Profiles Associated with Resistance Levels

Quantitative real-time PCR (qPCR) was used to investigate gene expression of selected P450 genes, which were most likely to be involved in resistance on the basis of characterization of whole genome P450 genes expression profiles among different populations, through susceptible, lower resistant and highly resistant (Yang and Liu, 2011). Five P450 genes, *CYP6BY7*, *CYP6CP1*, *CYP12F10*, *CYP6AA7* and *CYP4C52v1* were chosen to be tested at adult stage in terms of their significant up-regulation in the adult resistant *Culex* mosquitoes when compared to their expression in susceptible population. Respectively, six genes, *CYP9M10*, *CYP9J34*, *CYP6P14*, *CYP9J40*, *CYP6AA7* and *CYP4C52v1* were chosen at larval stage (Yang and Liu, 2011). As regards to single-egg-raft colonies, eight were selected, which distributed their resistance at different levels in the natural field population, $HAmCq^{G0}$, bearing different frequencies of A or T alleles. In the adult stage, all five P450 genes were expressed at relatively low levels in the colony, Cx-SERC4, while they were expressed at higher levels in the colony, Cx-SERC3. However, the expression profiles of these P450 genes varied in each of rest colonies but mostly no higher than the resistant population, $HAmCq^{G8}$ (Figure 4.1). Interestingly, at the larval stage, when permethrin selection was applied, the mosquitoes in colony Cx-SERC5, which was the most susceptible colony to permethrin, tended to have the lowest expression levels of all six selected P450 genes. The colonies, Cx-SERC3 and Cx-SERC6, with intermediate and relatively higher levels of resistance to permethrin, had these P450 genes expressed at higher levels. It tended that the higher the resistance level of a colony was, the higher the expression

levels of P450 genes were. The gene, *CYP9M10*, was expressed at a higher level in all the selected colonies except the most susceptible one, Cx-SERC5, compared to the field population, $HAmCq^{G0}$.

4.4 Discussions

Our previous studies have reported that both major mechanisms, sodium channel insensitivity and cytochrome P450-mediated detoxification, were observed to be involved in resistant mosquito strain $HAmCq^{G8}$ (Xu et al. 2006, 2011; Yang and Liu, 2011). Point mutations resulting in reduced sodium channel sensitivity were detected in permethrin-selected resistant mosquitoes and certain field collected individuals by SNP determination at the L-to-F *kdr* locus, revealing a strong correlation between the prevalence of the L-to-F allelic (T) expression at the RNA level and the level of resistance in those mosquitoes (Yang and Liu, 2013). However, these results may not indicate accurately that under the field situation, which mechanism is the predominant one to contribute resistance to each individual of mosquitoes following different resistance levels, or if both major mechanisms are equally involved in insecticide resistance in field mosquitoes. In the current study, our aim was to get insight into both molecular mechanisms conferring to insecticide resistance in eight single-egg-raft colonies with different permethrin resistance levels, by investigating the expression of A and/ or T alleles at the L-to-F locus and expression profiles of key P450 genes to determine the importance of both mechanisms involved in insecticide resistance.

In mosquito larvae bioassays, eight single-egg-raft *Culex* mosquito colonies distributed their resistance to permethrin approximately from 1.0- to 23-fold (Table 2). To investigate whether resistance was mediated by mutation of the permethrin target site, we amplified a 341 bp sodium

channel fragment for each individual mosquito. Comparing the frequency of alleles (A and/ or T) at the L-to-F *kdr* locus at the RNA level in each mosquito colonies revealed that colonies with lower resistance levels to permethrin tended to have most or all the individuals with A alleles, and that individuals in the colonies with relatively higher resistance levels, expressed more T allele (Table 3). Moreover, we investigated if metabolic detoxification also plays an important role in the permethrin resistance of the individuals in the field population. A number of P450 genes, which are considered to be most related to insecticide resistance, were chosen for gene expression study in both adults and larvae of individual mosquito colonies by qRT-PCR, because cytochrome P450s are the most important enzyme for metabolic detoxification of pyrethroid insecticides. In the adult mosquitoes, selected key P450 genes were all overexpressed in resistant strain HAmCq^{G8} compared with its parental strain HAmCq^{G0}, but in other single-egg-raft colonies, some were up-regulated and some were down-regulated. These results may result from that permethrin selection and bioassays were applied at larval stage, not at adult stage. The bioassay results may only reflect the resistance level accurately at the larval stage. In the adults, resistance level for each individual may be changed. However, in the mosquito larvae, those selected P450 genes tended to be expressed at lower level in the individuals with lower level of resistance, such as colonies Cx_SERC1, Cx_SERC5 and Cx_SERC8, and to be up-regulated in the individuals with higher level of resistance, such as Cx_SERC6 as well as resistant strain HAmCq^{G8}, suggesting that cytochrome P450-mediated detoxification also play a role in the development of insecticide resistance. Interestingly, these selected P450 genes were expressed at higher level in the colony Cx_SERC3 at both adult and larval stage of the mosquitoes, but its resistance level was 11-fold and the frequency of T allele was 0.45, suggesting that overexpression of P450 genes may not be the main mechanism conferring to resistance in this

colony. Studies on other insects also indicated that the interaction of multiple insecticide resistance mechanisms or genes is likely to be responsible for insecticide resistance (Carvalho et al., 2013; Pridgeon and Liu, 2003; Silva et al., 2012; Zhu et al., 2013).

In conclusion, the current study investigated two major mechanisms involved in pyrethroid resistance in eight single-egg-raft colonies isolated from a field population of *Cx. quinquefasciatus*, $HAmCq^{G0}$, in response to different levels of resistance to permethrin. The results indicated that multiple mechanisms/different mechanisms co-occur in conferring to insecticide resistance with or among the single colonies and may equally contribute to the development of insecticide resistance.

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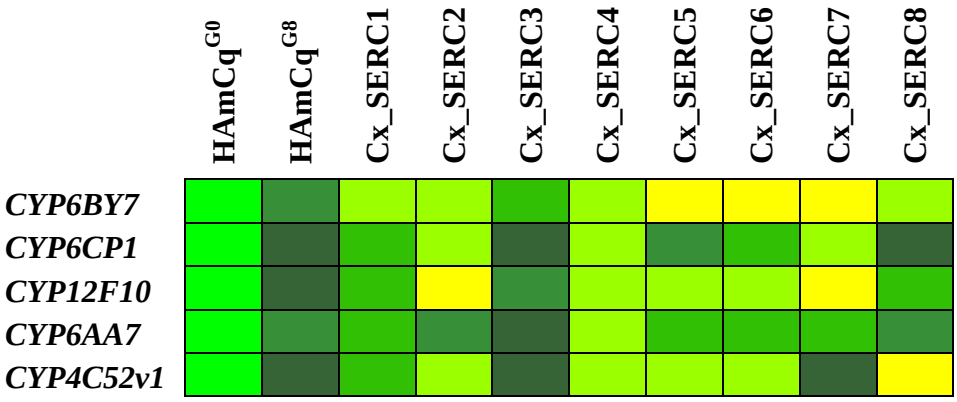
A



B

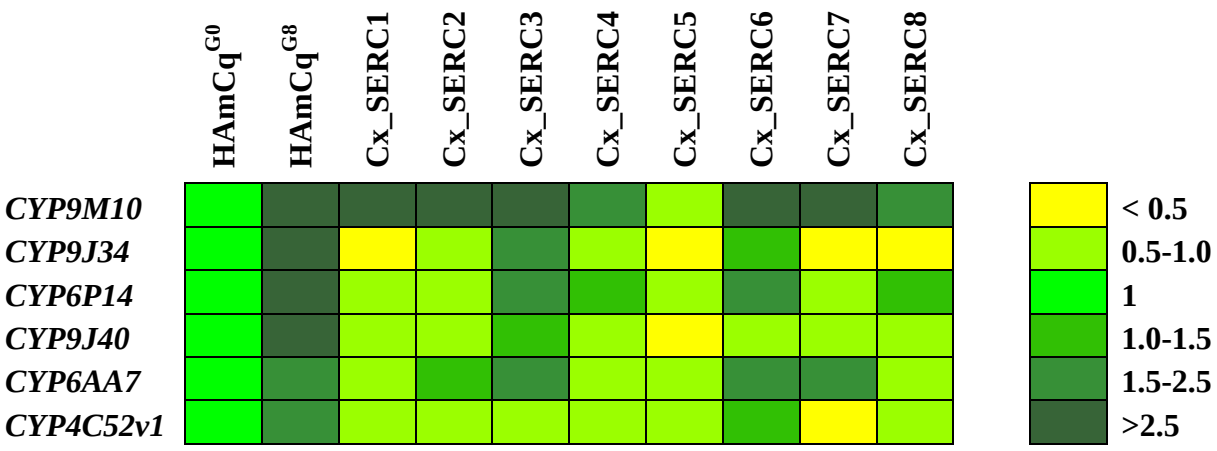


Figure 4.1 Target P450 gene expression levels associated with insecticide resistance in single-egg-raft colonies of *Culex quinquefasciatus*. The relative level of gene expression for $HAmCq^{G0}$ mosquitoes is 1.Gene expression levels were shown as mean fold relative to their levels in $HAmCq^{G0}$ (Student t-test, $P < 0.05$). A. P450 gene expression profiles at adult stage. B. P450 gene expression profiles at larval stage.

Table 4.1 Oligonucleotide primers used for amplifying the P450 qRT-PCR reactions

Transcript ID[a]	Accession No.	Gene[b]	Forward Primer (5′ to 3′)[c]	Reverse Primer (5′ to 3′)[c]
CPIJ003389	XM_001844782.1	CYP6BY7	AGGATTACATGGTGCGGTTCACGA	ACATCCTGAACTCACTGTTCGGCT
CPIJ005955	XM_001847351.1	CYP6P14	AAGGTGGAACCAGGTCTGACGATT	CCATCATTAGCCGCGATTGCCTTT
CPIJ005959	XM_001847355.1	CYP6AA7	ATGACGCTGATTCCCGAGACTGTT	TTCATGGTCAAGGTCTCACCCGAA
CPIJ010230	XM_001855550.1	CYP12F10	ATCCGGTAATGATGCAGCCGAAGA	AGTGATGAAAGTCACCCGGCATCT
CPIJ010543	XM_001855188.1	CYP9J40	AAAGGTACCCTGAAGCACCAGGAA	TCCAGCCAGGAAGAAGATCAAGCA
CPIJ010546	XM_001855208.1	CYP9J34	ATCCGATGTCGGTAAAGTGCAGGT	TGTACCTCTGGGTTGATGGCAAGT
CPIJ012640	XM_001863898.1	CYP6CP1	CATGCGTTACGTGGATTGGTGCAT	ATTCGCGAGTCCTAGCAATGGGAT
CPIJ014218	XM_001864262.1	CYP9M10	TGCAGACCAAGTGCTTCCTGTACT	AACCCACTCAACGTATCCAGCGAA
CPIJ018943	XM_001869165.1	CYP4C52v1	CACGCCGGCATTCCACTTTAAGAT	GGATTCCCATGGCGGTTTCACAAA

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence (http://c*quinquefasciatus*.vectorbase.org/)

[b]The annotation of the *Culex* P450 genes fromhttp://drnelson.utmem.edu/CytochromeP450.html [30]

[c]Specific primer pair designed according to each of the P450 gene sequences of the *Cx. quinquefasciatus* in vectorbase (http://c*quinquefasciatus*.vectorbase.org).

Table 4.2 Toxicity of Permethrin to mosquito colonies of *Culex quinquefasciatus*

Colony	df	n[a]	χ^{2b}	LC_{50}[c](CI)[d]	Slope(SE)	RR[e]
Cx_SERC1	5	114	2.0	0.02 (0.01-0.02)	2.7 (0.5)	5.7
Cx_SERC2	3	172	3.3	0.02 (0.01-0.03)	2.1 (0.3)	6.7
Cx_SERC3	4	114	4.8	0.03 (0.02-0.06)	1.7 (0.4)	11
Cx_SERC4	7	275	8.6	0.03 (0.03-0.05)	1.8 (0.2)	11
Cx_SERC5	5	171	12	0.003 (0.001-0.004)	3.1 (0.4)	1.0
Cx_SERC6	5	136	7.0	0.06 (0.04-0.08)	2.6 (0.4)	19
Cx_SERC7	6	155	5.0	0.07 (0.05-0.1)	1.6 (0.2)	23
Cx_SERC8	5	88	3.8	0.005 (0.002-0.007)	2.6 (0.6)	1.7
$HAmCq^{G0}$	3	150	1.3	0.07 (0.04-0.1)	2.4 (0.4)	22
$HAmCq^{G8}$	3	150	1.4	19 (9.9-30)	3.2 (0.5)	6,200

[a] Number of mosquito larvae tested.

[b] Pearson chi-square, goodness-of-fit test.

[c] LC_{50} value in ppm.

[d] 95% confidence interval, toxicity of permethrin is considered significantly different when the 95% CI fail to overlap.

[e] Resistance ratio between LC_{50} of each colony/strain and most susceptible colony Cx_SERC5.

Table 4.3 Allelic expression at the L-to-F locus of sodium channels in the single colonies of *Cx. quinquefasciatus*

Colony	n[a]	Resistance Phenotype[b]	Frequency of the specific alleles[c]		
			TTA	TTA/T	TTT
Cx_SERC1	33	5.7-fold	1	0	0
Cx_SERC2	38	6.7-fold	0.5	0.42	0.08
Cx_SERC3	33	11-fold	0.36	0.18	0.45
Cx_SERC4	32	11-fold	1	0	0
Cx_SERC5	30	1.0-fold	1	0	0
Cx_SERC6	30	19-fold	0	0	1
Cx_SERC7	36	23-fold	0.17	0.08	0.75
Cx_SERC8	33	1.7-fold	1	0	0
HAmCq^{G0}	30	22-fold	0.26	0.46	0.28
HAmCq^{G8}	30	6,223-fold	0	0.04	0.96

[a] The total number of tested flies.
[b]Resistance phenotype is the resistance ratio between LC_{50} of each colony/strain and most susceptible colony Cx_SERC5.
[c]The expression frequency of specific alleles at the L-to-F locus in the colonies; the nucleotides at the L-to-F locus changed in the RNA level are underlined.

Chapter5

Genome Analysis of Cytochrome P450s and Their Expression Profiles in Insecticide Resistant Mosquitoes, *Culex quinquefasciatus*

5.1 Abstract

Here we report a study of the 204 P450 genes in the whole genome sequence of larvae and adult *Culex quinquefasciatus* mosquitoes. The expression profiles of the P450 genes were compared for susceptible (S-Lab) and resistant mosquito populations, two different field populations of mosquitoes (HAmCq and MAmCq), and field parental mosquitoes (HAmCq ^{G0} and MAmCq^{G0}) and their permethrin selected offspring (HAmCq ^{G8} and MAmCq^{G6}). While the majority of the P450 genes were expressed at a similar level between the field parental strains and their permethrin selected offspring, an up- or down-regulation feature in the P450 gene expression was observed following permethrin selection. Compared to their parental strains and the susceptible S-Lab strain, HAmCq^{G8} and MAmCq^{G6} were found to up regulate 11and 6% of total P450 genes in larvae and 7 and 4% in adults, respectively, while 5 and 11% were down-regulated in larvae and 4 and 2% in adults. Although the majority of these up- and down-regulated P450 genes appeared to be developmentally controlled, a few were either up- or down-regulated in both the larvae and adult stages. Interestingly, a different gene set was found to be up- or down-regulated in the HAmCq^{G8} and MAmCq^{G6} mosquito populations in response to insecticide selection. Several genes were identified as being up- or down-regulated in either the larvae or adults for both HAmCq^{G8} and MAmCq^{G6}; of these, *CYP6AA7* and *CYP4C52v* were up -regulated and *CYP6BY3* was down-regulated across the life stages and populations of mosquitoes, suggesting a link with the permethrin selection in these mosquitoes. Taken together, the findings

from this study indicate that not only are multiple P450 genes involved in insecticide resistance but up- or down regulation of P450 genes may also be co-responsible for detoxification of insecticides, insecticide selection, and the homeostatic response of mosquitoes to changes in cellular environment.

Key Words: Cytochrome P450s; Up- and down regulation; Permethrin Resistance; *Culex quinquefasciatus*

5.2 Introduction

Cytochrome P450s have long been of particular interest as they are critical for the detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens. They are also involved in metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Basal and up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms, thus altering their pharmacological/toxicological effects [1]. Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides [2-4] and plant toxins [5,6]. While all insects probably possess some capacity to detoxify insecticides and xenobiotics, the degree to which they can metabolize and detoxify these toxic chemicals is of considerable importance to their survival in a chemically unfriendly environment [7] and to the development of resistance. A significant characteristic of insect P450s is their transcriptional up-regulation, resulting in increased P450 protein levels and P450 activities, which, in turn, cause enhanced metabolic detoxification of insecticides and plant toxins in insects, leading to the development of insecticide resistance [4,8-16] and a higher tolerance to plant toxins [17,18]. Insect P450s are also known to be an important part of the biosynthesis and degradation

pathways of endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH**)** [19-23] and thus play important roles in insect growth, development, and reproduction.

Cytochrome P450s are a superfamily that can take a number of related forms that frequently co-exist in the same cell type [24]. The rate at which a particular substrate is oxidized differs from one P450 to another, so that the overall metabolism of a specific substrate depends on the different forms present and varies between tissues, life stages, and sexes [25]. Because of the multiple cytochrome P450s expressed in each organism and the broad substrate specificity of some of these isoforms, P450s are capable of oxidizing a bewildering array of xenobiotics [25]. While the importance of P450s in insect physiology and toxicology is widely recognized, it is not yet clear how many P450 genes precisely are involved in insecticide resistance in a single insect such as the mosquito.

With the availability of the whole genome sequence for the mosquito *Culex quinquefasciatus* [26], we are now able to characterize the expression profiles of P450s in insecticide resistant mosquitoes and thus improve our understanding of the P450 gene interactions that play a role in the physiological and toxicological processes of insects. The current study focused on characterizing the expression profiles of these P450 genes from mosquito populations of *Cx. quinquefasciatus* bearing different phenotypes in response to permethrin (susceptible, intermediate and highly resistant) in order to pinpoint the key P450 genes involved in insecticide resistance.

5.3 Materials and Methods

5.3.1 Mosquito strains

Five strains of the mosquito *Cx. quinquefasciatus* were studied. $HAmCq^{G0}$ and $MAmCq^{G0}$ were field resistant strains collected from Huntsville and Mobile, respectively, from sites located >600 km apart in the state of Alabama, USA in 2002; the locations were not privately-owned or protected in any way, no specific permissions were required for these locations/activities, and the study did not involve endangered or protected species. Because *Cx. quinquefasciatus* is an important urban pest in Alabama, it has been a major target for several insecticides, including Bti, malathion, resmethrin, and permethrin, and control difficulties have been reported before the collection [27]. Both Field strains had the similar levels (10-fold compared with susceptible S-Lab) of resistance to permethirn [28]) and did not exposure to insecticides after established as colonies in the laboratory. $HAmCq^{G8}$ was the 8^{th} generation of permethrin-selected $HAmCq^{G0}$ offspring with a 2,700-fold level of resistance and $MAmCq^{G6}$ was the 6^{th} generation of permethrin-selected $MAmCq^{G0}$ offspring with a 570-fild level of resistance [29]. The permethrin selections for both $HAmCq^{G8}$ and $MAmCq^{G6}$ were performed at the 4^{th} instar larval stage [28, 29]. S-Lab was an insecticide susceptible strain provided by Dr. Laura Harrington (Cornell University).

All the mosquitoes were reared at $25 \pm 2^{o}C$ under a photoperiod of 12:12 (L:D) h and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

5.3.2 Quantitative real-time PCR (qRT-PCR)

The 4th instar larvae and 2-3 day-old adults (before blood deeding) of each mosquito population had their RNA extracted for each experiment using the acidic guanidine thiocyanate-phenol-chloroform method [8]. Total RNA (0.5 μg/sample) from each mosquito sample was reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR, which was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and a P450 gene specific primer pair designed according to each of the P450 gene sequences (http://c*quinquefasciatus*.vectorbase.org/, Table 5.5 with accession number for each of P450 genes) at a final concentration of 3-5 μM. All samples, including the A 'no-template' negative control, were performed in triplicate. The reaction cycle consisted of a melting step of 50 ℃ for 2 min then 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min. Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction using Dissociation Curves software [30]. Relative expression levels for the P450 genes were calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software [31]. The 18S ribosome RNA gene, an endogenous control, was used to normalize the expression of target genes [15,32, 33]. Preliminary qRT-PCR experiments with the primer pair (Table 5.5) for the 18S ribosome RNA gene designed according to the sequences of the 18S ribosome RNA gene had revealed that the 18S ribosome RNA gene expression remained constant among all 3 mosquito strains, so the 18S ribosome RNA gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated three to four times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a

Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant. Significant overexpression was determined using a cut-off value of a ≥2-fold change in expression [34].

5.4 Results

5.4.1 Cytochrome P450 genes in *Cx. quinquefasciatus*

The *Cx. quinquefasciatus* genome sequence has revealed 205 putative P450 (CYP) genes (including 8 Pseudogenes) in *Cx. quinquefasciatus* mosquitoes [26,35, http://c*quinquefasciatus*.vectorbase.org/]. The *Cx. quinquefasciatus* P450s fall into four major clans of CYP2, CYP3, CYP4, and mitochondrial (Figure 5.1), as do those identified in other insects [36]. Of the 204 *Cx. quinquefasciatus* P450s, the majority assemble in clans 3 and 4: 89 P450s were found in the clan CYP3, with 24 in the CYP9 family, 64 in the CYP 6 family and 1 in the CYP329 family, and 82 in the clan CYP4, with 34 in the CYP4 family, 47 in the CYP325 family, and 1 in the CYP326 family. Sixteen P450 genes were found in clan 2, with CYP families of 303 to 307, 18 and 15. The remaining 12 P450 genes were found in the mitochondrial clan with 6 P450 families of CYP12, CYP49, CYP301, CYP302, CYP314 and CYP315. Comparing this distribution with those of other insect species, *Cx. quinquefasciatus* showed a clear expansion of P450s in clans 3 and 4. This expanded P450 supergene family in the *Cx. quinquefasciatus* genome may provide a clue to the mechanisms that permit *Culex* mosquitoes to adapt to polluted larval habitats [26].

5.4.2 Dynamic changes of P450 gene expression in the mosquito populations of *Culex quinquefasciatus* following permethrin selection

To understand how the P450 gene expression profile changes following permethrin selection, we compared the gene expression of 204 P450 genes [26, http://c*quinquefasciatus*.vectorbase.org/, http://drnelson.utmem.edu/CytochromeP450.html] in both larvae and adults between susceptible and resistant *Culex* mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring using qRT-PCR. The accession numbers of the P450 genes were listed in Table 5.5. Mosquito populations bearing 3 different resistance phenotypes in response to permethrin were used, ranging from susceptible (S-Lab), through intermediate resistant ($HAmCq^{G0}$, field parental population) to highly resistant ($HAmCq^{G8}$, 8^{th} generation permethrin selected offspring of $HAmCq^{G0}$). Comparing the P450 gene expression profiles in both larvae and adults of permethrin selected $HAmCq^{G8}$ mosquitoes with those of their field parental population revealed that 69% of genes were expressed at a similar level in both $HAmCq^{G8}$ and $HAmCq^{G0}$ (Figure 5.2A), 11% were up-regulated in $HAmCq^{G8}$ larvae compared to $HAmCq^{G0}$, 7% were up-regulated in $HAmCq^{G8}$ adults, 1 gene was up-regulated in both larvae and adults, 5% were down-regulated in $HAmCq^{G8}$ larvae, 4% were down-regulated in $HAmCq^{G8}$ adults, and 2% were down-regulated in both larvae and adults of $HAmCq^{G8}$. Applying a cut off level of 2 [34], among the up-regulated P450 genes in larvae and adults of $HAmCq^{G8}$, the majority were expressed at 2- to 4-fold elevated levels compared with $HAmCq^{G0}$ and only 32% and 12% in larvae and adults, respectively, had >5-fold overexpression (Figure 5.2A).

Similar expression patterns were also identified in another permethrin selected mosquito strain, Here, MAmCq^{G6}, the 6th generation of permethrin selected field strain of MAmCq^{G0}, were compared with their parental strain of MAmCq^{G0}, which was collected at a location 600 km south of the collection site for the HAmCq^{G0} mosquitoes (Figure 5.2B). In MAmCq^{G0}, 6% of genes were found to be up-regulated in the larvae of MAmCq^{G6} compared with those of MAmCq^{G0} and S-Lab (Figure 5.2B), 4% were up-regulated in MAmCq^{G6} adults, 2 genes were up-regulated in both larvae and adults, 11% were down-regulated in larvae, 2% were down-regulated in adults, 2% were down-regulated in both larvae and adults, and 2% were down-regulated in larvae but up-regulated in adults. Taken together, these results revealed equally dynamic changes in abundance in both increased and decreased P450 gene expression in the two field mosquito strains of *Culex quinquefasciatus* following permethrin selection. Applying a cut off level of 2 [34], among the up-regulated P450 genes in larvae and adults of MAmCq^{G6} the majority exhibited 2- to 4-fold elevated levels compared with MAmCq^{G0} and only 7% and 27% in larvae and adults, respectively, of MAmCq^{G6} had >5-fold overexpression (Figure 5.2B).

5.4.3 P450 genes involved in up- and down-regulation in the larvae of resistant *Cx. quinquefasciatus*

Twenty five P450 genes were found to be up-regulated in the larvae stage (4th larval instar) of HAmCq^{G8} mosquitoes. The expression levels of these P450 genes were ≥2-fold higher in HAmCq^{G8} than that in both S-Lab and HAmCq^{G0} mosquito strains (Table 5.1). The genes were distributed in clans CYP3,CYP4, and mitochondria with 7 genes in family 9, 7 in family 6, 5 in family 4, 3 in family 325, 2 in mitochondria, and 1 without annotation. Except the six P450 genes *CYP6AG12*, *CYP6AA7*, *CYP4C38*, *CYP9J35*, *CYP6Bz2*, and *CYP9M10* whose expression levels in parental HAmCq^{G0} mosquitoes were 2.2-, 2.8-,2.1-, 11- 2.0- and 5-fold higher than in

susceptible S-Lab mosquitoes, the expression levels of other genes were similar or lower in HAmCq^{G0} compared with the susceptible S-Lab strain (Table 5.1). Similar patterns were observed when comparing the changes in P450 expression in the larvae of MAmCq^{G6} with those of both the S-Lab and MAmCq^{G0} mosquito strains. Fifteen P450 genes were found to be up-regulated in the larvae of MAmCq^{G6} mosquitoes. The expression levels of these P450 genes in MAmCq^{G6} were ≥2-fold higher than those in both the S-Lab and MAmCq^{G0} mosquito strains (Table 5.1). These genes were distributed in clans CYP2, CYP3, and CYP4 with 7 genes in family 9, 5 in family 6, and 1 in each of families 4, 306, and 307. The expression of these genes was similar or lower in MAmCq^{G0} compared with the susceptible S-Lab strain (Table 5.1) except for *CYP9M10* and *CYP6AA7,* whose expression levels in the parental MAmCq^{G0} mosquitoes were 8.9- and 4.5-fold higher, respectively.

Beside the up-regulation of P450 genes identified in the larvae of *Cx. quinquefasciatus* following permethrin selection, a number of P450 genes were found to be down-regulated in larvae of permethrin selected *Cx. quinquefasciatus*. Sixteen P450 genes were down-regulated in the larvae (4th larval instar) of HAmCq^{G8} mosquitoes. The expression levels of these P450 genes in HAmCq^{G8} were ≤2-fold lower than that in HAmCq^{G0} mosquitoes (Table 5.2). These down-regulated genes were distributed in clans CYP3 and CYP4, with 2 genes in family 9, 10 in family 6, and 2 in each of families 4 and 325. The expression of the majority of these genes in HAmCq^{G8} was at similar or lower levels compared with that in susceptible S-Lab mosquitoes, even though most were expressed at higher levels in HAmCq^{G0} than in S-Lab (Table 5.2). Although the similar P450 down-regulation patterns were also found in the larvae of MAmCq^{G6} compared with both S-Lab and MAmCq^{G0}, we did notice extended numbers and distribution of

these genes in the CYP clans compared with HAmCq mosquitoes. Thirty P450 genes were down-regulated in the larvae (4th larval instar) of MAmCq^{G6} mosquitoes. The expression levels of these P450 genes in MAmCq^{G6} were ≤2-fold lower than in that in MAmCq^{G0} mosquitoes (Table 5.2). The genes were distributed in clans CYP2, CYP3,CYP4, and mitochondria with 3 gene in family 9, 2 in family 6, 11 in family 4, and 8 in family 325, 1 in family 326, 2 in family 12, 1 in each of families 304 and 18, and 1 without annotation.. The expression levels of these genes were again similar or lower in MAmCq^{G6} compared with susceptible S-Lab mosquitoes, even though most were expressed at higher levels in MAmCq^{G0} than in S-Lab (Table 5.2).

5.4.4 P450 genes involved in up- and down-regulation in resistant *Cx. quinquefasciatus* adults

The expression of 204 *Culex* P450 genes in the adults of the 5 mosquito populations was examined using qRT-PCR. Seventeen P450 genes were found to be up-regulated in the adult stage (2-3 day old) of HAmCq^{G8} mosquitoes. The expression levels of these P450 genes in HAmCq^{G8} were ≥2-fold higher than that in both S-Lab and HAmCq^{G0} mosquito strains (Table 5.3). The overexpression levels of the up-regulated P450 genes in all the mosquito populations tested were closely correlated with their levels of resistance and were higher in permethrin-selected mosquitoes than in their parent field strain. These genes were mainly distributed in clans CYP3 and CYP4, with 3 genes in family 9, 5 in family 6, 5 in family 4, and 3 in family 325. One gene was in mitochondria clan, family 12. The expression of all these genes in HAmCq^{G0} was similar or lower than in susceptible S-Lab mosquitoes (Table 5.3). Similar changes in the P450 gene expression were also found in MAmCq^{G6} adults compared with their S-Lab and MAmCq^{G0} counterparts. Fifteen P450 genes were up-regulated in adult MAmCq^{G6} mosquitoes. The

expression levels of these P450 genes were ≥2-fold higher than those in both S-Lab and MAmCq^{G0} adults (Table 5.3). As in the HAmCq^{G8} mosquitoes, the genes whose expression changed in MAmCq^{G6} mosquitoes following permethrin selection were also distributed in clans CYP3 and CYP4, with 1 gene in family 9, 2 in family 6, 1 in family 4, and 11 in family 325. The expression of these genes was similar or lower in MAmCq^{G0} compared with susceptible S-Lab mosquitoes except for *CYP325BF1v2* and *CYP325K3v*, which were 2.4- and 3-fold higher, respectively, in MAmCq^{G0} (Table 5.4).

As in the mosquito larvae, a number of P450 genes were down-regulated in adult *Cx. quinquefasciatus* following permethrin selection. Fourteen P450 genes were down-regulated in adult HAmCq^{G8} mosquitoes. The expression levels of these P450 genes in HAmCq^{G8} were ≤2-fold lower than that in HAmCq^{G0} strain (Table 5.4). These genes were distributed in clans CYP3 and CYP4, with 3 genes in family 9, 4 in family 6, 3 in family 4, and 4 in family 325. Apart from *CYP6M12*, whose expression was ~2-fold higher in HAmCq^{G8} than in the susceptible S-Lab strain, all were expressed at lower levels in HAmCq^{G8} than in S-Lab adults even though most of the P450 genes in HAmCq^{G0} were expressed at higher levels than in S-Lab mosquitoes (Table 5.4). Similar down-regulation patterns for P450 were again found in MAmCq^{G6} adults compared with both S-Lab and MAmCq^{G0} adults. Nine P450 genes were down-regulated in MAmCq^{G6} mosquitoes, the expression levels of these 9 P450 genes were ≤2-fold lower in MAmCq^{G6} than that in MAmCq^{G0} mosquitoes (Table 5.4). The genes were distributed in clans CYP2, CYP3, and CYP4, with 2 genes in family 304, 3 in family 9, 1 in family 6, and 3 in family 4. All these genes had lower expression levels in MAmCq^{G6} than in S-Lab adults; the expression of these

genes in the MAmCq^{G0} mosquitoes was similar to that in the S-Lab strain except for *CYP9J46*, whose expression was much lower (Table 5.4).

5.5 Discussions

Two hundred four putative P450 (CYP) genes in *Cx. quinquefasciatus* mosquitoes [26, 35, http://c*quinquefasciatus*.vectorbase.org/] have put them in the largest P450 repertoire for any insect genome that has been reported so far; it is larger than that of *Anopheles gambiae* (111 P450s [37]), *Aedes aegypti* (160 P450s [34]), *Drosophila melanogaster* (90 P450s [38]), *Nasonia vitripennis* (jewel wasp, 92 P450s, [39]), *Bombyx mori* (silk moth, 86 P450s [40]), honeybee *Apis mellifera* (46 P450s [41]), *Tribolium castaneum* (red flour beetle, 134 P450s [42], [143 were reported by Dr. nelson, http://drnelson.utmem.edu/CytochromeP450.html]), pea aphid *Acyrthosiphon pisum* (83putative/ 58 complete P450, [43]), green peach aphid *Myzus persicae* (115 P450s, [43]), *Pediculus humanus* (human body louse, 37 P450s, [44]) and ants (http://drnelson.utmem.edu/CytochromeP450.html).

Our previous studies have indicated that P450s may be one of the primary enzymes involved in detoxifying permethrin and conferring permethrin resistance in *Culex* mosquitoes [45]. In order to examine the possible role of P450 genes, as a whole, in the development of insecticide resistance in *Culex quinquefasciatus* mosquitoes, we, for the first time, examined the expression profiles of a total of 204 P450 genes in both larvae and adults of *Cx. quinquefasciatus* by comparing the profiles for susceptible and resistant mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring. Insecticide resistance is generally assumed to be a pre-adaptive phenomenon, where prior to

insecticide exposure rare individuals carrying an altered (varied) genome already exist, thus allowing the survival of those carrying the genetic variance after insecticide selection [46]. We therefore expected that the number of individuals carrying the resistance P450 genes or alleles should increase in a population following selection and become predominant under severe selection pressure. The approach adopted for this study, which compared P450 gene expression among different mosquito populations and between two parental field populations, $HAmCq^{G0}$ and $MAmCq^{G0}$, and their permethrin selected offspring, $HAmCq^{G8}$and $MAmCq^{G6}$, for different levels of insecticide resistance highlighted the importance of P450 genes in resistance by detecting the changes in their expression within each population following permethrin selection. Our results showed a dynamic change in the P450 genes expressed in both of the field mosquito strains of *Cx. quinquefasciatus* following permethrin selection. Interestingly, most of these up- and down-regulated P450 genes in *Cx. quinquefasciatus* were found to be developmentally regulated following selection: changes in the level of expression (either increasing [up-regulation] or decreasing [down-regulation]) in the larval stage of mosquitoes following the selection were not found in the adult stage and vice versa. However, several genes were identified that had up- or down-regulation patterns that not only reflected the permethrin selection but were also consistent in both the larval and adult stages of the mosquitoes, suggesting the importance of these genes in response to insecticide resistance over the mosquitoes' whole life span. Comparison of the P450 gene expression between two different field mosquito populations following permethrin selection revealed that although both mosquito populations had a similar number of the P450 genes that were up- and down-regulated, the two populations for the most part regulated a different gene set in response to the insecticide selection. However, several genes were identified as being up- or down-regulated in either the

larvae or adults for both HAmCq^{G8} and MAmCq^{G6}; of these, *CYP6AA7* and *CYP6BY3* were up- and down-regulated, respectively, across all the life stages and populations of mosquitoes, suggesting that these genes are these genes are indeed related to insecticide selection. These results further propose that different mechanisms and/or P450 genes may be involved in the response to insecticide pressure for different developmental stages of mosquitoes and in different populations of mosquitoes [28]; some are specific to certain development stages and others provide protection throughout the insect's life cycle.

Basal and up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their pharmacological/toxicological effects [1]. In many cases, increased P450-mediated detoxification has been found to be associated with enhanced metabolic detoxification of insecticides, as evidenced by the increased levels of P450 proteins and P450 activity that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects [4,9,10,13-16,47-50]. In addition, multiple P450 genes have been identified as being up-regulated in several individual resistant organisms, including house flies and mosquitoes [12-14,16,49], thus increasing the overall expression levels of P450 genes. These findings suggest that overexpression of multiple P450 genes is likely to be a key factor governing increased levels of detoxification of insecticides and insecticide resistance. Nevertheless, although their importance in insect physiology and toxicology is widely recognized, there are gaps in our knowledge of insect P450s. One crucial piece of information that has been missing up until now is the issue of how many P450 genes are involved in insecticide resistance in a single organism, in this case the mosquito. The availability of the whole genome sequence of mosquitoes *Culex*

quinquefasciatus [26] has enabled us to address this question by characterizing the expression profiles of P450s in insecticide resistant mosquitoes at a genome-wide level.

Our comparison of P450 gene expression profiles between two field mosquito populations following permethrin selection has revealed that although both mosquito populations have similar numbers of P450 genes that are up-regulated, for the most part the mosquito populations regulate an array of P450 genes that differ from each other. However, several P450 genes are up- and down-regulated across the two different field mosquito populations of HAmCq and MAmCq in the same way and these are distributed in families 9, 6, 4, and 325. This finding is in agreement with previous studies on the expression levels of P450 transcripts, which have often reported up-regulated expression of the P450 genes in insecticide resistant strains in *CYP* families 4, 6, and 9 [2-4,9,10,13,14,16,51-54] and suggested this to be a factor in the detoxification of insecticide. Unlike the previous studies, however, our study has for the first time uncovered abundant genes in CYP family 325 that are up-regulated in resistant mosquitoes in the same way as those in families 4, 6, and 9. In addition, a few of genes from clans 2 and mitochondria were up-regulated. This discovery brings new information to bear on the issue of which P450 genes and families might be involved in insecticide resistance. A previous study by our group [16] has indicated that four P450 genes, *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*, from mosquitoes *Cx. quinquefasciatus* are up-regulated and the overexpression levels of these four P450 genes are closely correlated to their levels of resistance, being markedly higher in $HAmCq^{G8}$ compared to the parent strain $HAmCq^{G0}$. The overexpression of *CYP9M10* has also been reported in a resistant *Culex* mosquito strain in Japan and has been tentatively linked with pyrethroid resistance in *Culex* mosquito **[49,50,55**]. These four P450 genes have,

again, been identified as being overexpressed in resistant mosquitoes across two different field populations, strongly suggesting a common feature of these P450 genes in pyrethroid resistance in *Culex quinquefasciatus*. The significant change in the expression of these P450 genes between field parental and permethrin selected highly resistant mosquito offspring, along with the sound correlation with the levels of P450 gene expression following permethrin selection, provides a strong case further supporting the importance of these P450 genes, particularly in families 9, 6, 4, and 325, in the response to permethrin selection of resistant mosquitoes and in the development of insecticide resistance.

Our study has also revealed a down-regulation characteristic of P450 gene expression following permethrin selection in *Culex* mosquitoes. The number of down-regulated P450 genes. The clans and CYP families over which these genes were found to be distributed were similar to the up-regulated P450 genes, mainly in families 9, 6, 4 and 325. It has been pointed out that expression of many P450s is suppressed in response to various endogenous and exogenous compounds and this is also true for P450 suppression in vertebrates in response to pathophysiological signals **[56-**61]. Compared with our knowledge of P450 up-regulation involved in resistance, however, the mechanisms involved in P450 down-regulation and its relevance relating to resistance are poorly understood. It has been suggested that decreases in CYP gene expression could be an adaptive or homeostatic response [62,63]. A number of mechanisms have been proposed for P450 down-regulation, including: 1) an adaptive homeostatic response to protect the cell from the deleterious effects of P450 derived oxidizing species, nitric oxide, or arachidonic acid metabolites [63,64]; 2) a homeostatic or pathological response to inflammatory processes [62]; and/or 3) a need for the tissue to utilize its transcriptional machinery and energy for the synthesis of other

components involved in the inflammatory response [65]. These hypotheses all offer reasonable explanations for our observation of both up- and down-regulation of multiple P450 genes in the resistant mosquitoes following permethrin selection. P450 down-regulation could, for example, be linked to the homeostatic response that insects need to protect the cell from the toxic effects of extra P450 derived oxidizing species and metabolites from the up-regulated P450s and thus balance the usage of energy, O_2, and the other components needed for the syntheses proteins (including up-regulated P450s) that play important roles in insecticide resistance. It has been previously reported that some organophosphate insecticides require an oxidative biotransformation into more toxic structures that inhibit acetylcholinesterase, a process that is mediated by some P450 enzymes [2]. In such cases, a decrease in the expression levels of these CYP genes would be an advantage in the presence of an organophosphate insecticide by preventing its bioactivation by P450 enzymes. However, this argument may not apply to the permethrin used here for the selection of resistant mosquitoes [28,29].

5.6 Conclusions

The expression profiles of a total of 204 P450 genes in both larvae and adults of *Cx. quinquefasciatus* were compared between susceptible and resistant mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring. The results provide direct evidence that up- and down-regulation of multiple P450 genes co-occur in the genome of *Culex quinquefasciatus* following permethrin selection. These genes are mainly distributed in clans CYP3 and CYP4. These findings have important implications as they demonstrate that not only are multiple genes involved in insecticide resistance, but also multiple mechanisms are involved in P450 gene regulation. Both up- and

down regulation of P450 genes may be co-responsible for the detoxification of insecticides, evolutionary insecticide selection, and the homeostatic response of mosquitoes to changing cell environments.

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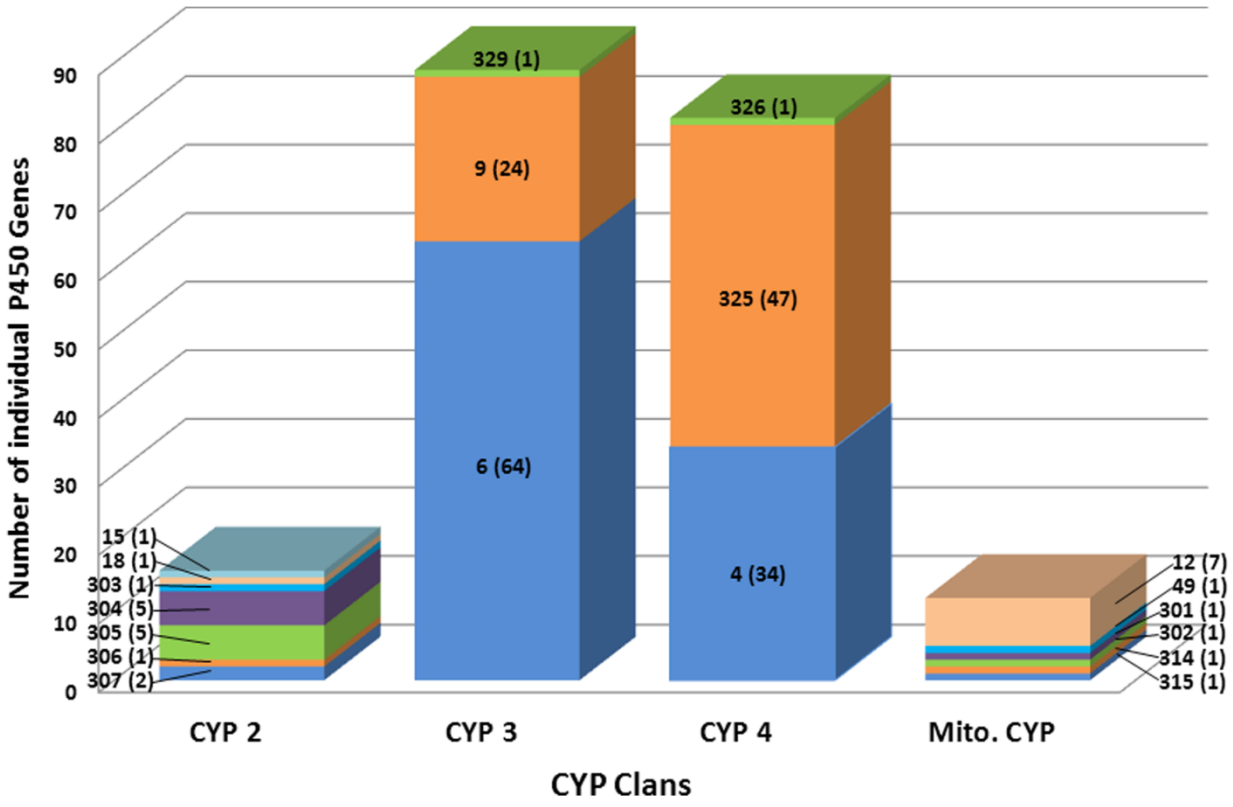

Figure 5.1 Number, family and clan distribution of cytochrome P450 genes in mosquitoes, *Culex quinquefasciatus*. The number shown along each column represents the P450 family and the number in parenthesis is the number of individual genes in the corresponding family. The P450 gene sequence information generated is from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/].

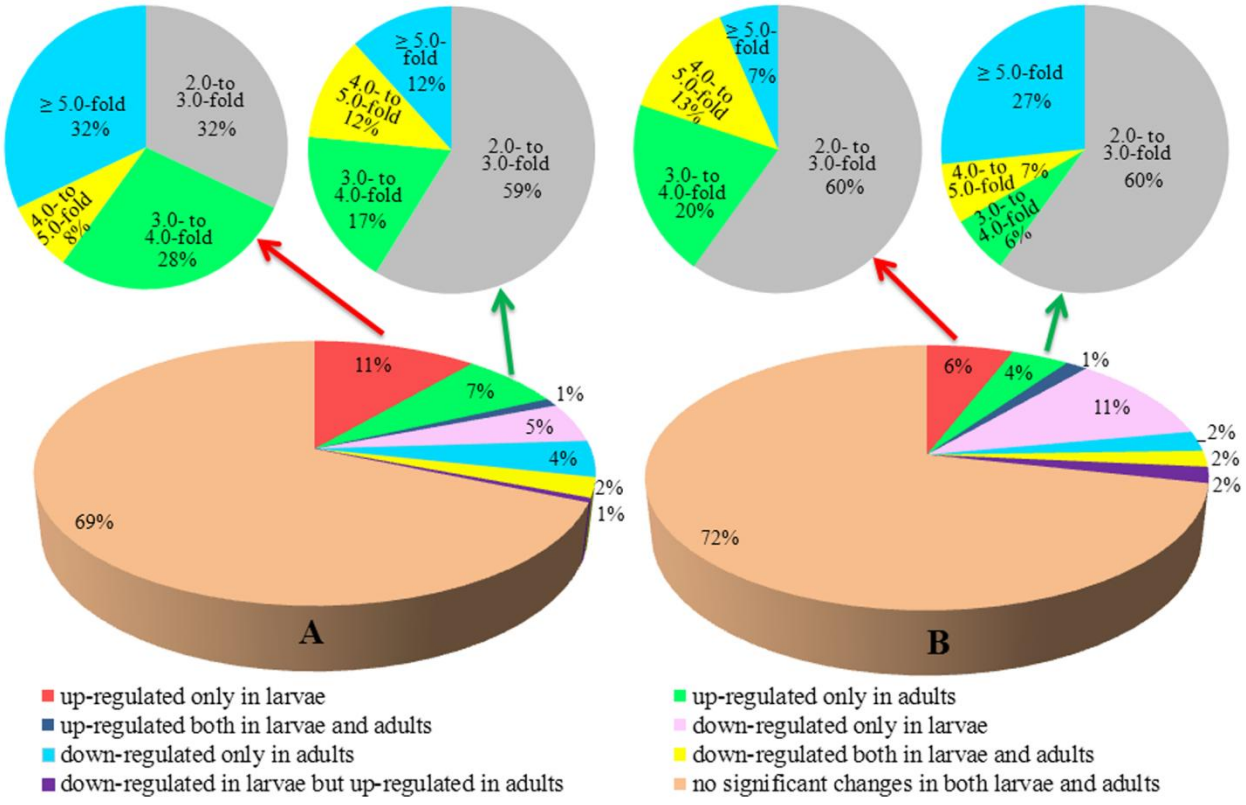

Figure 5.2 Diagrammatic representation of the analysis of the P450 gene expression profiles in both larvae and adults of permethrin selected mosquito populations $HAmCq^{G8}$ and $MAmCq^{G6}$ compared with their corresponding field parental populations $HAmCq^{G0}$ and $MAmCq^{G0}$. The statistical significance of the gene expressions was considered to be a p value ≤ 0.05. Significant overexpression was analyzed using a cut-off value of a ≥2-fold change in expression [29]. A. P450 gene expression profiles in $HAmCq^{G8}$. B. P450 gene expression profiles in $MAmCq^{G6}$.

Table 5.1 Up-regulation of P450 genes in larvae of permethrin selected offspring of the field populations of *Culex quinquefasciatus*

Mosquito Population	Transcript ID[a]	Gene	Relative Gene expression ± SE[b]		Ratio[e]
			Parental strain[c]	Resistant strain[d]	
HAmCq (25)	CPIJ002538	CYP6AG12	2.2 ±0.3	4.7 ±1.8	2.1
	CPIJ005959*[#]	CYP6AA7	2.8 ±0.9	5.9 ±0.6	2.1
	CPIJ003082	CYP9J42	1.3 ±0.2	2.9 ±1.5	2.2
	CPIJ001810	CYP4C38	2.1 ±0.6	5.1 ±0.3	2.4
	CPIJ015957	CYP325G4	1.2 ±0.2	3.3 ±0.3	2.8
	CPIJ005957	CYP6AA9	0.9 ±0.04	2.5 ±0.6	2.8
	CPIJ007091	CYP325Y6	1.1 ±0.3	3.1 ±1.2	2.8
	CPIJ010546*	CYP9J34	0.8 ±0.3	2.3 ±0.3	2.9
	CPIJ000926[¶]	-	0.7 ±0.1	2.2 ±0.2	3.1
	CPIJ016847	CYP6CQ1	0.9 ±0.2	2.8 ±0.9	3.1
	CPIJ009478	CYP4D42v1	1.3 ±0.1	4.1 ±0.9	3.2
	CPIJ010540	CYP9J35	11 ±2.0	39 ±10	3.5
	CPIJ005956	CYP6BZ2	2.0 ±0.6	7.3 ±1.1	3.7
	CPIJ010537*	CYP9J45	0.6 ±0.3	2.3 ±1.3	3.8
	CPIJ012470*	CYP9AL1	0.6 ±0.2	2.3 ±0.4	3.8
	CPIJ014218*	CYP9M10	5.0 ±1.2	21 ±4.0	4.2
	CPIJ005954	CYP6CC2	0.6 ±0.004	2.5 ±0.6	4.2
	CPIJ010225	CPY12F7	0.5 ±0.06	2.6 ±0.5	5.2
	CPIJ010227	CYP12F13	0.5 ±0.06	2.6 ±0.5	5.2
	CPIJ010543	CYP9J40	0.6 ±0.2	3.6 ±1.3	6.0
	CPIJ018943*[#]	CYP4C52v1	0.5 ±0.04	3.1 ±1.8	6.2
	CPIJ005955*	CYP6P14	0.6 ±0.1	3.8 ±0.6	6.3
	CPIJ001759	CYP4H40	0.3 ±0.09	2.1 ±0.6	7.0
	CPIJ020229	CYP4D42v2	0.4 ±0.1	2.8 ±2.4	7.0
	CPIJ017021	CYP325K3v1	0.2 ±0.02	2.1 ±0.2	11
MAmCq (15)	CPIJ014218*	CYP9M10	8.9 ±1.6	14 ±3.9	1.6
	CPIJ010548[#]	CYP9J39	0.9 ±0.4	2.0 ±0.3	2.2
	CPIJ005958	CYP6AA8	0.7 ±0.001	1.8 ±0.8	2.6
	CPIJ001039	CYP306A1	1.0 ±0.1	2.6 ±0.06	2.6
	CPIJ005959*[#]	CYP6AA7	4.5 ±1.0	12 ±4.2	2.7
	CPIJ005332	CYP9J43	0.9 ±0.08	2.5 ±0.02	2.8
	CPIJ004411	CYP6Z12	1.4 ±0.2	4.0 ±1.9	2.9
	CPIJ005955*	CYP6P14	1.6 ±0.1	4.6 ±0.5	2.9

CPIJ008566	CYP6Z15	0.7 ±0.06	2.1 ±0.7	3.0
CPIJ010546*	CYP9J34	1.1 ±0.4	3.4 ±1.1	3.1
CPIJ010537*	CYP9J45	1.0 ±0.3	3.1 ±1.3	3.1
CPIJ012470*	CYP9AL1	0.9 ±0.2	3.4 ±0.2	3.8
CPIJ010544	CYP9J33	0.6 ±0.1	2.9 ±1.0	4.8
CPIJ000989	CYP307B1	0.5 ±0.2	2.5 ±1.2	5.0
CPIJ018943*[#]	CYP4C52v1	0.2 ±0.02	2.7 ±1.7	14

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/]

[b]The relative level of gene expression represents the ratio of the gene expression in each permethrin selected strain compared with that in the susceptible S-Lab strain. The relative level of gene expression for S-Lab is 1

[c]Parental strain for HAmCq population is $HAmCq^{G0}$ with a 10-fold level of resistance to permethrin compared with S-Lab and for MAmCq is $MAmCq^{G0}$ with a 10-fold level of resistance to permethrin [28].

[d]Permethrin selected strain for HAmCq population is $HAmCq^{G8}$ with a 2700-fold level of resistance to permethrin and for MAmCq is $MAmCq^{G6}$ with a 570-fold level of resistance to permethrin [28].

[e]The ratio of the relative gene expression in each permethrin selected strain compared its parental strain

*The genes that are up regulated in both larvae of $HAmCq^{G8}$ and $MAmCq^{G6}$

[#]The genes that are up regulated in both larvae and adults of $HAmCq^{G8}$ and/or $MAmCq^{G6}$

[¶]No annotation in Dr. Nelson's P450 homepage
http://drnelson.utmem.edu/CytochromeP450.html

Table 5.2 Down-regulation of P450 genes in larvae of permethrin selected offspring of the field populations of *Culex quinquefasciatus*

Mosquito Population	Transcript ID[a]	Gene	Relative Gene expression ±SE[b]		Ratio[e]
			Parental strain[c]	Resistant strain[d]	
HAmCq (16)	CPIJ009085	CYP6AG13	1.3 ±0.07	0.6 ±0.04	-2.2
	CPIJ019586[§]	CYP6Z13P	2.8 ±2.2	1.3 ±0.3	-2.2
	CPIJ006950	CYP325BG1	6.7 ±1.9	3.0 ±0.7	-2.2
	CPIJ016852	CYP6N19	2.9 ±0.9	1.0 ±0.3	-2.9
	CPIJ005683[#]	CYP325Y10	5.3 ±2.7	1.8 ±0.5	-2.9
	CPIJ008972[#§]	CYP6F5P	2.9 ±0.7	0.9 ±0.1	-3.2
	CPIJ018716*	CYP4C38	1.0 ±0.06	0.3 ±0.1	-3.3
	CPIJ014219[§]	CYP9M10-de1b	2.6 ±0.5	0.8 ±0.2	-3.3
	CPIJ009473[#]	CYP4D41	2.1 ±0.8	0.6 ±0.01	-3.5
	CPIJ017462	CYP6E1	0.7 ±0.02	0.2 ±0.0	-3.5
	CPIJ011129	CYP6N25	3.5 ±1.5	0.9 ±0.1	-3.9
	CPIJ000299	CYP6AH3	4.8 ±0.7	1.1 ±0.2	-4.4
	CPIJ010547[#]	CYP9J47	1.6 ±0.3	0.3 ±0.08	-5.3
	CPIJ003377*	CYP6BY5	2.9 ±0.008	0.5 ±0.08	-5.8
	CPIJ003361	CYP6BY2	1.9 ±0.3	0.2 ±0.005	-9.5
	CPIJ003375*[#]	CYP6BY3	1.7 ±0.2	0.05 ±0.02	-34
MAmCq (30)	CPIJ017351	CYP4C50v1	1.4 ±0.3	0.7 ±0.07	-2.0
	CPIJ018854	CYP4C50v2	1.4 ±0.3	0.7 ±0.07	-2.0
	CPIJ010542	CYP9J38	1.2 ±0.2	0.6 ±0.2	-2.0
	CPIJ017198	CYP325BF1-de1b	1.4 ±0.2	0.7 ±0.06	-2.0
	CPIJ010228	CYP12F12	1.2 ±0.2	0.6 ±0.3	-2.0
	CPIJ017243	CYP304B4	2.3 ±0.4	1.1 ±0.2	-2.1
	CPIJ007090	CYP325Y5	2.2 ±0.07	1.0 ±0.07	-2.2
	CPIJ014579	CYP4AR3	2.1 ±1.2	0.9 ±0.08	-2.3
	CPIJ010231	CYP12F9	1.4 ±0.2	0.6 ±0.3	-2.3
	CPIJ019765[#]	CYP9M14	0.7 ±0.8	0.3 ±0.1	-2.3
	CPIJ007091	CYP325Y6	1.5 ±0.1	0.6 ±0.2	-2.5
	CPIJ015953	CYP325BF1v2	1.5 ±0.2	0.6 ±0.004	-2.5
	CPIJ015318	CYP325V5v2	3.3 ±0.5	1.3 ±0.09	-2.5
	CPIJ018944	CYP4C51v1	8.9 ±0.6	3.4 ±0.2	-2.6
	CPIJ003377*	CYP6BY5	2.7 ±0.6	1.0 ±0.3	-2.7
	CPIJ011843	CYP325BH1	9.8 ±2.5	3.6 ±2.5	-2.7

CPIJ001038	CYP18A1	5.2 ±1.6	1.9 ±1.4	-2.7
CPIJ009569	CYP326BK1	3.1 ±1.4	1.1 ±0.2	-2.8
CPIJ001757[#]	CYP4H39	3.6 ±1.2	1.2 ±0.03	-3.0
CPIJ001754	CYP4J6	4.5 ±0.4	1.5 ±0.6	-3.0
CPIJ001755	CYP4J19	0.9 ±0.1	0.3 ±0.0	-3.0
CPIJ009477	CYP4D19	4.3 ±0.7	1.4 ±0.6	-3.1
CPIJ018716*	CYP4C38	1.9 ±0.8	0.6 ±0.05	-3.2
CPIJ009475	CYP4D43	1.7 ±0.4	0.5 ±0.3	-3.4
CPIJ014220	CYP9M12	1.4 ±0.1	0.4 ±0.1	-3.5
CPIJ015961	CYP325BE1	2.8 ±0.6	0.7 ±0.09	-4.0
CPIJ009471[¶]	-	1.2 ±0.07	0.2 ±0.04	-6.0
CPIJ003375*[#]	CYP6BY3	2.8 ±0.06	0.3 ±0.01	-9.3
CPIJ001810[#]	CYP4C38	16 ±2.5	1.4 ±0.3	-11
CPIJ017200	CYP325N3v2	6.0 ±1.5	0.5 ±0.1	-12

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/]

[b]The relative level of gene expression represents the ratio of the gene expression in each resistant strain compared with that in the susceptible S-Lab strain. The relative level of gene expression for S-Lab is 1

[c]Parental strain for HAmCq population is HAmCq^{G0} with a 10-fold level of resistance to permethrin compared with S-Lab and for MAmCq is MAmCq^{G0} with a 10-fold level of resistance to permethrin [28].

[d]Permethrin selected strain for HAmCq population is HAmCq^{G8} with a 2700-fold level of resistance to permethrin and for MAmCq is MAmCq^{G6} with a 570-fold level of resistance to permethrin [28].

[e]The ratio of the relative gene expression in each permethrin selected strain compared its parental strain

*The genes that are down-regulated in both larvae of HAmCq^{G8} and MAmCq^{G6}

[#]The genes that are down-regulated in both larvae and adults of HAmCq^{G8} and/or MAmCq^{G6}

[¶]No annotation in Dr. Nelson's P450 homepage
http://drnelson.utmem.edu/CytochromeP450.html

[§]pseudogene

Table 5.3 Up-regulation of P450 genes in adults of permethrin selected offspring of the field populations of *Culex quinquefasciatus*

Mosquito Population	Transcript ID	Gene	Relative Gene expression ±SE		Ratio
			Parental strain	Resistant strain	
HAmCq (17)	CPIJ017199	CYP325BF1v1	0.9 ±0.1	1.8 ±0.3	2.0
	CPIJ019587	CYP6Z14	1.1 ±0.3	2.3 ±0.7	2.1
	CPIJ010536	CYP9J44	0.9 ±0.06	1.9 ±0.2	2.1
	CPIJ005959*#	CYP6AA7	1.4 ±0.2	3.2 ±1.0	2.3
	CPIJ015959	CYP325BJ1	1.9 ±0.1	4.3 ±0.2	2.3
	CPIJ015318	CYP325V5v2	0.9 ±0.02	2.2 ±0.5	2.4
	CPIJ010548*	CYP9J39	1.0 ±0.1	2.5 ±0.1	2.5
	CPIJ016284	CYP4J4	0.8 ±0.05	2.0 ±0.2	2.5
	CPIJ011127	CYP4H34	0.9 ±0.08	2.4 ±0.3	2.7
	CPIJ010203	CYP9AM1	0.9 ±0.1	2.7 ±0.6	3.0
	CPIJ001758	CYP4H38	0.7 ±0.4	2.2 ±0.7	3.1
	CPIJ009085	CYP6AG13	0.6 ±0.2	1.9 ±0.2	3.2
	CPIJ012640	CYP6CP1	0.9 ±0.08	3.3 ±1.1	3.7
	CPIJ018943*#	CYP4C52v1	0.8 ±0.02	3.6 ±2.0	4.5
	CPIJ006721	CYP4H37v1	0.6 ±0.05	2.9 ±0.8	4.8
	CPIJ003389	CYP6BY7	0.5 ±0.2	2.7 ±0.08	5.4
	CPIJ010230	CYP12F10	1.8 ±0.01	10 ±2.0	5.6
MAmCq (15)	CPIJ005957	CYP6AA9	0.9 ±0.09	1.9 ±0.4	2.1
	CPIJ015953	CYP325BF1v2	2.4 ±0.8	5.3 ±1.8	2.2
	CPIJ007092	CYP325Y7	0.9 ±0.01	2.1 ±0.4	2.3
	CPIJ015961	CYP325BE1	1.1 ±0.2	2.5 ±1.3	2.3
	CPIJ010548*#	CYP9J39	0.9 ±0.04	2.2 ±0.2	2.4
	CPIJ007091	CYP325Y6	1.3 ±0.3	3.2 ±1.7	2.5
	CPIJ007090	CYP325Y5	0.8 ±0.03	2.0 ±1.1	2.5
	CPIJ005959*#	CYP6AA7	1.3 ±0.5	3.4 ±1.0	2.6
	CPIJ015954	CYP325N3v1	0.7 ±0.2	1.9 ±0.5	2.7
	CPIJ006952	CYP325BG3	1.7 ±0.07	5.9 ±0.8	3.5
	CPIJ010272	CYP325BK2	0.9 ±0.07	4.1 ±1.0	4.6
	CPIJ014730	CYP325AA2	0.3 ±0.2	1.9 ±0.6	6.3
	CPIJ017021	CYP325K3v1	3.0 ±0.09	20 ±1.6	6.7
	CPIJ005685	CYP325BB2	0.9 ±0.1	6.1 ±2.0	6.8
	CPIJ018943*#	CYP4C52v1	0.2 ±0.02	2.8 ±0.7	14

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/]

[b]The relative level of gene expression represents the ratio of the gene expression in each resistant strain compared with that in the susceptible S-Lab strain. The relative level of gene expression for S-Lab is 1

[c]Parental strain for HAmCq population is $HAmCq^{G0}$ and for MAmCq population is $MAmCq^{G0}$

[d] Permethrin selected strain for HAmCq population is $HAmCq^{G8}$ and for MAmCq population is $MAmCq^{G6}$

[e]The ratio of the relative gene expression in each permethrin selected strain compared its parental strain

*The genes that are up regulated in both adult of $HAmCq^{G8}$ and $MAmCq^{G6}$

[#]The genes that are up regulated in both larvae and adults of each of $HAmCq^{G8}$ and $MAmCq^{G6}$, or both

Table 5.4 Down-regulation of P450 genes in adults of permethrin selected offspring of the field populations of *Culex quinquefasciatus*

Mosquito Population	Transcript ID[a]	Gene	Relative Gene expression ±SE[b]		Ratio[e]
			Parental strain[c]	Resistant strain[d]	
HAmCq (14)	CPIJ008972[#§]	CYP6F5P	0.8 ±0.02	0.4 ±0.04	-2.0
	CPIJ010547[#]	CYP9J47	2.0 ±0.01	0.9 ±0.2	-2.2
	CPIJ016849	CYP6M12	5.1 ±0.9	2.2 ±0.5	-2.3
	CPIJ003376	CYP6BY4	1.9 ±0.4	0.8 ±0.1	-2.4
	CPIJ005332	CYP9J43	3.2 ±0.1	1.2 ±0.2	-2.7
	CPIJ000294*	CYP4J13	1.6 ±0.09	0.6 ±0.1	-2.7
	CPIJ010542	CYP9J38	0.8 ±0.1	0.3 ±0.2	-2.7
	CPIJ006951	CYP325BG2P	1.1 ±0.4	0.4 ±0.1	-2.8
	CPIJ014730	CYP325AA2	0.6 ±0.2	0.2 ±0.1	-3.0
	CPIJ009473[#]	CYP4D41	3.3 ±0.2	0.9 ±0.04	-3.7
	CPIJ007093	CYP325Y8	2.3 ±0.3	0.6 ±0.06	-3.8
	CPIJ005683[#]	CYP325Y10	2.7 ±0.2	0.5 ±0.2	-5.4
	CPIJ010480	CYP4J20	1.1 ±0.2	0.1 ±0.04	-11
	CPIJ003375*[#]	CYP6BY3	0.4 ±0.04	0.02 ±0.007	-20
MAmCq (9)	CPIJ001757[#]	CYP4H39	1.7 ±0.1	0.8 ±0.1	-2.1
	CPIJ019765[#]	CYP9M14	2.3 ±0.4	1.1 ±0.3	-2.1
	CPIJ017245	CYP304B6	1.3 ±0.3	0.6 ±0.06	-2.2
	CPIJ010545	CYP9J41	1.4 ±0.3	0.6 ±0.02	-2.3
	CPIJ003375*[#]	CYP6BY3	0.7 ±0.02	0.3 ±0.03	-2.3
	CPIJ000294*	CYP4J13	1.3 ±0.2	0.5 ±0.05	-2.6
	CPIJ017242	CYP304C1	2.9 ±0.3	1.1 ±0.1	-2.6
	CPIJ010538	CYP9J46	0.02 ±0.01	0.005 ±0.003	-4.0
	CPIJ001810[#]	CYP4C38	2.3 ±0.1	0.1 ±0.02	-23

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/]

[b]The relative level of gene expression represents the ratio of the gene expression in each resistant strain compared with that in the susceptible S-Lab strain. The relative level of gene expression for S-Lab is 1

[c]Parental strain for HAmCq population is $HAmCq^{G0}$ and for MAmCq population is $MAmCq^{G0}$

[d] Permethrin selected strain for HAmCq population is $HAmCq^{G8}$ and for MAmCq population is $MAmCq^{G6}$

[e]The ratio of the relative gene expression in each permethrin selected strain compared its parental strain

*The genes that are down-regulated in both adult of HAmCq^{G8} and MAmCq^{G6}
#The genes that are down-regulated in both larvae and adults of each of HAmCq^{G8} and MAmCq^{G6}, or both
§pseudogene

Table 5.5 Oligonucleotide primers used for amplifying the P450 qRT-PCR reactions

Transcript ID[a]	Accession No.	Gene[b]	Forward Primer (5′ to 3′)[c]	Reverse Primer (5′ to 3′)[c]
CPIJ000067	XM_001841684.1	CYP49A1	ACGGTAACGAAGAGTCCAAACGGA	TCCATGGCAATGTTGATGTGTCGC
CPIJ000293	XM_001841814.1	CYP4J18	TTGACGCCCACGTTCCATTTCAAC	ACAAAGGTTGTAGCTCGGTGACGA
CPIJ000294	XM_001841815.1	CYP4J13	AGCAGTTTCTGCAGGACTCGACAT	CTTCATCAATGGCGTTGTCCGCTT
CPIJ000298	XM_001841819.1	CYP6AH2	TCCGATCATCAAGAATGTGGCGGA	ATTCCAAACGCCGAACTTGACACC
CPIJ000299	XM_001841820.1	CYP6AH3	TCCGCAGCTTGATCGAGAATGTCA	TCAGAAACGTGACCGAGTGACCAA
CPIJ000655	XM_001842366.1	CYP329B1	ACGTGTTCAACGTGATCTCTGGGT	TTACCGTAAACCAGCGAACTCCCA
*CPIJ000924	XM_001842495.1	CYP325X5P	AAGGGTCTCTTCACGGCCAAGTAT	CACACTTGACAAACAACGGCACGA
CPIJ000925	XM_001842496.1	CYP325X6	TGCGCTACACCGAAATGTTCCTGA	ACCATCTTCTCGGCACACTTGACA
#CPIJ000926	XM_001842497.1	-	GCAAGCAGTACAAATTCGCCGACA	TGATCAGATCGGTCCGTTGCAAGT
CPIJ000927	XM_001842498.1	CYP325X7	ATGCAGAACCGCCTCTTTGAGGAA	ACGCAATTGTTTCAGCATCTCGGG
*CPIJ000928	XM_001842499.1	CYP325X8P	ACAGGCGAAAGTACAGCTTCGCTA	TCAATCGATCGCTCCTGCTGAACA
CPIJ000929	XM_001842500.1	CYP325X9	TGCTTCAACACGCGAATTCTGCAC	TCACCGACGTGTACTGGTGAATGT
CPIJ000989	XM_001842607.1	CYP307B1	GCAGTACTTTGCCAGCCATCGTTT	TCCATGCGCTTCAGATTACGGTGA
CPIJ001038	XM_001842655.1	CYP18A1	TCCAAATGATGCAGGTGATTGCGG	GCAGCATAAACACGTTCAGCCACA
CPIJ001039	XM_001842656.1	CYP306A1	GTGTTTGCCTTTGATCCTGC	GGCAGGAAATTGACCGCCAT

CPIJ001380	XM_001843226.1	CYP314A1	ACCA ACTACGGGTTCAAGCTGTGG AAGT	TGAA GCTCTTCTTGCAGCGCTTCG TTTA
CPIJ001754	XM_001843567.1	CYP4J6	ACCGTCAAGAACCTGGAGG ACTTT	ATCGATCTGGTTTCGTGCTT CGGA
CPIJ001755	XM_001843568.1	CYP4J19	CATTAAGCAACGCAGGGAG CTGTT	ATCTGATTACGTGCCTCGGA TGCT
CPIJ001757	XM_001843570.1	CYP4H39	AAAGCCACTGAGTCGTGAG GACAT	TTCTTGTCCTTGCCCAGAAT CCGA
CPIJ001758	XM_001843571.1	CYP4H38	TTACCGTTGAGGGTAAGCCG TTGA	ACAACTTCTGCTGAACGTCC TGGT
CPIJ001759	XM_001843572.1	CYP4H40	TTTCCTGTATCCTTTCGCACG GGA	TGTAAACGTCCTCTTCCTGC TGCT
CPIJ001810	XM_001843432.1	CYP4C38	ACTTCAACATCCTGGACGGG TTCT	TCATGGCCGTCTCGCAGATA ATGT
CPIJ001886	XM_001843611.1	CYP4H31	TCGAATGACGTTCCTGGACC TGTT	TTCCTGCACATCTGGATGCT TTGC
CPIJ002535	XM_001844152.1	CYP6AG9	CGTAAATGGCCTCCTGCATT TGCT	TCGGGAAAGTGATGTTCGTC CAGT
CPIJ002536	XM_001844153.1	CYP6AG10	AACTCGCAGCCAAATTCACC ACAG	TGAGTAGCTTGGCAAGTTTC GGGA
CPIJ002537	XM_001844154.1	CYP6AG11	ACGACACTCTACTGGAATTG CCCT	CAAGTTCGATGGGTTCCGTG CATT
CPIJ002538	XM_001844155.1	CYP6AG12	AAGCCCTTTCAACGCTGGTT GATG	GAGCGTCGATGGCAAACAC ACAAT
CPIJ003082	XM_001844416.1	CYP9J42	TGCAAGCTAGAGCTGGTCTG CTAA	TGCTTGTGCGATCATTTCCG TGTC
CPIJ003361	XM_001844754.1	CYP6BY2	ACGGCGTTGAGAGGAATGA CTTCT	ACGACGAAGTCTCGAATCCA CCAA
CPIJ003375	XM_001844768.1	CYP6BY3	ATTGGGAGTTGTGCCTTTGG CATC	TGGCCGCTTCCTCTCTAAAC ACTT
CPIJ003376	XM_001844769.1	CYP6BY4	GTTGTGCAACGATTCACCAC GGAT	AACAATCGCTTCTTCCCGGA ATGC

CPIJ003377	XM_001844770.1	CYP6BY5	TGGTGCTGAACTTGAGGTGAAGGA	TTCCAGCAGATATGGGCTCGTTGA
CPIJ003378	XM_001844771.1	CYP6BY6	TTTCCTACCTTGAAGGCGGTTGGA	CAATTCCAAACGCACAGCTTCCGA
CPIJ003389	XM_001844782.1	CYP6BY7	AGGATTACATGGTGCGGTTCACGA	ACATCCTGAACTCACTGTTCGGCT
CPIJ004410	XM_001845944.1	CYP6Z11	TTACGGAAATATCCGGGACTGCCA	AAGTACCGTTCGTCCTTGGCGTAA
CPIJ004411	XM_001845945.1	CYP6Z12	GCCGGCAAGAAGCTGGAACATTAT	TCCGGGTTGTTCAACGTATCCACT
CPIJ005332	XM_001846722.1	CYP9J43	TGTGCCTTCGGTGTTAGACTGGAT	TCGATTCCAAGTTTCCCGGTGAGT
CPIJ005683	XM_001847180.1	CYP325Y10	CTGGCAATGAACCCGAACATCCAA	AGCCGAAGCGACTCTTTGATGACT
CPIJ005684	XM_001847181.1	CYP325BB1	ATGATCGGACCACTTCCGGTGATT	AAAGTGCGATTCAGTAGCCTCCGA
CPIJ005685	XM_001847182.1	CYP325BB2	TCAAATGACGGAAGCGTATCGGGA	AACTCATCTTCTTCGGGTGCCTGT
*CPIJ005899	XM_001847672.1	CYP6N26P	TAATTGATCCGGACGTGTCGCAGT	AAATCATTCCGCTCAACGCGGTTC
CPIJ005900	XM_001847673.1	CYP6N23	CGCTGTGCGTGAAACGGTTGAATA	GAAGCCGGCCAGAAAGAACACAAA
CPIJ005952	XM_001847348.1	CYP6BB4	TACGAGCTGGCCCTTAATCCGTTT	AGACTTTCCGCAGGTGGGTACTTT
CPIJ005953	XM_001847349.1	CYP6BB3	AGCGTTCGCTTTGACAACGACATC	TGAAGTCGTTACGCTGTGATCCGT
CPIJ005954	XM_001847350.1	CYP6CC2	ACGTGTTCTTCTCGCCATCGATCA	AGCTTATCCCTGTACAAACCGCGA
CPIJ005955	XM_001847351.1	CYP6P14	AAGGTGGAACCAGGTCTGACGATT	CCATCATTAGCCGCGATTGCCTTT
CPIJ005956	XM_001847352.1	CYP6BZ2	ACCATGGCGTCAGCTAAGGATGAA	TCCGTGGTGAATCCGACTAGCAAA
CPIJ005957	XM_001847353.1	CYP6AA9	TCACCTGTGACGTCATTGGA	AGGAAGCTGTCGAAGACCC

CPIJ005958	XM_001847354.1	CYP6AA8	TCGT AAAGGCGTTGGCGAAGAGA TTCAC	AACTT TCGTCAGCAGTTCCGAACCT TCAT
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CPIJ008936	XM_001850970.1	CYP4H41	TCCAGCAGAAGCTGTACGACGAAA	AATGCGGAGGATCTCCTTGATGGT
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*CPIJ008972	XM_001850501.1	CYP6F5P	CGCTTGGCGTGTACGTTCTTTGTT	ATCCTGTCGGGATACGTTGTTGCT
CPIJ008980	XM_001850555.1	CYP301A1	AAAGTGTACGAAGAGCTGCGACGA	TGTAAACCCGCAGAACCTCCTTGA
CPIJ009085	XM_001850916.1	CYP6AG13	ACCTCGAGCAGGTCATCAACGAAA	CCAAAGGGAATGACCGCACAAACA
CPIJ009170	XM_001850606.1	CYP303A1	TTCATCGTCCGGCATCTGAAGGAA	ATGAGTGCGGCAAAGTCTTGTTGG
#CPIJ009415	XM_001851032.1	-	GGCTTTGACTACGCAATGGCTGTT	TCATGTTGAAGAAGAGGTCCGGGT
CPIJ009468	XM_001851364.1	CYP4K3	TACCAAGCAGTACAAGCGGTTCCA	AGCTTCTTCTTCCTGGTCCCACAA
CPIJ009469	XM_001851365.1	CYP4AR4	AGAAGCGAGATGGAATCTGCCGAA	TGACCTGGTCCTTGATTTGCTCGT
#CPIJ009471	XM_001851367.1	-	AGATCGACGTGTGCAATACCCACT	TTGCGATGTTTGAACCAGTTCGGG
CPIJ009473	XM_001851369.1	CYP4D41	TGGCCGGAAGATGATGGAGAATCA	TTGCTCTTCTCAGCGGAGGTTTCT
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CPIJ009475	XM_001851371.1	CYP4D43	AGATCCTGGAGCAGTTTGTGGAGA	AGTCGGAGTGAATCTGTGCGTTGA
*CPIJ009476	XM_001851372.1	CYP4D44	GGATGGTGGATCCGGACATAGTTT	ATTTGTTCGTCAGGTTGCGGTTCG
CPIJ009477	XM_001851373.1	CYP4D19	ACCTGACGAACAAATCCGAC	TCCGATCAAACACCTCCACG

CPIJ009478	XM_001851374.1	CYP4D42v1	GAGT TCAACTATCTGGTTCGGGAT GCGA	AACT ACTTCCGGCTGAGGTTCGTT ATGA
CPIJ009569	XM_001851144.1	CYP326BK1	TCAAGTGCTTGAGATGGACG GTGA	GCCAGCTGCCACAAACGTCA TAAT
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CPIJ009587	XM_001851162.1	CYP325K3v2	GGCCTGCAACATGCTGGAAT ACAT	AATGTTGAAGATGCGCTTGC CGAG
CPIJ010075	XM_001870633.1	CYP4H35	TTAACGCGCAGGATAATCCC GACT	TAAAGCTGTGCAGGACTGCC AATG
CPIJ010125	XM_001851431.1	CYP12F14	TCAGCGAATGTCCGTTGAGG AAGA	TGACGTGGATCATCGTAGCA CCAA
CPIJ010175	XM_001870674.1	CYP9J48	ATGATTGCGTTCAAGCTGTT CCCG	TGGTGCTTTAGCGTACCTTT CCGA
CPIJ010203	XM_001870875.1	CYP9AM1	AGATGTTTCAGCTAGCGCTG GGAT	ACTTCCGCCCAAATAAGCAA CACG
CPIJ010225	XM_001855523.1	CPY12F7	TGGACAAGAATCCGAGCCA AGACA	TCTCCGGGTTCTTTGCTAAG CAGT
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CPIJ010228	XM_001855539.1	CYP12F12	AGACTACGGCGATCTGTTGG TGAT	CCGGCCGCACATTCTTTCGA TAAT
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CPIJ010230	XM_001855550.1	CYP12F10	ATCCGGTAATGATGCAGCCG AAGA	AGTGATGAAAGTCACCCGGC ATCT
CPIJ010231	XM_001855553.1	CYP12F9	AGCTGGATGAGGTTTCCAGG GATT	AAAGCCAGCACTCCGATCAT CTCA
CPIJ010272	XM_001870820.1	CYP325BK2	TTCTGGAGCGCGAAGGTAGT CAAA	TGCTCGACGAACCTTGATGG TCTT

CPIJ010480	XM_001854859.1	CYP4J20	ACGAAGAACATCGACAAGAGCCGA	GCGTCTTCGATGCAACCACTTTGA
CPIJ010536	XM_001855158.1	CYP9J44	TGAAGACTCGGCAAGAGCAGAACA	ACTTGGGATTCTTCGACAGTGGCA
CPIJ010537	XM_001855163.1	CYP9J45	TCAGCGGTACGGAAACGATGTGAT	AGTCCATGTTGGTCTTCTGTCCCA
CPIJ010538	XM_001855166.1	CYP9J46	ATCCAACGTGGGAAAGTCCACTCA	TGCCGAGTTCACTGAAAGCTCGTA
CPIJ010539	XM_001855170.1	CYP9J36	TCCACATGTTGATGGAGGTGCAGA	ATCAGCTCGTTCTCCGTCCAAACT
CPIJ010540	XM_001855175.1	CYP9J35	TTGCCCAATGCTTCCTGTTCTTCC	GTTTCCGCCAAGTGCTTGTTCTGT
CPIJ010541	XM_001855180.1	CYP9J37	TCTTGCTGGGAAGCCACTCAACTA	TCGTGGGAATCCATATGGTGCGAT
CPIJ010542	XM_001855184.1	CYP9J38	AGATGCTGGATTTGCTACCGTGGA	AGGAAGAACAGGAAGCACTGGGAA
CPIJ010543	XM_001855188.1	CYP9J40	AAAGGTACCCTGAAGCACCAGGAA	TCCAGCCAGGAAGAAGATCAAGCA
CPIJ010544	XM_001855191.1	CYP9J33	TCCGGCCTTTACTGGTTCCAAGAT	AAAGGCACACGTCGCAATCACATC
CPIJ010545	XM_001855203.1	CYP9J41	GATGTGATCGCAACCTGTGCGTTT	ATCCAGTCCGGAAACAGTCGGAAT
CPIJ010546	XM_001855208.1	CYP9J34	ATCCGATGTCGGTAAAGTGCAGGT	TGTACCTCTGGGTTGATGGCAAGT
CPIJ010547	XM_001855211.1	CYP9J47	AGGTCCAGCTTTCTCGACCAATGA	ACGGACCAACTCGTAGACTGCAAA
CPIJ010548	XM_001855214.1	CYP9J39	AGGTACTTGTGTGTGGTTTCCGGT	TGTTTGTTCTCATCGCTGAACCGC
CPIJ010810	XM_001856162.1	CYP325BC2	ACCGAGCGAACTTTCATCGGTACT	ACTCTTCTGGTTGATCGTCGGCAT
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CPIJ010858	XM_001861680.1	CYP6F1	CTGAGATTGGGCAAGCTGCA	ACACCTTAATACCTCCAACC

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CPIJ011835	XM_001862136.1	CYP325BM1	AGTCCCGTTCAGCGATCAGG AAAT	TGAACTCGTCGTAAACCTTC CGCT
CPIJ011836	XM_001862137.1	CYP325BN1	ATGTCCTTCTACGCGCTTCA TCGT	TGCAGTTCCGAGAACCACCA CTAA
CPIJ011837	XM_001862138.1	CYP325V2	TTATTCCGTTCAGTGGAGGC ACGA	TTCAGCAGTGCTTCAAACCG GAAG
CPIJ011838	XM_001862139.1	CYP325V3	GCGCAAAGCGATTAATCCCA CCTT	CATCTGCAAATGCTCCCAGG GTTT
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CPIJ011843	XM_001862144.1	CYP325BH1	GGTTTGTGGTACAACGCTGG GAAT	GCGGAAATTTGGTGTGTGGC GATA
CPIJ012470	XM_001862711.1	CYP9AL1	TGAACGTCCTTAGGGATGGC GAAA	TTGCTAGTCGCGGAAACGAA CTGA
CPIJ012640	XM_001863898.1	CYP6CP1	CATGCGTTACGTGGATTGGT GCAT	ATTCGCGAGTCCTAGCAATG GGAT
CPIJ012685	XM_001862837.1	CYP315A1	AACGATGCTGTGCGTCTTGG AAAC	ACTCAGGTAGCGGATGTTTG GGAT
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CPIJ014221	XM_001864265.1	CYP9M13P	TCTTCGGAGGAATCGAAAGCACGA	AATCTCCGCGTGAAGTTTGGCTTG
CPIJ014336	XM_001864768.1	CYP9M14(partial)	GTGATTCCGCCACAGGTGTTCATA	TTGGGCTACGTTCTCAGTCCAGTT
CPIJ014579	XM_001864938.1	CYP4AR3	GATTGTGGAAACGGCCATGGGAAT	TGTCAAACATCCTCCGGAACACGA
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CPIJ014943	XM_001864993.1	CYP305A11	ATGGACACTTACCACAGGTCAGCA	ACTGCCGGTTAAAGTCGACCAGTA
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CPIJ015428	XM_001865445.1	CYP6Z10	TGAAACACTTGCGAAGTCCAACGG	TCTGCGTTCCCTTCTTGATGACCA
CPIJ015681	XM_001866290.1	CYP4H37v2	TGAGTGAGCTGATACTGTTGCG	TGTTTGCGTCGCTTCTGAATGACC
CPIJ015953	XM_001866307.1	CYP325BF1v2	CTAACGGCGTGCTGGATTTCCAAT	GCAGTGTTTCGAGTTCAGCACTGT
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CPIJ015957	XM_001866311.1	CYP325G4	TGCC CGAGGTGCACGCTGGATTTG ATTT	CGTC AGTCTTGTGGTAAACCCAGT CCGT
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CPIJ015960	XM_001866314.1	CYP325BD1	AGTGCATTCGGAGGTCCTTC ATGT	AGACTTGTCACCAGCTTATC GGCA
CPIJ015961	XM_001866315.1	CYP325BE1	TTCCAGGAAATCCGGGACAC ACTT	GCATCGTTTCCTTCAGCACC ATGT
CPIJ015963	XM_001866317.1	CYP325L2	AGTACTGCAACGCAACCCGA TACT	ATTCTTTCTCGATCGCATCCC GGT
CPIJ016284	XM_001866492.1	CYP4J4	ACACGGCGAGAATACGTTAT GCCT	TTGAGCTGTTCAGCCAGAAT CGGT
*CPIJ016355	XM_001866596.1	CYP6AK1-de1	GGAACTTTCTGCAGCCACTG TTGA	CAGCGCAATCCCAAACAGCT TACT
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CPIJ016846	XM_001867236.1	CYP6M13	TTCAAAGTGGTGCGTGAAAC GGTG	TCGAACCCAGCCAAGTAGA ACACA
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CPIJ016848	XM_001867238.1	CYP6M14	GAAATGCCAAGCCCATGGG AAGAA	GCAAAGACGCACTTCAGCA GTTCA
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CPIJ016853	XM_001867243.1	CYP6N21P	TATTCGACGATTACGGCGGTTGGT	TTGTTACGCAAGCTGTTGCACTCC
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CPIJ016855	XM_001867245.1	CYP6N20v1	CTGGCGATGAACCAAGACCTTCAA	AGGATTGATGCTGGCGGGTAGATT
CPIJ016856	XM_001867246.1	CYP6N18	AGTGCGTAAGGGATGCTGTGAAGA	TCATGCCCTTGTGGAAGATGAGGT
CPIJ016857	XM_001867247.1	CYP6S4	GGGCAGGTTAAAGGCCATGTTTCA	CAATCCCAAACGCACACGATCCAA
CPIJ017014	XM_001868044.1	CYP6AG14	GCTGACGCTTTCGACGATGGATTT	ACGATTGTGGGTACTCCACGGTTT
CPIJ017021	XM_001867284.1	CYP325K3v1	AAGGTCAAGTTTGCCGAGTCGTTG	AAATCTCGTCCGAACTCTTCCCGA
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CPIJ017199	XM_001867779.1	CYP325BF1v1	AATTTAACCCGGATCGCTTCCTGC	GCAGCACGTGCACCATAATGATCT
CPIJ017200	XM_001867780.1	CYP325N3v2	GCAAGCTTGTCCAGCAAAGGATCA	TCGCCGGTATGGAGTTCTTGTCAT
CPIJ017242	XM_001867596.1	CYP304C1	GGTGTTCTTTGCGCTGTTCTCCAA	TTCTTGCCAACTTCGAACAGCACG
CPIJ017243	XM_001867597.1	CYP304B4	TGTTCCCAAACGGTACCCACTACA	ATTCTCCTTGGGCTCGGTCTTCTT
CPIJ017244	XM_001867598.1	CYP304B5	TAGCTAAAGATGTGTCCGTGCCGT	CGAATGGCCGCTGCTTCAAACTAA
CPIJ017245	XM_001867599.1	CYP304B6	ACTGGAAGTGGAAACAGACTGCGA	CAAACAGCACATGCGGACACTTGA
CPIJ017246	XM_001867600.1	CYP304B7	AACTGGTTATGATCCTGGCGGACT	GGCCACAAACGTTGTCGATTTCCT
CPIJ017351	XM_001867494.1	CYP4C50v1	ATCAAATCGGTGGCATCGTCCAGA	TTTCTGCTTGCGGATCTCCTCCTT
CPIJ017462	XM_001867845.1	CYP6E1	TTTGGTATTGTCGGACCCTC	ACTCACATCGTTTGGTAGGA

CPIJ017609	XM_001867905.1	CYP6CD3	CGTTAGTGATGGGAAGGAAGGTGTTCCA	CGCATTTCGTTCCTTGAGCAGTCCCGAT
CPIJ018494	XM_001869103.1	CYP6CD2	TGGTCAAGGACTTTGGCAGCTTTG	ATCTTGCTCACGGTGAAGATCGGT
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CPIJ018716	XM_001868893.1	CYP4C38	CTGCTCGAGGAGAAGAGTGTCATT	CCGCGTAATTAGCTCGTGGTTGAT
CPIJ018854	XM_001869293.1	CYP4C50v2	ATCAAATCGGTGGCATCGTCCAGA	TTGTTGGACAGTTTCTGCTTGCGG
CPIJ018943	XM_001869165.1	CYP4C52v1	CACGCCGGCATTCCACTTTAAGAT	GGATTCCCATGGCGGTTTCACAAA
CPIJ018944	XM_001869166.1	CYP4C51v1	TCGTGAACCGTCCATGATCGAACT	TTTCCACCTGGACATCCTCGGTTA
CPIJ019395	XM_001869675.1	CYP4C52v2	TGGAGGAACAGCTTGGAAATCGGA	TTAGCTGGTGGGCTCGAACGTATT
*CPIJ019586	XM_001869902.1	CYP6Z13P	GAAGAGTGCGCAGCGAATGTGAAT	AGCTGATCTCACCACCTGATTGCT
CPIJ019587	XM_001869903.1	CYP6Z14	AGTGATTTCGGGCTCTCGTTGGAA	TGAACCTTCTGCATGAGATCCGGT
CPIJ019673	XM_001869873.1	CYP6AG15	CAGCGACAATGAGTTTGCCGAGTT	TTAGTCATACGCGACGCAAGCTCT
CPIJ019700	XM_001870137.1	CYP6M15	GCGTGTTTGCGAAGGACTTTCAGT	CGACAACTTGTGCCGCAGATTCTT
CPIJ019701	XM_001870138.1	CYP6CQ2	ACCGATTCCGGTGTTGATTCGAGA	ACGGTTCGCGTTTGGACTTTGATG
CPIJ019702	XM_001870139.1	CYP6M16	GAAATGCCAAGCCCATGGGAAGAA	GCAAAGACGCACTTCAGCAGTTCA
CPIJ019703	XM_001870140.1	CYP6Y6	AAGAACACCGGAAGGTTGGAGGAA	TTCCCTGTTCATGGCGAGTTCGTA
CPIJ019704	XM_001870141.1	CYP6N24	TGTCATTGGAACGTGTGCGTTTGG	AAATCCATCGTCGAAAGCGTCAGC

CPIJ019705	XM_001870142.1	CYP6N27P	GCACAGCTGCTACGAACAACTCAA	AGCGAGCGGTCGTGAAAGTACAAA
CPIJ019751	XM_001869920.1	CYP6AG16	TGGAGCCATCGTTTGGATCTCTGT	GCCCGTTTGATTGAGTTTGCCTCA
CPIJ019765	XM_001869935.1	CYP9M14	TGACTCAGTGCAAGATCCGGACAA	AATATGAACACCTGCGGCGGAATC
CPIJ020018	XM_001870574.1	CYP6Z16	TGTCCAAGTTTCGGTTCGAGGCTA	AGGTGATGGCATCCGTTGAGGTAT
CPIJ020019	XM_001870603.1	CYP6Z17	TGGAATCTGTGAAGGCAGACCACA	TTCCGTTGGTCAAGATCTGCGTGT
CPIJ020082	XM_001870617.1	CYP6F6	ACCTGGAGAACTGCATCGACGAAA	AGTACTGGGATGATGACGGTGGTT
CPIJ020199	XM_001870352.1	CYP6N20v2	GCTGTTGGATGTGTTCAAGCCGTT	AGCACCTTCACGCTCTTTCGGTAA
CPIJ020229	XM_001870408.1	CYP4D42v2	GAGCTTCAAATTTGGCCCGCCATT	ACTTCCGGCTGAGGTTCGTTATGA

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence [http://c*quinquefasciatus*.vectorbase.org/]
[b]The annotation of the *Culex* P450 genes from http://drnelson.utmem.edu/CytochromeP450.html [36]
[c]Specific primer pair designed according to each of the P450 gene sequences of the *Cx. quinquefasciatus* in vectorbase (http://c*quinquefasciatus*.vectorbase.org)
*Pseudogene http://drnelson.utmem.edu/CytochromeP450.html
[#]No annotation in Dr. Nelson's P450 homepage http://drnelson.utmem.edu/CytochromeP450.html

Chapter 6

Functional Study of Multiple Cytochrome P450 genes Associated with Pyrethroid Resistance in the Southern House Mosquito, *Culex quinquefasciatus*

6.1 Introduction

Insecticide resistance and resistance development have become a world-wide problem in most insect pests, including mosquitoes, due to repeated application of insecticide. Pyrethroid insecticides are the most widely used insecticides for mosquito control, based on their high insecticidal effectiveness and low mammalian toxicity (Khambay and Jewess, 2004). However, mosquitoes have been reported to develop their resistance to pyrethroids, which in turn, can lead to the emergence of resistant population and resurgence of diseases (Brogdon and McAllister, 1998). The causes of pyrethroid insecticide resistance mainly fall into two types of mechanisms, which are cytochrome P450-mediated detoxification and voltage-gated sodium channel insensitivity. Insect cytochrome P450s are very important not only in metabolizing endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH), but also in detoxifying xenobiotics such as insecticides and plant toxins (Berenbaum, 1991; Schuler, 1996; Sutherland et al., 1998; Scott, 1999; Gilbert, 2004; Niva et al., 2004; Feyereisen, 2011). Insect cytochrome P450s confer insecticide resistance by their transcriptional up-regulation, leading to increased P450 protein levels and activities, in turn, resulting in enhanced metabolic detoxification and development of insecticide resistance (Liu and Scott, 1998; Zhu and Liu, 2008; Zhu et al., 2008a; Zhu et al., 2010; Liu et al. 2011). Understanding the molecular basis of resistance mechanisms related to cytochrome P450-mediated detoxification, such as getting

insight to the function of such P450 genes, is of importance in developing novel resistance control strategies.

So far, the powerful and robust tools performed in investigation of genes function have been focused on RNA interference (RNAi) technology and *Drosophila* transgenic technology. RNAi technology has been applied successfully on many insect species, and also on resistant genes silencing to insecticide by double-stranded RNA (dsRNA) injection and disruption of target mRNA (Fire et al., 1998; Lycett et al., 2006; Baum et al., 2007). To understand the function of a gene, not only can we knock down the gene in the organism to investigate the response, but also we can transform the target exogenous gene into the genome of *Drosophila melanogaster* to exploit the changes of the host. In the current study on the function of several target up-regulated P450 genes related to insecticide resistance, we introduced these two reliable technologies to elucidate the effect. According to our previous research, we found several P450 genes that were overexpressed in the resistant mosquito strains, $HAmCq^{G8}$ and $MAmCq^{G6}$, which were assumed to be in response to permethrin exposures and responsible for development of insecticide resistance (Yang and Liu, 2010). In this paper, we selected up-regulated cytochrome P450 genes and described their detoxification roles by using RNA interference (RNAi) and *Drosophila* transgenic technology. Our findings elucidated functional importance of these P450 genes on insecticide resistance.

6.2 Materials and Methods

6.2.1 Mosquito strains

Two permethrin highly resistant strains, $HAmCq^{G8}$ and $MAmCq^{G6}$, were used for P450 gene functional study by RNA interference. $HAmCq^{G8}$ and $MAmCq^{G6}$ strains, respectively, were the

8^{th} and 6^{th} generation of permethrin-selected offspring of two field-collected parental strains, $HAmCq^{G0}$ and $MAmCq^{G0}$, with resistance levels at 2,700-fold and 570-fold, compared with a lab susceptible strain, S-Lab (Xu et al., 2006). The permethrin selections for both $HAmCq^{G8}$ and $MAmCq^{G6}$ were performed at the 4^{th} instar larval stage (Li and Liu, 2010). The parental strains, $HAmCq^{G0}$ and $MAmCq^{G0}$ were field strains collected from Huntsville and Mobile, respectively, from sites located >600 km apart in the state of Alabama, USA in 2002; the locations were not privately-owned or protected in any way, no specific permissions were required for these locations/activities, and the study did not involve endangered or protected species. Both field strains had the similar levels (10-fold compared with susceptible S-Lab strain) of resistance to permethirn) and did not exposure to insecticides after established as colonies in the laboratory (Liu et al., 2004). S-Lab was an insecticide susceptible strain provided by Dr. Laura Harrington (Cornell University). All the mosquitoes were reared with yeast in the larval stage and sugar water in the adult stage, both at $25 \pm 2^{o}C$ under a photoperiod of 12:12 (L:D) h. Adult females were fed blood samples from horses for egg development(Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

6.2.2 RNA extraction

A total of 16 4^{th} instar mosquito larvae were flash frozen on dry ice and immediately processed for RNA extraction. Total RNA was extracted using the acidic guanidine thiocyanate (GIT)-phenol-chloroform method for the dsRNA synthesis experiments or, as the replications of qRT-PCR experiments for evaluation of gene expression level after RNAi of target P450 genes.

6.2.3 Quantitative real-time PCR (qRT-PCR)

Total RNA (0.5 μg/sample) from each injected/non-injected mosquito sample was reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR, which was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (15 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and a P450 gene specific primer pair designed according to each of the P450 gene sequences (http://c*quinquefasciatus*.vectorbase.org/, Table 6.1 with accession number for each of P450 genes) at a final concentration of 3-5 μM. All samples, including the 'no-template' negative control, were performed in triplicate. Relative expression levels for the P450 genes were calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software (Livak and Schmittgen, 2001). The 18S ribosome RNA gene, an endogenous control, was used to normalize the expression of target genes (Aerts et al., 2004; Zhu et al., 2010). Preliminary qRT-PCR experiments with the primer pair (Table 6.1) for the 18S ribosome RNA gene designed according to the sequences of the 18S ribosome RNA gene had revealed that the 18S ribosome RNA gene expression remained constant among all mosquito strains, so the 18S ribosome RNA gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated at least three times with independent biological samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

6.2.4 Double-stranded RNA synthesis and RNA interference

The dsRNAs of the P450 genes (targets) and a green fluorescent protein (GFP-pMW1650) gene (control) were synthesized by *in vitro* transcription using MEGAscrip T7 High Yield Transcription kit (AB applied biosystems) with the manufacture's instruction. A~500 bp PCR product were generated complimentary to the cDNA sequences of target P450 genes and a pMW1650 plasmid, respectively, with T7 promoter sequences (5'-TAATACGACTCACTATAGGG-3') appended the 5' ends of both sense and antisense of each PCR product (Table 6.1). This PCR product was used as a template to synthesize dsRNA. For dsRNA purification, phenol/chloroform extraction followed by ethanol precipitation method was applied. dsRNA was diluted in nuclease-free water to 3~4 μg/μL for injection into late 3rd or early 4th instar larvae of *Cx. quinquefasciatus*. To avoid gene injection affects for target gene expression, dsRNA of the GFP gene were served as the control, and the mosquitoes, from HAmCq^{G8} and MAmCq^{G6} strains, without injection were served as the calibrator.

The late 3rd or early 4th instar larvae were anaesthetized in ice-cold water for about 5 minutes prior to injection. Anaesthetized larvae were then placed on dry filter paper under dissection microscope. The dsRNA (~400 ng) was injected vertically to the body axis in the thoracic region with an injection needle pulled out from a glass capillary tube using needle puller (Sutter Instrument) by the program: Heat 545, Pull 30, Vel 120, Time 125. The manual microinjection procedure was mastered with Nanojects II (Drummond Scientific Company). After injection, the larvae were immediately removed from the filter paper back into distilled water at normal rearing conditions and fed with regular food. Total RNAs were extracted from 48~72 h post-injected and non-injected 4th instar larvae for evaluation of gene expression levels. Each experiment was repeated 3 times with 3 independently isolated RNA samples from mosquitoes.

6.2.5 Mosquito larvae bioassays

Stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) for the insecticide bioassays were prepared in acetone. The bioassay method used for the larvae was as described in previous studies (Liu et al. 2004a, 2004b; Liu et al. 2009, Li et al. 2009, Li and Liu 2010); each bioassay consisted of 4^{th} instar mosquito larvae in regular tap water and 1% insecticide solution in acetone at the required concentration, using at least four concentrations that resulted in >0 and <100% mortality. Control groups received only 1% acetone. Mortality was assessed after 24 h. At least 3 replications of the bioassay were performed. Bioassay data were pooled and analyzed by standard probit analysis, as described by Liu et al. (2004a, 2004b). Statistical analysis of LC_{10}, $LC_{50,}$ and LC_{90} values was based on non-overlapping 95% confidence intervals (CI).

6.2.6 Construction of transgenic *Drosophila* flies

The full lengths of the candidate up-regulated P450 genes from the $HAmCq^{G8}$ strain of *Cx. quinquefasciatus* were amplified from cDNA of *Cx. quinquefasciatus* using platinum Taq DNA polymerase High Fidelity (invitrogen) with specific primer pairs (Table 6.1) based on the 5' and 3' end sequences of the genes. PCR products of the full length genes were purified using a QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were ligated into pCR 2.1 vector using the Original TA Cloning kit (Invitrogen) as described by the manufacture instruction. The full lengths of the P450 genes were cloned in One Shot TOPO 10F' cells using the One Shot TOP10F' Chemically Competent *E. coli* kit (Invitrogen). Cloning and sequence analyses of the cDNAs were repeated at least three times and three TA clones from each

replication were verified by sequencing. The clones were then sub-cloned into a pUASTattB vector (a gift from Dr. Johannes Bischof, University of Zurich). The plasmid of each pUASTattB-up-regulated P450 gene was transformed into the germline of the *D. melanogaster* 58A and 86Fb strains using standard P-element-mediated (ΦC31) transformation techniques (Rainbow Transgenic Flies Inc.). The female offspring were chosen for further analysis.

6.2.7 *Drosophila* contact bioassays

Female *Drosophila* at 2~3 days posteclosion were used to conduct permethrin toxicity contact bioassays. A serial concentrations of permethrin solution in acetone, ranging from 3ng/µl to 80ng/µl that gave >0 and <100% mortality to the tested insects were prepared, 200 ul of each permethrin solution were evenly coated on the inside of individual 20 ml glass scintillation vials (Plapp et al., 1987). Fifteen female flies were transferred to each of the prepared vials, which were plugged with cotton balls soaked with 5% sucrose. The vials for the control groups were coated with acetone alone and plugged with identical 5% sucrose-soaked cotton balls. The mortality was scored after 24 hr exposure to permethrin. Once the concentration range had been determined, a single concentration (20 ng/µl, 4 µl/vial) that showed a significant difference in mortality between the transgenic and non-transgenic flies was selected for further permethrin bioassays. For each bioassay, a total of four vials containing 15 2-3-day old adult female *D. melanogaster* each were treated with 200 ul of 20 ng/µl permethrin solution for each strain. Each bioassay was independently replicated three times using only flies that exhibited the correct morphological marker (orange-pink) eyes. The *D. melanogaster* strain with no transgene from *Cx. quinquefasciatus* was used as the control reference strain. All tests were run at 27^{o}C and mortality was assessed after 24 h. The average survival values from each bioassay were tested

using a one way analysis of variance followed by a Welch's t-test in R to separate the means that were significantly different at the α=0.01 level of significance (R Core Team, 2012). All *D. melanogaster* were reared on Jazz-Mix Drosophila food (Fisher Scientific, Kansas City, MO) at 26±2°C under a photoperiod of 12:12 (L: D) h.

6.3 Results

6.3.1 Identification of cytochrome P450 genes involved in up-regulation in resistant *Cx. quinquefasciatus*

On the basis of our previous study, Several P450 genes were found to be up-regulated in either larval stage (4th instar) or adult stage (3-day-old) of both highly resistant strains of *Cx. quinquefasciatus*, $HAmCq^{G8}$ and $MAmCq^{G6}$ (Yang and Liu, 2010; Table 6.1). Five of them, *CYP6P14*, *CYP9AL1*, *CYP9J34*, *CYP9J45*, and *CYP9M10*, were up-regulated specifically in larval stage of both resistant strains. Meanwhile, one P450 gene, *CYP9J40*, was up-regulated only in the larvae of $HAmCq^{G8}$ strain; one P450 gene, *CYP9J39*, was up-regulated in the adults of both mosquito strains. Two P450 genes, *CYP4C52v1* and *CYP6AA7*, were identified to be up-regulated across life stages and both populations of mosquitoes, suggesting a link with the permethrin selection in these mosquitoes.

6.3.2 Knockdown of individual cytochrome P450 gene suppressed larval resistance of *Cx. quinquefasciatus* to permethrin

To further examine the function of up-regulated P450 genes, we introduced RNAi technique to knock down the expression of target P450 genes in the late 3rd or early 4th instar larvae of two resistant mosquito strains, $HAmCq^{G8}$ and $MAmCq^{G6}$. We first conducted RNAi on six P450

genes, *CYP4C52v1*, *CYP6AA7*, *CYP6P14*, *CYP9J34*, *CYP9J40*, and *CYP9M10*, in the larvae of HAmCq^{G8} strain. After 48~72 h of injection of dsRNAs of these six P450 genes respectively, bioassays using the standard procedure for LC_{50} determination were applied. The results indicated that mRNA levels of all target P450 genes decreased by 30~46% (Figure 6.1), suggesting that these genes were successfully silenced by RNAi. To determine whether knocking down the expression of these P450 genes resulted in the decrease of the resistance to permethrin in the HAmCq^{G8} strain, we performed bioassays to compare resistance levels among non-injected HAmCq^{G8} larvae and P450 gene dsRNA and GFP dsRNA injected HAmCq^{G8} larvae. As shown in Figure 6.1, the resistance ratios, which were the comparison of LC_{50} from target P450 gene injected mosquito larvae or GFP gene injected mosquito larvae to that from non-injected HAmCq^{G8} larvae, were dramatically decreased. The resistance levels after injection of target P450 genes were suppressed by 64~75% among six P450 gene injected mosquito strains, compared with non-injected mosquitoes. The dose response showing HAmCq^{G8} larvae injected with GFP dsRNA to permethrin was observed similar as HAmCq^{G8} non-injected larvae. However, the HAmCq^{G8} mosquito larvae injected with target P450 gene dsRNA showed a much higher susceptiblility to permethrin.

Similarly, we applied RNAi-mediated knockdown of the expression of two P450 genes, *CYP6AA7* and *CYP9M10*, in the other permethrin resistant strain, MAmCq^{G6} to confirm the role of these two genes, which were also up-regulated in the MAmCq^{G6} larvae, in the resistance to permethrin (Figure 6.2). The result showed that the mRNA levels of *CYP6AA7* and *CYP9M10* were reduced by 41% and 30% respectively in the larvae injected with respective dsRNA as compared with those in the non-injected mosquitoes after 48~72 h. Correspondingly, as both the *CYP6AA7* and *CYP9M10* mRNA levels were significantly suppressed in the MAmCq^{G6} larvae by

RNAi, we examined the resistance levels of the target P450 gene injected mosquitoes to permethrin. The LC_{50} of dsRNA of *CYP6AA7* and *CYP9M10* injected $MAmCq^{G6}$ larvae were also reduced by 55% and 68% respectively (Figure 6.2).

These data indicated that RNAi-mediated knockdown of the expression of up-regulated P450 genes enhanced the larval susceptibility of both the $HAmCq^{G8}$ and $MAmCq^{G6}$ strains to permethrin, suggesting the importance of these P450 genes related to permethrin resistance in *Culex* mosquitoes.

6.3.3 Knockdown of combination of *CYP6AA7* and *CYP9M10* genes decreased resistance of *Cx. quinquefasciatus* to permethrin

To investigate whether there was synergistic effect on reduction of the resistance of mosquito larvae to permethrin when two P450 genes were knocked down at one time, we injected the mixture of dsRNA of both *CYP6AA7* and *CYP9M10* genes into 4^{th} instar mosquito larvae in the $HAmCq^{G8}$ strain. A reduction of the expression of each gene was also observed (Figure 6.3). After injection of the combination of both genes for 48~72 h, the expression level of *CYP6AA7* gene was suppressed by 30%, while the expression level of *CYP9M10* gene was decreased by 34%. Then we conducted bioassays for determination of resistance level of the injected mosquito larvae to permethrin. The $HAmCq^{G8}$ larvae exhibited a higher susceptibility to permethrin by a 69% reduction of resistance level compared to non-injected $HAmCq^{G8}$ larvae. However, the reduction rate was similar as that of single gene knockdown of *CYP6AA7* and *CYP9M10* genes respectively (Figure 6.3).

6.3.4 Transgenic expression of *CYP6AA7* and *CYP9M10* in *Drosophila melanogaster*

To determine whether the expression of up-regulated P450 genes is sufficient to cause permethrin resistance, we selected two P450 genes, *CYP6AA7* and *CYP9M10*, for transforming *D. melanogaster* (Rainbow Transgenic Flies, Inc., Camarillo, CA). These two genes were chosen for the further examination of their function in insecticide resistance or tolerance to insecticides because there were highly overexpressed in both resistant mosquito strains, $HAmCq^{G8}$ and $MAmCq^{G6}$ compared to their parental strains, $HAmCq^{G0}$ and $MAmCq^{G0}$, and also showed the most remarkable difference between the resistant strains and laboratory susceptible strain, S-Lab. We characterized the sensitivities of the transgenic lines of *D. melanogaster* to permethrin compared to the sensitivities of the non-transgenic lines of *D. melanogaster*. The contact bioassay results showed that both *CYP6AA7* and *CYP9M10* transgenic lines of *D. melanogaster* had increased their levels of tolerance to permethrin with lower mortalities when the transgenic *D. melanogaster* line was exposed to 4 μg/per vial of permethrin compared to the level of tolerance for the non-transgenic *D. melanogaster* lines after exposure to the same challenge (Figure 6.4). The mortality ranged from 35% to 59% for the *CYP6AA7* transgenic lines (Figure 6.4A) and 29% to 61% for the *CYP9M10* transgenic lines (Figure 6.4B) compared to 97% mortality for the control (non-transgenic *D. melanogaster*) when exposed to the same permethrin concentration.). These results show that both up-regulated P450 genes in resistant mosquitoes are capable of conferring permethrin tolerance for *D. melanogaster*, strongly suggesting the importance of these up-regulated genes, *CYP6AA7* and *CYP9M10*, in detoxification of permethrin and insecticides resistance.

6.4 Discussions

Our previous research have indicated that multiple P450s may be co-working on and co-responsible for detoxifying permethrin and conferring permethrin resistance in *Culex* mosquitoes (Liu et al., 2011; Xu et al., 2005; Yang and Liu, 2011). In this study, our goal was to elucidate functions of several candidate key P450 genes, which were up-regulated and considered to be involved in permethrin selection and insecticide resistance in both resistant mosquito strains, $HAmCq^{G8}$ and $MAmCq^{G6}$. We introduced the powerful tools, RNA interference (RNAi) technique and *Drosophila melanogaster* transgenic technique to determine the roles of key P450 genes in conferring to insecticide resistance and the molecular basis of resistance mechanisms in the mosquito, *Culex quinquefasciatus*. Cytochrome P450s are known to be as a superfamily of metabolic enzymes found in all living organisms (Feyereisen, 2005). Therefore, identification and characterization of new cytochrome P450 genes have become a very attractive research area. Our previous study and other research suggested that multiple P450s associated with insecticide resistance through up-regulation in resistant strains. Zhu et al. (2008b) observed that several P450 genes, *CYP4D4v2*, *CYP4G2*, *CYP6A38*, and *CYP6A36* were up-regulated by induction or constitutive expression in a permethrin resistant housefly strain ALHF. Two duplicated P450s, *CYP6P4* and *CYP6P9*, were overexpressed in pyrethroid resistance *Anopheles funestus* strain (Wondji et al., 2009).

Based on the studies, we utilized RNAi technique to knockdown six candidate P450 genes, *CYP4C52v1*, *CYP6AA7*, *CYP6P14*, *CYP9J34*, *CYP9J40* and *CYP9M10* in forth instar larvae of both $HAmCq^{G8}$ and $MAmCq^{G6}$ mosquitoes. The results showed that injections of dsRNA transcribed *in vitro* from those candidate P450 genes led to remarkable and specific gene silencing 48~72h after injection through a decrease of gene expression level for all P450 genes. The delivery of dsRNA could achieve successful and effective RNAi, which, as a valuable tool

for gene functional study, has been applied in many insect species (Alves et al., 2010; Chen et al., 2010; Fauce and Owens, 2009; Guo et al., 2012; Jaubert-Possamai et al., 2007; Liu et al., 2010; Qiu et al., 2012; Tang et al., 2012; Zhu et al., 2010; 2012). Correspondingly, bioassay results for dsRNA injected forth instar larvae also showed obvious gene knockdown effect on resistant mosquito strains via decreased resistance to permethrin. The survival rate of mosquito larvae injected with dsRNA of target P450s after exposed to permethrin were decreased to around 20%~40% compared with non-injection mosquitoes. Thus, our results showed increased mortality of the mosquito larvae after each of the target P450 genes was silenced by RNAi. These cytochrome P450 genes are likely to be involved in permethrin detoxification in the *Culex* mosquitoes.

To investigate whether there was synergistic effect on reduction of the resistance of mosquito larvae to permethrin when two P450 genes were silenced at one time, we injected the mixture of dsRNA of both *CYP6AA7* and *CYP9M10* genes into forth instar mosquito larvae in the $HAmCq^{G8}$ strain. Reductions of the expression level of each gene and resistance level to permethrin were also observed. However, the reduction rate was similar as that of single gene knockdown of *CYP6AA7* and *CYP9M10* genes respectively. No synergistic effect was observed after mixture of dsRNA of both genes injected. This phenomenon may result from the insufficiency of dsRNA of either P450 gene approaching target mRNA, since the insect body can only afford certain amount of dsRNA solution injected into the hemolymph.

Transgenic overexpression of P450 genes in *D. melanogaster* was successfully exploited to evaluate the function of fruit fly P450s in insecticide resistance. For example, in one study, eight *D. melanogaster* P450s were expressed in the midgut, Malpighian tubules, and fat body, and some of them conferred resistance to multiple insecticides (Daborn et al., 2007). In the current

study, two *Culex* P450 genes, *CYP6AA7* and *CYP9M10*, expressed in the whole body of *D. melanogaster*. The results showed that the transformation of these two P450 genes conferred resistance to permethrin with transgenic fruit flies exhibiting lower level of mortalities. Taken together, this study suggested that transgenic *Drosophila* technique could be exploited as a powerful tool for studying the function of P450s from target insects.

In summary, our findings indicated that up-regulated key P450 genes are responsible for detoxification of pyrethroid insecticides, conferring resistance to permethrin in the mosquito, *Culex quinquefasciatus*. Moreover, cytochrome P450-mediated detoxification does play an important role in insecticide selection and development of insecticide resistance.

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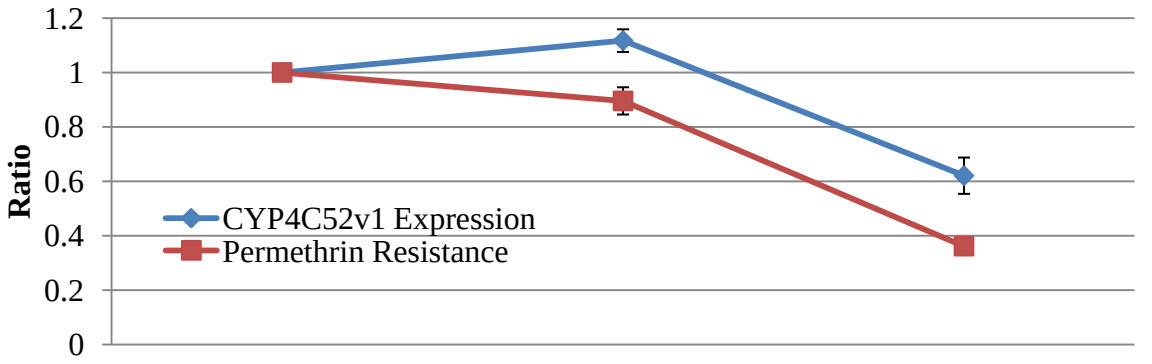
A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Permethrin Resistance

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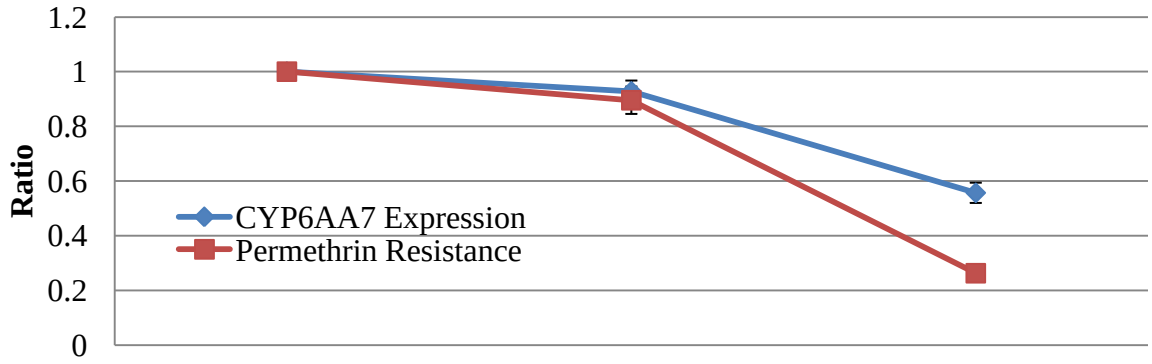
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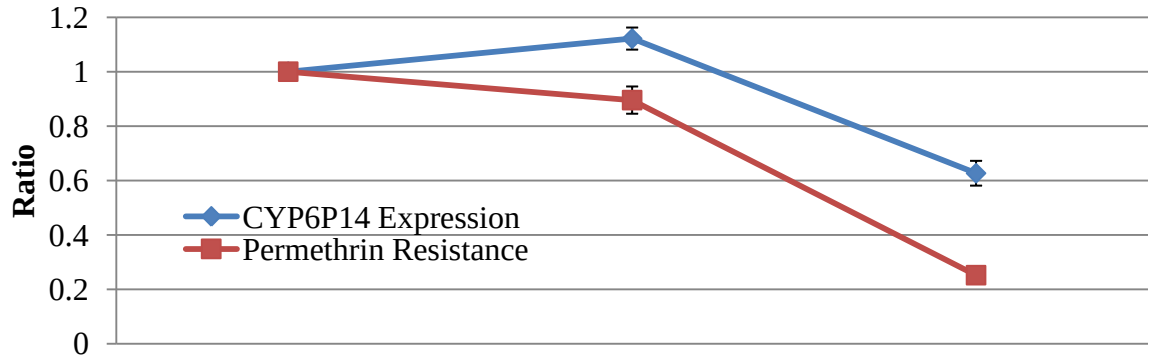
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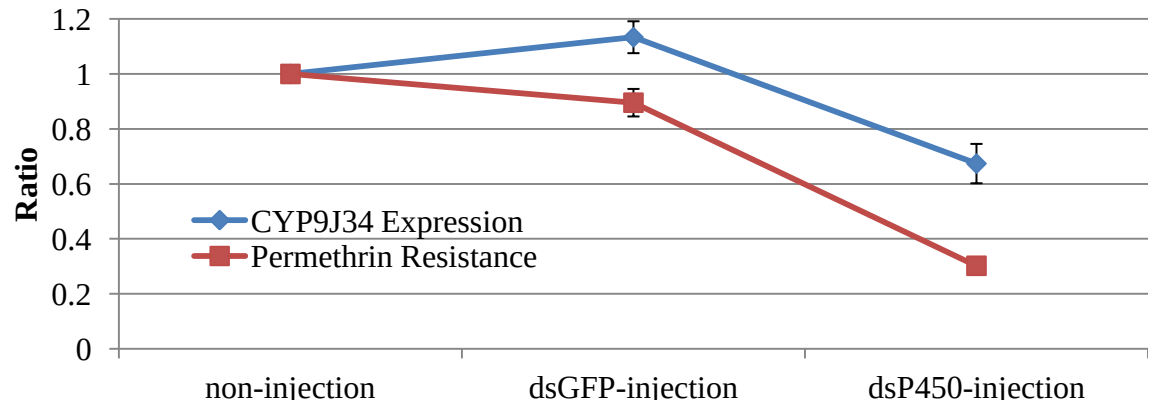
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dsGFP-injection
dsP450-injection

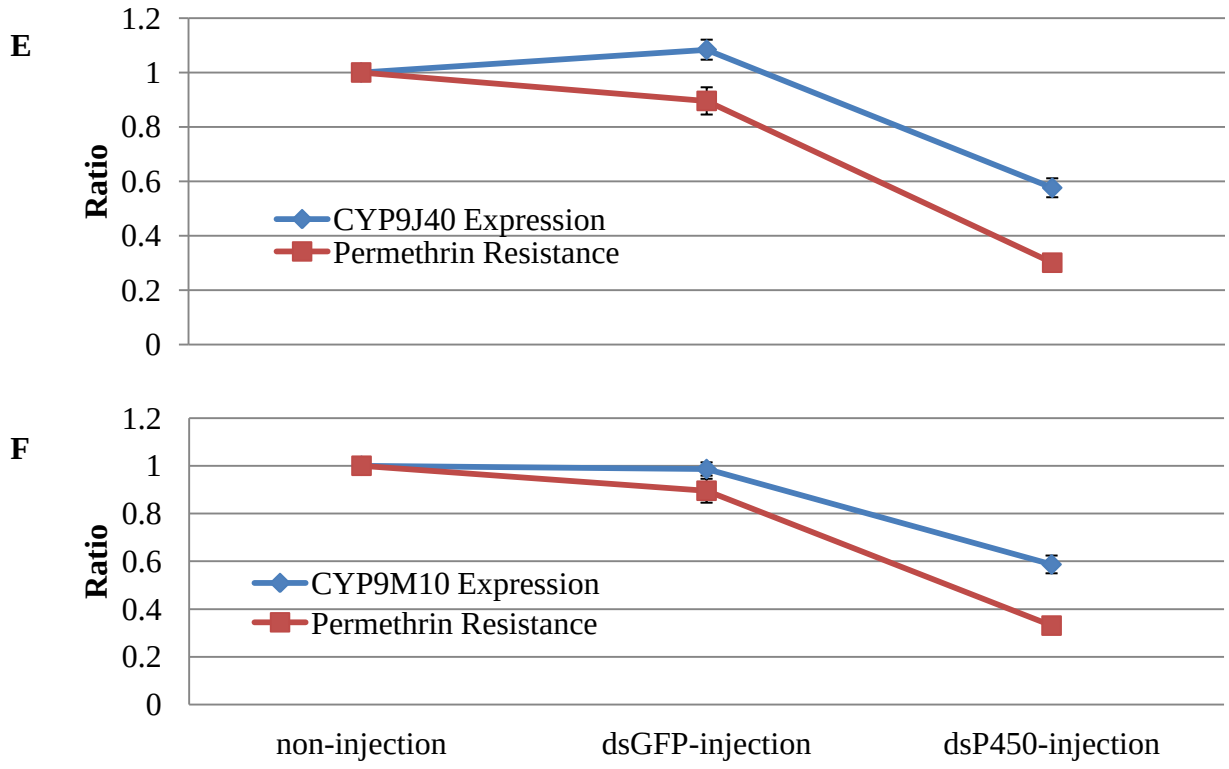

Figure 6.1 Differential gene expression ratio and permethrin resistance ratio after knockdown of target P450 genes in *Cx. quinquefasciatus* resistant strain HAmCq^{G8} larvae. The target gene expression ratio represents the level of target gene expression in dsGFP-injected and dsP450-injected mosquitoes compared with that in non-injection mosquitoes. The relative level of gene expression for non-injection mosquitoes is 1. The permethrin resistance ratio represents the resistance level of dsGFP-injected and dsP450-injected mosquitoes compared with that in non-injection mosquitoes to permethrin. The resistance ratios were calculated by comparing LC_{50} values obtained with dsGFP-injected and dsP450-injected mosquitoes with the LC_{50} values obtained with non-injection mosquitoes. The permethrin resistance ratio for non-injection mosquitoes is 1.The data shown are the mean ± SEM ($n \geq 3$). A. *CYP4C52v1*. B. *CYP6AA7*. C. *CYP6P14*. D. *CYP9J34*. E. *CYP9J40*. F. *CYP9M10*.

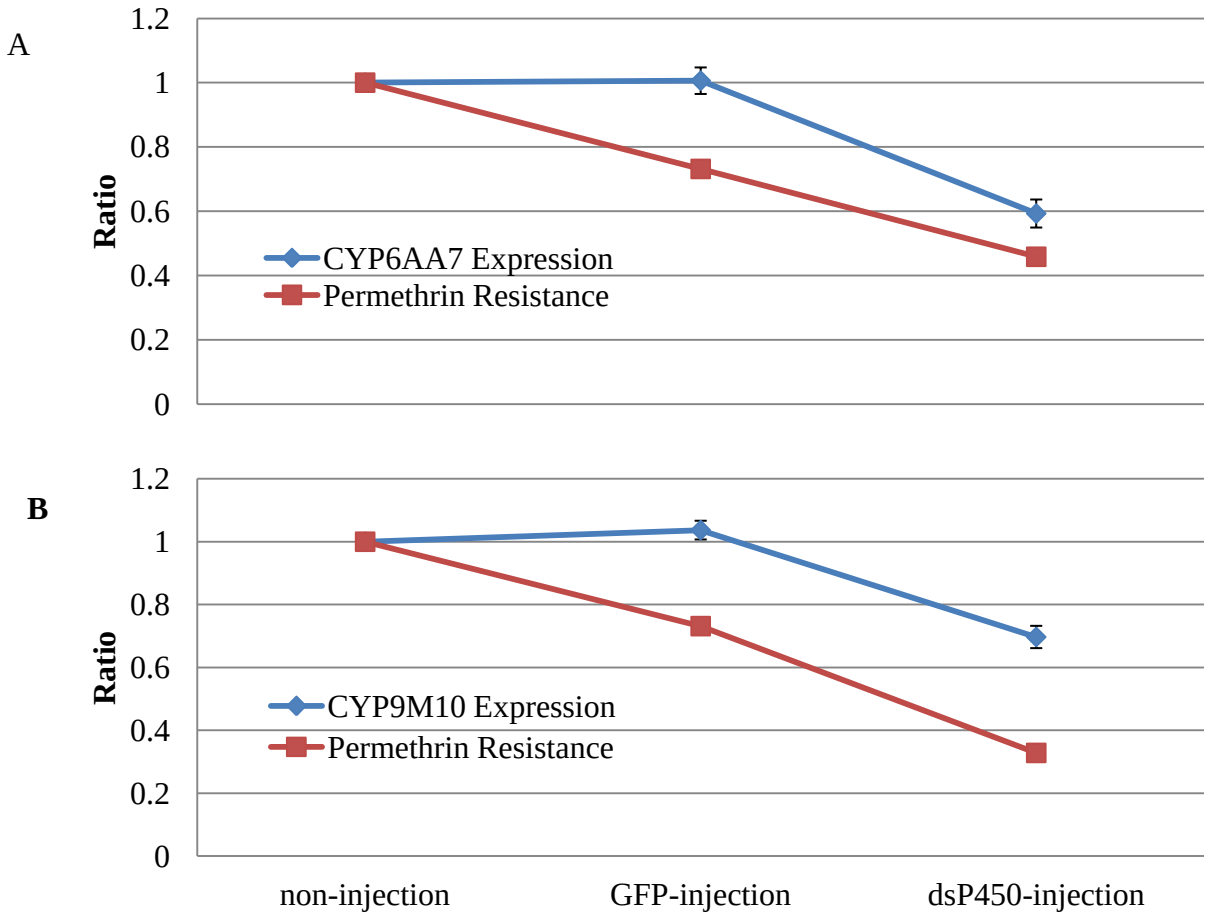

Figure 6.2 Differential gene expression ratio and permethrin resistance ratio after knockdown of target P450 genes in *Cx. quinquefasciatus* resistant strain MAmCq^{G6} larvae. The target gene expression ratio represents the level of target gene expression in dsGFP-injected and dsP450-injected mosquitoes compared with that in non-injection mosquitoes. The relative level of gene expression for non-injection mosquitoes is 1. The permethrin resistance ratio represents the resistance level of dsGFP-injected and dsP450-injected mosquitoes compared with that in non-injection mosquitoes to permethrin. The resistance ratios were calculated by comparing LC_{50} values obtained with dsGFP-injected and dsP450-injected mosquitoes with the LC_{50} values obtained with non-injection mosquitoes. The permethrin resistance ratio for non-injection mosquitoes is 1.The data shown are the mean ± SEM ($n \geq 3$). A. *CYP6AA7*. B. *CYP9M10*.

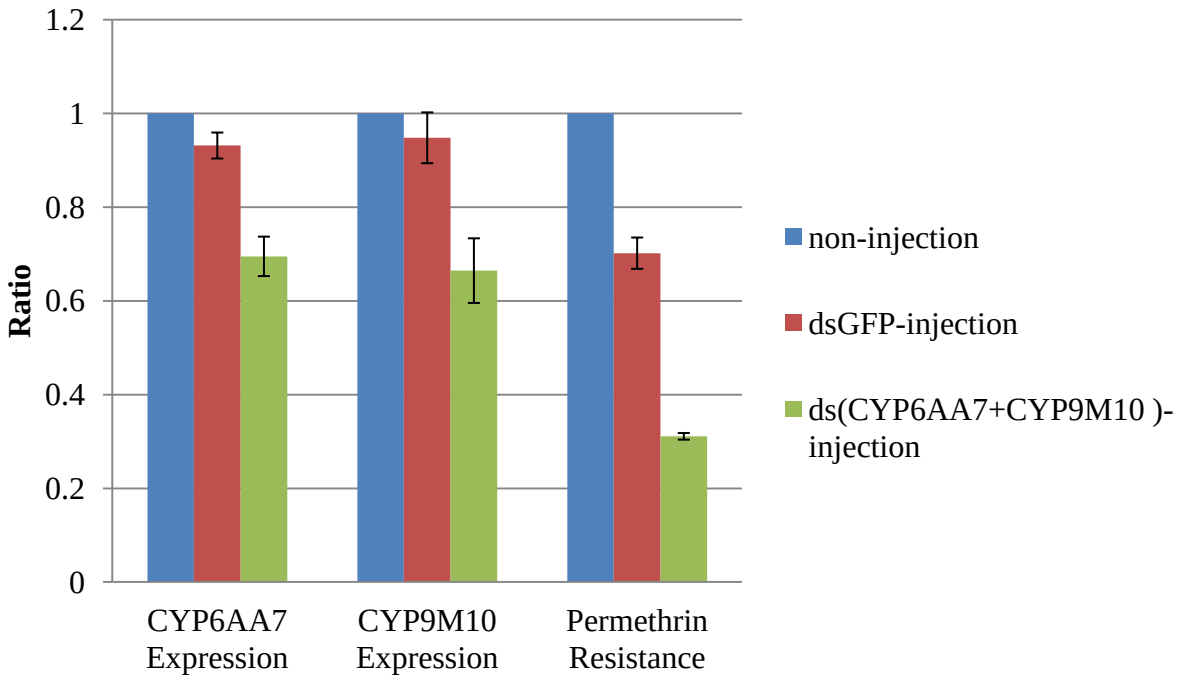

Figure 6.3 Differential gene expression ratio and permethrin resistance ratio after knockdown of the combination of two P450 genes *CYP6AA7* and *CYP9M10* in *Cx. quinquefasciatus* resistant strain HAmCq^{G8} larvae. The gene expression ratio represents the level of gene expression in dsGFP-injected and ds(*CYP6AA7*+*CYP9M10*)-injected mosquitoes compared with that in non-injection mosquitoes. The relative level of gene expression for non-injection mosquitoes is 1. The permethrin resistance ratio represents the resistance level of dsGFP-injected and ds(*CYP6AA7*+*CYP9M10*)-injected mosquitoes compared with that in non-injection mosquitoes to permethrin. The resistance ratios were calculated by comparing LC_{50} values obtained with dsGFP-injected and ds(*CYP6AA7*+*CYP9M10*)-injected mosquitoes with the LC_{50} values obtained with non-injection mosquitoes. The permethrin resistance ratio for non-injection mosquitoes is 1.The data shown are the mean ± SEM ($n \geq 3$)

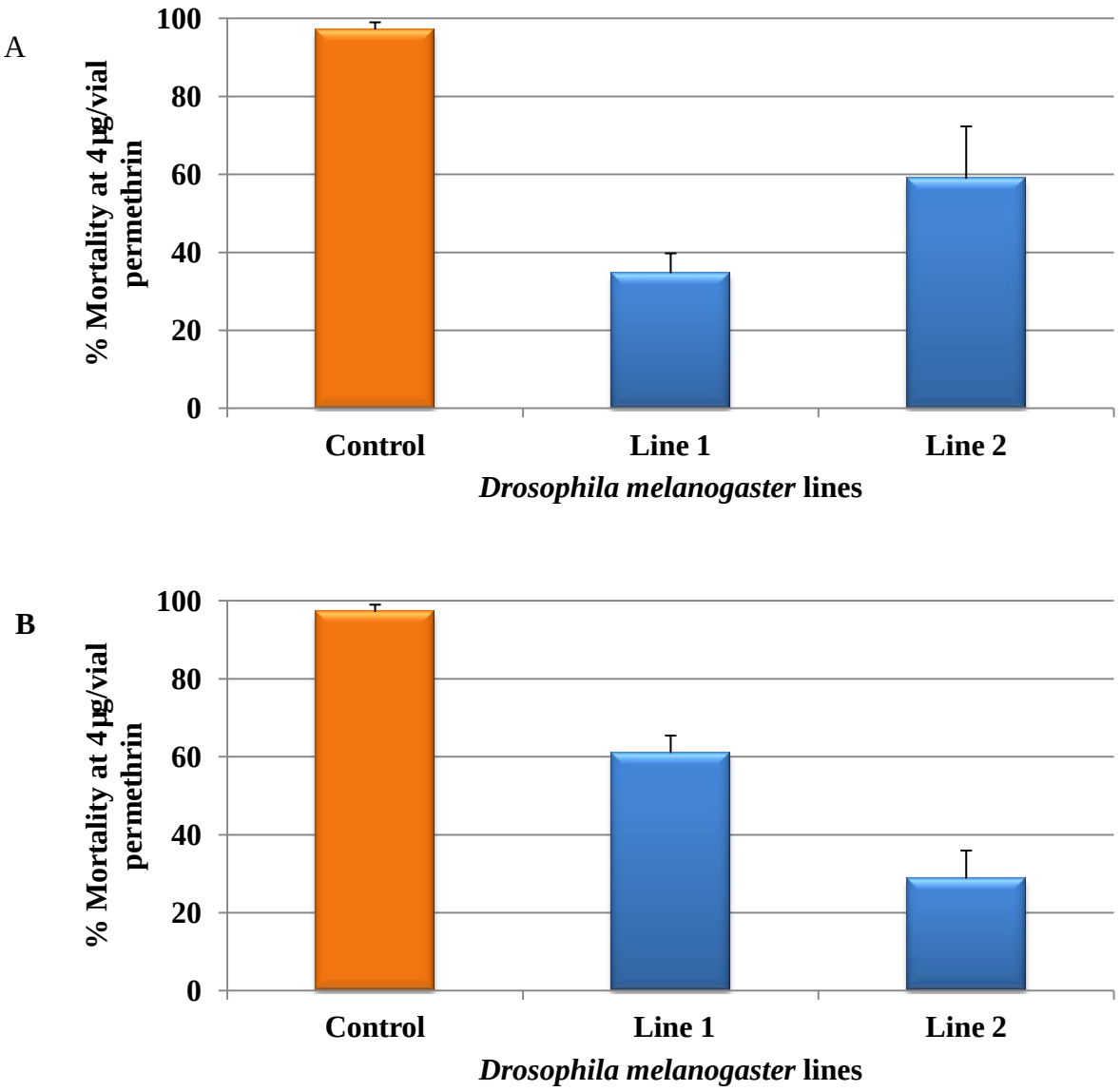

Figure 6.4 The effects of *Cx. quinquefasciatus* P450 genes on the tolerance of transgenic *D. melanogaster* to permethrin. The percent mortality of control strain and two transgenic lines exposed to 4 µg permethrin/vial. The data shown are mean + SEM ($n \geq 3$). A. *CYP6AA7* transgenic lines B. *CYP9M10* transgenic lines

Table 6.1 Up-regulated cytochrome P450 genes, primers used for qRT-PCR, dsRNA synthesis, and transgenic expression

Transcript ID[a]	Accession No.	Gene[b]			Primer (5′ to 3′)[c]
CPIJ010543	XM_001855188.1	CYP9J40	qRT-PCR	Forward	AAAGGTACCCTGAAGCACCAGGAA
				Reverse	TCCAGCCAGGAAGAAGATCAAGCA
			dsRNA	Forward	TAATACGACTCACTATAGAGCTAATCAAGAA GGTCGCCGTGA
				Reverse	TAATACGACTCACTATAGATGTCCGGTCGAA TGATTCCGTGA
CPIJ005955	XM_001847351.1	CYP6P14	qRT-PCR	Forward	AAGGTGGAACCAGGTCTGACGATT
				Reverse	CCATCATTAGCCGCGATTGCCTTT
			dsRNA	Forward	TAATACGACTCACTATAGCAACTCGCTTAAA CAGCGCAACCT
				Reverse	TAATACGACTCACTATAGTTGGCTAGCTGAA ACGGTGTTTGC
CPIJ012470	XM_001862711.1	CYP9AL1	qRT-PCR	Forward	TGAACGTCCTTAGGGATGGCGAAA
				Reverse	TTGCTAGTCGCGGAAACGAACTGA
			dsRNA	Forward	TAATACGACTCACTATAGGGACCCACGACGA CTTCGTATC
				Reverse	TAATACGACTCACTATAGGGTCGATTACCCA AGCATAGCC
CPIJ010546	XM_001855208.1	CYP9J34	qRT-PCR	Forward	AGTGTTCGGGATGATGGATATG
				Reverse	GGATGATCGTACTCGGGATTTC
			dsRNA	Forward	TAATACGACTCACTATAGGGGATCGTCGAGT GCAGTGAAA
				Reverse	TAATACGACTCACTATAGGGACACGGTATCA AAACCAGCC
CPIJ010537	XM_001855163.1	CYP9J45	qRT-PCR	Forward	ACCCACAGTACTTCCCAGA
				Reverse	CCGACAGCACCAACCTAAA
			dsRNA	Forward	TAATACGACTCACTATAGGGGCGGGACTACA ACAACGAAT

				Reverse	TAATACGACTCACTATAGGGCATGTTGGGGGACCACTTAC
CPIJ014218	XM_001864262.1	CYP9M10	qRT-PCR	Forward	TTTGACCACTTTGCGGATCACACG
				Reverse	AGATGAGGAACATGTTTGCGCTGC
			dsRNA	Forward	TAATACGACTCACTATAGAACAACGACCGTCATCTGCTTTGC
				Reverse	TAATACGACTCACTATAGGCTTGTTCAGCTTGATCGGCACAT
			transgenic expression	Forward	CCGGAATTCCAAAATGACCTCACTCGAGTGGTTGCT
				Reverse	CTAGTCTAGATTTCTTCATCTCCCCTCTCGGAAC
CPIJ010548	XM_001855214.1	CYP9J39	qRT-PCR	Forward	AGGTACTTGTGTGTGGTTTCCGGT
				Reverse	TGTTTGTTCTCATCGCTGAACCGC
			dsRNA	Forward	TAATACGACTCACTATAGGGTTCCGGTTCACGGAATACAC
				Reverse	TAATACGACTCACTATAGGGTTGCGCTTCACCAGTTGTA
CPIJ005959	XM_001847355.1	CYP6AA7	qRT-PCR	Forward	AAGGTTCGGGTTGAAGTCAGCTCT
				Reverse	TTGCGAGCTACGAAAGGAACTGGT
			dsRNA	Forward	TAATACGACTCACTATAGTACGAAGAACGGGAAGAAGCCGTT
				Reverse	TAATACGACTCACTATAGCCGGATTGTTGGCCAGTTCAAACA
			transgenic expression	Forward	CCGGAATTCCAAAATGTCTCTGCTGAACACGCTGCT
				Reverse	CTAGTCTAGACTCTTTGGAGTTGATTTTGTTCAC
CPIJ018943	XM_001869165.1	CYP4C52v1	qRT-PCR	Forward	AATGCCTCCAAGTTCTGCACTCCT
				Reverse	ATCGTTGTTGTTGTTGAGGTCGGC
			dsRNA	Forward	TAATACGACTCACTATAGCGGCAAAGATCGCAAATCTGCTCA

	Reverse	TAATACGACTCACTATAGTTCCAAGCTGTTC CTCCAGCTTCT

[a]The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence[http://c*quinquefasciatus*.vectorbase.org/]
[b]The annotation of the *Culex* P450 genes from http://drnelson.utmem.edu/CytochromeP450.html
[c]Specific primer pair designed according to each of the P450 gene sequences of the *Cx. quinquefasciatus* in vectorbase (http://c*quinquefasciatus*.vectorbase.org)

Chapter 7

Summary and Future Research Perspectives

7.1 Research summary

My doctoral research projects were mainly focused on the mechanisms involved in the resistance to pyrethroid insecticide in the mosquito, *Culex quinquefasciatus*. Specifically, my work focused on the investigation and characterization of cytochrome P450-mediated detoxification in high-level resistant *Culex* mosquitoes. I isolated 104 single-egg-raft colonies from a natural field mosquito population and examined the resistance levels for each of them. By evaluating the results of LC_{50} using insecticide bioassays, I found that most mosquitoes had evolved some degree of resistance to permethrin compared with our laboratory susceptible population. Furthermore, to determine the mechanisms conferring resistance in those mosquitoes, I identified the frequency of L-to-F *kdr* allelic expression of sodium channels, which are the target sites of pyrethroid insecticides, by SNP determination in randomly selected single-egg-raft colonies. In addition, Icharacterized the expression tendency of some up-regulated key P450 genes, which were reported to be related to insecticide detoxification, by qRT-PCR following different resistant levels of the mosquitoes bearing different *kdr* alleles. These results indicated that multiple mechanisms were involved in insecticide resistance and were also co-responsible for the development and evolution of insecticide resistance. There was a clear correlation between the prevalence of allelic expression of A or T in the L-to-F *kdr* locus at the RNA level and the level of their susceptibility or resistance to permethrin. Moreover, mosquitoes with higher levels of resistance to permethrin tended to have higher expression levels of up-regulated P450 genes. Based on these findings, I then I concentrated my research on one of the major

mechanisms contributing insecticide resistance in mosquitoes, which is cytochome P450-mediated detoxification. I conducted genome analysis of a total of 204 P450 genes identified in the mosquito, *Cx. quiquefasciatus*, and compared expression profiles of P450 genes in both larvae and adult mosquitoes among susceptible population, field parental populations, and resistant offspring populations. My findings demonstrated that multiple P450 genes were involved in insecticide resistance, also multiple mechanisms were involved in P450 gene regulation, and that both up- and down regulation of P450 genes might be co-responsible for the detoxification of insecticides. To clarify the function of key P450 genes that were considered to play important role in detoxification of insecticides, double-stranded RNA-mediated gene interference (RNAi) techniques and *Drosophila melanogaster* techniques were introduced into gene functional studies. Interestingly, silencing expression of up-regulated genes in mosquitoes resulted in a reduction of resistance. Increased levels of tolerance to permethrin in transgenic *Drosophila* lines were also found. These results from my dissertation research clearly demonstrated that multiply mechanisms co-confer insecticide resistance and that up-regulation of P450 genes play important roles in insecticide detoxification. My data provide us new insight into the mechanism of insecticide resistance which will be important for the future studies on characterization of P450 gene regulation.

7.2 Future studies

According to the findings demonstrated in my doctoral researches, which are the characterization of total 204 P450 gene expression profiles and functional studies of up-regulated key P450 genes, I concluded that cytochrome P450s are very important for insecticide detoxification, insecticide resistance development and insecticide selection by up-regulating their

expression levels. However, the regulatory elements of up-regulated P450 genes which are mostly found upstream from the promoter regions and are responsible for binding regulatory proteins are still unknown. For my future studies, I will focus on characterization of the molecular mechanisms involved in regulation of P450 gene expression associated to insecticide resistance in *Culex* mosquitoes. Electrophoretic mobility shift assay (EMSA), also known as gel shift assay, will be applied for investigation and determination of the regulatory elements of up-regulated P450 genes (Figure 7.1).

First, I will compare the promoter region sequences of target up-regulated P450 genes between susceptible and resistant mosquitoes to determine the locus/location where the nucleotides are different. The promoter sequences showing the difference between susceptible and resistant mosquitoes, which are considered as candidate regulatory elements, then will be selected for further investigation of their significance in the interaction with transcriptional regulatory factors, which can be extracted from nuclear if exist. Several assumed fragments from the promoter region of up-regulated P450 genes will be designed to synthesize the radioactive probe DNA with both ends labeled [γ-^{32}P] ATP. After incubation for binding reaction of radioactive probe DNA and nuclear protein, electrophoresis and autoradiograph development will be performed. If the radioactive probe DNA can bind with the regulatory factors, the DNA-protein complex will be resolved by electrophoresis on a non-denaturing polyacrylamide gel and visualized by autoradiography by exposure to X-ray film with intensifying screens. In this way, I can determine the interaction between the regulatory elements at the promoter region of target P450 genes and the transcriptional regulatory factor by the mobility of the molecules and signal appearing on the film after autoradiography (Figure 7.1).

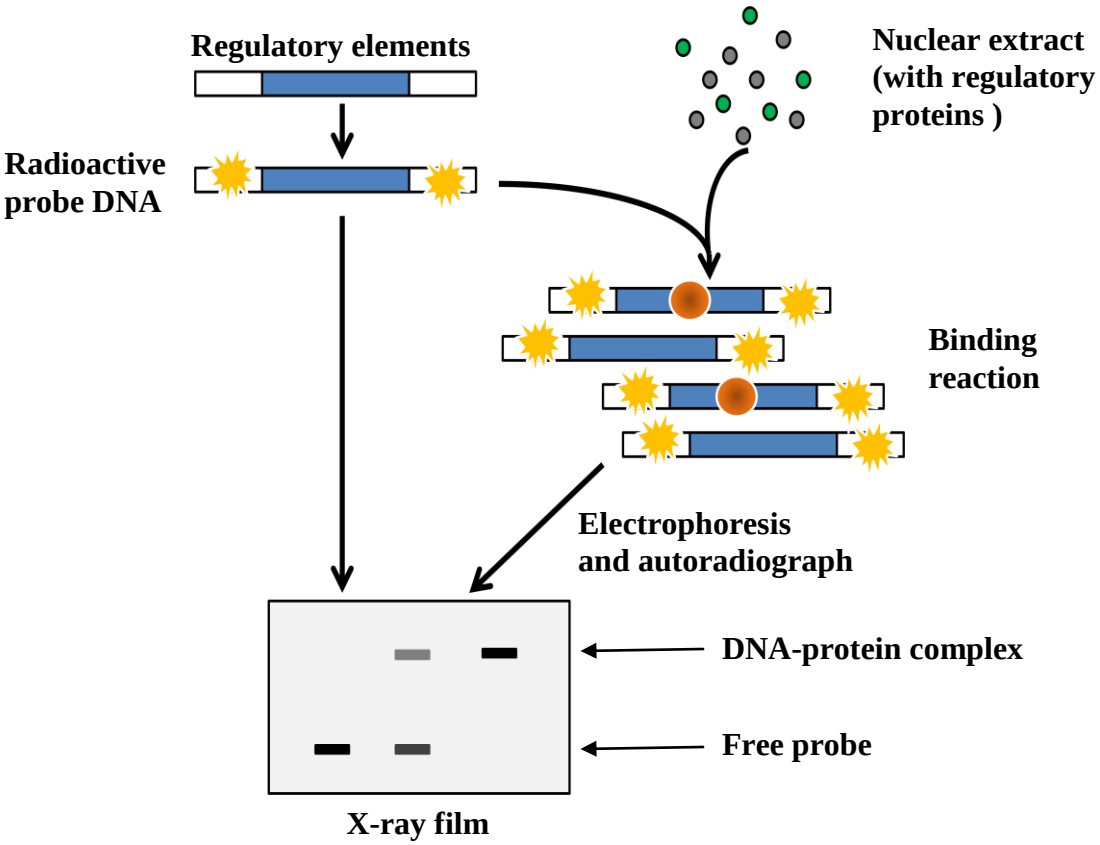

Figure 7.1 The scheme of Electrophoretic mobility shift assay (EMSA)