MOLECULAR CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF CYTOCHROME P450 GENES IN THE YELLOW FEVER MOSQUITO *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

by

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Abstract

Cytochrome P450 monooxygenases (P450s) are important enzymes involved in the metabolism of a variety of xenobiotics, including insecticides and plant allelochemicals, and endogenous compounds, including juvenile hormones, ecdysteroids and fatty acids, in insects. Despite rapid advances in revealing various P450 genes in insects, our knowledge on the role of these genes in detoxification of insecticides is very limited. This research was to perform a genome-wide analysis of P450 genes and evaluate the role of selected P450 genes in detoxification of three commonly used pyrethroid insecticides in the yellow fever mosquito (*Aedes aegypti*).

Our genome-wide analysis of revealed 159 P450 genes that can be classified into 18 families and 63 subfamilies. These genes are distributed in four clans, including 11 genes in the CYP2 clan, 80 in the CYP3 clan, 58 in the CYP4 clan and 10 in the mitochondrial CYP clan. The largest families are CYP6, CYP9, CYP4 and CYP325. The intron-exon organization of the genes is very diverse among the gene families, and the highest conservation of gene structures was observed in the CYP6 and CYP9 families predominantly containing single-intron genes. The phylogenetic analysis suggested that the CYP6 and CYP9 families might be derived from a common ancestor. The expression patterns of five transcripts including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* were investigated in various tissues and at different developmental stages of the mosquito. Our results indicated differential expressions of these transcripts in different tissues and at different developmental stages examined. Furthermore, the exposure of the mosquitoes (larvae and adults) to each of three pyrethroid insecticides (permethrin, cypermethrin and deltamethrin) resulted in either down or up-regulation of these transcripts.

Functional analyses of the selected P450 transcripts were conducted by using RNA interference (RNAi) followed by insecticide bioassay. RNAi was achieved by feeding mosquito larvae with chitosan/double stranded RNA (dsRNA) nanoparticles or injecting dsRNA to the adults. For the larvae, we obtained relatively low repressions of the P450 transcripts but the repressions were sufficient for carrying out our functional studies. Our study showed increased mortalities by 41.2% to cypermethrin when *CYP6AA5* was silenced and 46.0% to permethrin when *CYP9J32* was silenced. Similarly, the injection of dsRNAs in adults resulted in significant repressions of the P450 transcripts, and subsequent insecticide exposures led to a 29.3% increase in the adult mortality to cypermethrin when *CYP6AA5* was silenced. Our further analysis of the nuclear receptor *HR96* in the up-regulation of the P450 genes showed that when *HR96* was silenced by RNAi, the up-regulation of *CYP4J16B* by cypermethrin was reduced by 10.1-fold but silencing *HR96* did not affect the up-regulation of other P450 genes examined. These results suggest that *HR96* is likely involved in regulating the expression of *CYP4J16B* in *Ae. aegypti*. However, different regulatory mechanism (s) may be involved in the up-regulation of other P450 genes examined.

Model structure of CYP6AA5 was created by homology modeling and insecticides substrates were docked into the active site of this protein . Our results indicate that all three insecticides can fit into the catalytic pocket. The interaction distances between the heme iron and the putative aromatic hydroxylation site were 9.2, 9.4 and 7.2 Å for permethrin, cypermethrin and deltamethrin, respectively, whereas for aliphatic hydroxylation site these distances were 5.3, 2.8 and 2.9 Å. These results showed that CYP6AA5 may be able to metabolize cypermethrin and deltamethrin preferentially by aliphatic hydroxylation as indicated by the close interaction with the heme iron.

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Dedication

This thesis is dedicated to my parents and my entire family for their loving support and encouragements.

Chapter 1 - Literature Review

Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (P450s) represent a large superfamily of enzymes involved in many metabolic pathways and found in almost all kingdoms of life, from bacteria to protists, plants, fungi, animals (Werck-Reichhart and Feyereisen, 2000; Feyereisen, 2012) and even in some virus (Lamb et al., 2009). These enzymes are extremely important because of their involvement in the detoxification of xenobiotics, such as drugs, pesticides, and plant toxins; and in the regulation of endogenous compounds such as hormones, fatty acids, and steroids (Scott, 1999).

A brief history

Cytochrome P450 enzyme was first reported in 1958 by Garfinkel and Klingenberg when they independently observed a spectral absorbance peak at 450 nm in rat liver microsornes that had been exposed to carbon monoxide (Garfinkel, 1958; Klingenberg, 1958). Omura and Sato (1962) later showed that the protein responsible for this spectral property at 450 nm in rat liver microsomes was in fact a hemoprotein capable of binding molecular oxygen and temporarily named it cytochrome P450. It was later found that the term was inappropriate since these enzymes do not carry electrons, but rather function as oxidases (Coon et al., 1992). However, the term become widely used in the literature so that it is retained today. After this initial discovery, it was believed that there was a single P450 enzyme; however, subsequent studies have shown the presence of multiple isoforms in any given organism (Nelson and Nebert, 2011).

Classification and nomenclature

The term cytochrome P450 was given to the first enzyme found in rat liver microsomes (Omura and Sato, 1964), and subsequently used for all its isoforms (Estabrook, 1996). Several name have been used in the literature to designate P450 enzymes including cytochrome P450 monooxygenases, mixed function oxidases, polysubstrate monooxygenases, microsomal oxidases, and heme-thiolate proteins (Feyereisen, 2012). The classification of cytochrome P450s is based on amino acid sequence identity. Those sequences having more than 40% identity are grouped as a family and those having more than 55% identity as a subfamily (Nebert et al., 1987, 1991). The newly discovered P450s are named by the Cytochrome P450 Nomenclature Committee and deposited in the P450 database at the University of Tennessee (http://drnelson.uthsc.edu/CytochromeP450.html).

The genes encoding P450 enzymes and the enzymes themselves are designated by the abbreviation CYP (stands for cytochrome P450) followed by a numeral number corresponding to the gene family. This family number may be associated with the function of the enzyme as in CYP21, where the enzyme acts as a steroid 21-hydroxylase, or it may have been chosen arbitrarily. The family name is followed by a capital letter that designates the subfamily and another numeral number to designate the individual gene or enzyme. For instance the gene *CYP9J32* is described in Figure 1.1. However, some P450 gene or enzyme names may differ from this nomenclature, denoting the catalytic activity and the name of the compound used as substrate. For instance, the gene *CYP5A1*, responsible for thromboxane A2 synthesis, is abbreviated by *TBXAS1* (ThromBoXane A2 Synthase 1).

As many divergent families of P450 enzymes from various organisms were being named, it became apparent that a higher level of organization was needed. For this, it has been suggested that P450 families be grouped into clans. According to this scheme, families of P450s that

consistently grouped together on phylogenetic tree of P450 from different species be grouped in the same clan (Nelson, 1998). In general, insect P450 enzymes are classified into four clans that are named after the closely related families in vertebrates (CYP3, CYP4, CYP2 clans) or their subcellular locations (mitochondrial CYP clan) (Feyereisen, 2012).

Structure and diversity

The recent advancement in genome sequencing technology has tremendously increased the number of known P450 enzymes. Currently, more than 21,000 sequences of P450 genes from all kingdoms of life have been deposited in the P450 database (http://drnelson.uthsc.edu/CytochromeP450.html). All P450 enzymes exhibit similarity in their structure and general mechanism of action. In general, five conserved motifs are found in their protein sequences. These include the C-helix motif (WxxxR) located at the C-terminal, followed by the Helix I motif (GxE/DTT/S), the motif (ExLR) in the K-helix, the PERF motif (PxxFxPE/DRF) also located in the K helix, and the heme-binding motif (PFxxGxRxCxG/A), which contain a cysteine residue involved in the binding of heme iron in the fifth coordination site (Feyereisen, 2005). The heme is a prosthetic group consisting of an iron ion coordinated by four nitrogen atoms of porphyrin, and is linked to the apoprotein via a conserved cysteine residue (Urlacher and Girhard, 2012), although some P450 enzymes lack this residue (Sezutsu et al., 2013). The heme binding motif is the most conserved portion of the protein, and often considered as signature sequence for P450 enzymes.

A common feature of the substrates for P450 enzymes is their hydrophobic nature, where the enzymes act as a monooxygenase to insert an oxygen atom into the substrate thereby increasing its water solubility. This is toxicologically critical for survival due to the fact that an organism must quickly ride its body of toxic chemicals before they reach their target sites, by

rendering them more water soluble and less reactive so that they can be easily excreted. It is important to note that not all P450 enzymes carry monooxygenation reactions. For instance, in the fungus *Fusarium oxysporum,* CYP55A1, a key enzyme involved in the reduction of nitric oxide into nitrous oxide, does not have monooxygenase activity (Shoun and Tanimoto, 1991). Similarly, not all monooxygenases are P450s enzymes (Mansuy and Renaud, 1995). In general monooxygenases are enzymes that catalyze the insertion of a single oxygen atom into their substrates, and there are many types of monooxygenases depending on which cofactor is present in the enzyme, or whether none is present (Torres Pazmiño et al., 2010).

The accumulation of information about P450s form different organisms reveal that these enzymes can be grouped into three different categories based on their subcellular localization, including microsomal, mitochondrial and cytosolic (Fulco, 1991; Feyereisen, 2012). Most of the mammalians P450s are microsomal, embedded in the membrane of the smooth endoplasmic reticulum. A few number of P450 enzymes are found the inner membrane of mitochondria. They represent the majority of eukaryote P450 enzymes. Mitochondrial P450s on the other hands are typically found embedded in the inner membrane of the mitochondria. These two groups of P450s are specific of eukaryotic organisms. In prokaryotic organisms all P450 enzymes are soluble and found in the cytoplasm (Feyereisen, 2012; Lamb and Waterman, 2013).

Catalytic mechanism

The main function of P450 enzymes is to activate molecular oxygen to yield a reactive species that can attack functional groups of relatively inert chemicals in order to introduce hydroxyl groups into these structures (Feyereisen, 2012). The generally accepted catalytic mechanism for the P450 catalytic cycle (Figure 1.2) derived from works on bacterial and mammalian P450 enzymes (Ortiz de Montellano, 2005). The P450 enzyme (P450-Fe^{3+}) binds to

the inert substrate (RH) through the ferric heme group to form a complex of P450-substrate (P450- Fe^{3+}-RH). The complex P450-RH receives a single electron from a redox partner (P450 reductase or adrenodoxin) to form ferrous P450 (P450-Fe^{2+}) which then binds molecular oxygen. A second electron is provided to the complex either by P450 reductase or in some cases cytochrome b5, or from adrenodoxin to into water molecule. The basic stoichiometry of a P450-catalyzed hydroxylation reaction is represented by the following equation:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

Function of cytochrome P450 monooxygenases in insects

In insects, P450 enzymes play important functions in metabolizing various endogenous compounds such as ecdysteroid hormones (ecdysone, juvenile hormone) involved in the regulation of insect metamorphosis, development and reproduction (Helvig et al., 2004a; Rewitz et al., 2007). P450 enzymes are also responsible for the metabolism of fatty acids (Helvig et al., 2004b), pheromones and many other signaling molecules that are critical for insect communication and defense (Maibeche-Coisne et al., 2004). Moreover, insect P450 enzymes are responsible for the metabolism of xenobiotics (insecticides and plant allelochemicals), resulting in detoxification in most cases.

Metabolism of endogenous compounds

P450 enzymes are involved in the metabolism of many biologically active compounds in insects. The insect molting hormone 20-hydroxyecdysone is responsible for triggering the molting process and many other developmental processes crucial for insect growth. The sequential steps that lead to the formation of this hormone are all catalyzed by a set of P450 enzymes known as Halloween genes (Feyereisen and Durst, 1978; Williams et al., 1997; Gilbert

et al., 2002). These genes include phantom coding for *CYP306A1*, disembodied coding for *CYP302A1*, shadow coding for *CYP315A1*, and shade coding for *CYP314A1*. Mutations of these genes in insects prevent the formation of normal cuticle resulting in lethality (Wieschaus et al., 1984). Similarly, juvenile hormone (JH) plays a critical role in insect development, metamorphosis, reproduction, and many other physiological processes (Sutherland et al., 1998; Helvig et al., 2004a).

The involvement of P450 enzymes in the metabolism of JH has been demonstrated in the giant cave roach *Blaberus giganteus* (Hammock, 1975) and the locust *Locusta migratoria* (Feyereisen et al., 1981). In the house fly *Musca domestica*, Andersen et al. (1997) have shown that CYP6A1 was able to metabolize JH I and JH III. Several P450 genes were found to be predominantly expressed in the antennae of several insect species. This suggests their probable role in olfactory function which is important for insect communication (Maibeche-Coisne et al., 2005; Ono et al., 2005).

Metabolism of xenobiotics

Insects encounter a wide variety of xenobiotics (insecticides, plant allelochemicals) in their environment, and P450s along with others enzyme systems (esterases, glutathione *S*-transferases) are largely responsible for metabolizing these toxic substances (Feyereisen, 2012). The detoxification of most classes of insecticides can be carried out by P450 enzymes, given their number in most insect species and their broad substrate specificity. The ability of several insect species to feed on toxins-producing plants is believed to be mainly conferred by P450s (Krieger et al., 1971; Berenbaum et al., 1990; Kumar et al., 2013).

Detoxification of insecticides: The function of insect P450 enzymes associated with detoxification of insecticides represents the most known function and has been widely reported

in insect species. P450-mediated detoxification of insecticides is an important resistance mechanism that can cause significant level of resistance to insecticides (Brattsten et al., 1977; Scott and Georghiou, 1986a) and cross-resistance to unrelated compounds due to the wide variety of substrates that cytochrome P450s can metabolize (Scott, 1991; 1993). Sometimes, other enzyme systems, such as esterases and glutathione *S*-transferases, intervene in the metabolic detoxification of insecticides (Hemingway et al., 2004; Feyereisen et al., 1989) leading to resistance. The basic principal of P450-mediated detoxification of insecticide is trough insertion of an oxygen atom into the structure of an insecticide, thereby increasing its solubility and subsequent elimination through feces or for further processing by other enzyme systems such as esterases and glutathione *S*-transferases (Feyereisen 1999).

Although P450-mediated metabolism results in detoxification in most cases, it can also result in insecticide activation, especially with certain organophosphates (Feyereisen, 1999; Scott, 1999). For instance, the substitution of sulphur by oxygen in organophosphorodithioates catalyzed by P450 enzymes will result in an increased toxicity of these insecticides due to the increased potency to inhibit acetylcholinesterases in insects. A typical example is the oxidative activation of dimethoate to omethoate leading to higher toxicity due to increased inhibitory potency of omethoate to acetylcholinesterase (Anderson and Zhu, 2004).

Metabolism of plant allelochemicals: Plants synthesize a broad range of secondary metabolites, including alkaloids and terpenoids that are toxic to herbivorous organisms and pathogens, and so act as defense chemicals. Insect P450 enzymes play important roles in the metabolism of these compounds (Cohen et al., 1992), resulting in most cases in detoxification and thereby in the adaptation to feeding on plants. Several studies have reported the involvement of P450 enzymes in the detoxification of plants toxins. Two species of swallowtails, *Papilio*

polyxenes and *Papilio multicaudatus*, are known to specifically feed on furanocoumarins-containing plants. These furanocoumarins are xanthotoxins known to crosslink with DNA strands and prevent the occurrence of transcription and replication (Berenbaum, 1978; Berenbaum et al., 1990). Scott et al. (1998) found that *CYP6B* expression could be induced in these swallowtail species by host plant toxins and in turn to metabolize these toxins, allowing these insects to adapt to furanocoumarins-containing host plants. Similarly, the tobacco hornworm *Manduca sexta*, feeds on nicotine-containing and excrete this nicotine through feces. Kumar et al. (2013) have shown that CYP6B46 in *Manduca sexta* redirects a part of ingested nicotine (0.65%) from the midgut into hemolymph, from which nicotine is exhaled through the spiracles as an antispider signal. Silencing this gene by plant-mediated RNA interference caused the larvae to become more susceptible to spider attacks. These results indicate that CYP6B46may be involved in the metabolism of nicotine.

Contribution to insecticide resistance

Insecticide resistance is functionally defined as the ability of an insect population to survive exposure to dosages of a given compound that are lethal to the majority of individuals of a susceptible lineage of the same species (Beaty and Marquardt, 1996). It is a dynamic and evolutionary phenomenon that arose as a result of the combination of genetic makeup and selection pressure by xenobiotics such as insecticides and allelochemicals. The phenomenon of resistance was first found in the San Jose scale *Quadraspidiotus pernicious* (Comstock) resistant to lime sulfur in 1908 (Melander 1914) and subsequently found in most target species for virtually all classes of insecticides (Zhu, 2008). Insect resistance to insecticides gained much attention in the mid-1940s following the introduction and widespread use of DDT and other

organic insecticides that transformed the issue to a more global agricultural and public health problem.

There are several mechanisms through which insects develop resistance to insecticides, including behavioral change, decreased penetration, target site insensitivity and increased metabolic detoxification (ffrench-Constant et al., 2004; Li et al., 2007). Among these mechanisms, increased metabolic detoxification is the most important, and is mainly conferred by three detoxification enzyme systems, including carboxylesterases, glutathione *S*-transferases and P450 enzymes (Scott, 1999; Hemingway et al., 2004; Feyereisen, 2012). Although it is possible that more than one of these enzymes act together in a given insect to metabolize a particular insecticide, there is growing evidence that the initial step of the metabolism of most insecticides usually requires processing by P450 enzymes, making them crucial in the detoxification process (Brattsten et al., 1977; Scott and Georghiou, 1985, 1986; Feyereisen 1999).

P450-mediated insecticide resistance was first reported by Eldefrawi et al. (1960) when they noted that carbaryl resistance in house flies can be reduced by the P450 inhibitor sesamex. Since then, many P450 enzymes and genes have been identified from insecticide resistant strains and their involvement in resistance has been well documented. In fact, the first insect P450 gene, *CYP6A1,* was cloned and sequenced from the diazinon resistant strain of house fly (Rutgers) (Feyereisen et al., 1989). The expression of *CYP6A1* in this strain 10-fold higher than that of a susceptible strain (Carino et al., 1994). In the Jpal-per strain of southern house mosquito *Culex quinquefasciatus,* resistance to permethrin was estimated to be 2,500-fold as compared with a susceptible strain. This high level of resistance was reduced to 43-fold by treatment with piperonyl butoxide, suggesting that P450 mediated detoxification was an important mechanism

in permethrin-resistant strain (Kasai et al., 1998). Synergism studies in *Blattella germanica* indicated that P450-mediated detoxification was responsible for the development of insecticide resistance (Wei et al., 2001).

Although both constitutive and overexpression of P450 genes are thought to contribute to the overall resistance to insecticides (Liu et al., 2012), enhanced metabolism by P450 enzymes mostly results from overexpression of specific P450 genes, leading to the production of increased amounts of the same enzymes. The mechanisms through which P450s are upregulated involve alterations of the regulatory regions of these genes or via indels or mutations in cis-acting elements (Liu, 2012). In house fly, for instance, Rutgers strain resistant to organophosphates and carbamates has been known for the upregulation of two P450 genes, *CYP6A1* and *CYP6D1*,due to mutations in negative trans-regulatory loci (loss-of-function mutation) on chromosome 2 (Carino et al., 1994; Liu and Scott 1996). The insertion of the transposable element, Accord in the 5' end of *CYP6g1*, leads to the upregulation of this gene in *Drosophila* (Catania et al., 2004). Another means of increased detoxification by P450 has been described as being a mutation in the coding sequence that leads to a more active enzyme. Amichot (2004) found that several mutations (Ar335 to Ser, Leu336 to Val and Val476 to Leu) in *CYP6AG2* gene of DDT-resistant strain of *Drosophila* led to higher catalytic activity.

Use of RNA interference to reveal the detoxification function of P450 genes

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism that targets the mRNA of a specific gene for destruction, thereby, preventing the formation of functional gene product (usually a protein) (Fire et al., 1998). When a long double-stranded RNA (dsRNA) is introduced into a cell, it is recognized and cleaved by dsRNA-specific endonuclease called dicer into short fragments (21 bp) known as small interfering RNAs (siRNAs). A siRNA is

assembled into a complex called RISC (RNA-induced silencing complex) by an enzyme called argonaute. This complex mediates the sequence-specific degradation of targeted mRNA (Price and Gatehouse, 2008). This natural and conserved mechanism was reported for the first time in the nematode, *Caenorhabditis elegans* (Fire et al., 1998), and is now known in various organisms including fungi, plants, insects and mammals (Mello and Conte, 2004).

RNAi techniques have been widely used in recent years to address the questions of gene function, regulation and interaction at cellular and organismal levels in various organisms particularly in insects (Zhu, 2013). RNAi has been used to study the role of P450 genes in the detoxification of insecticides in many insect species, and present a real advantage in identifying P450 genes contributing to the detoxification of insecticides (Bautista et al., 2009; Mao et al., 2007; Guo et al., 2012; Tang et al., 2012).

In the diamondback moth, *Plutella xylostella*, *CYP6BG1* was found to be overexpressed in fourth instars of a permethrin-resistant strain and inducible in the susceptible one. To investigate the possible role of this gene in the detoxification of permethrin, Bautista et al. (2009) used dsRNA droplet feeding to knockdown *CYP6BG1* in the larvae and exposed them to permethrin. They observed a reduction in LC_{50} values in dsRNA-fed larvae compared to the control. Moreover, resistance was reduced by 61.4% and 53.0%, after exposure to permethrin for 24 and 48 h, respectively. These results showed the involvement of *CYP6BG1* in the detoxification of permethrin. In another study, Guo et al. (2012) silenced *CYP409A1* and *CYP408B1* in the nymphs of *Locusta migratoria* by injection of dsRNA. Subsequent exposure of injected locusts to deltamethrin led to increased susceptibility of the insect to this insecticide by 21.1% and 16.7% when *CYP409A1* and *CYP408B1* were, respectively, silenced. This result clearly showed the involvement of these genes in the detoxification of deltamethrin.

Furthermore, silencing *CYP6AE14* by plant-mediated RNAi in the cotton bollworm, *Helicoverpa armigera*, a serious agricultural pest, has shown to impair larval tolerance of gossypol (Mao et al., 2007).

Studies by Zhang et al. (2010) have shown that overexpression of *CYP6B7* was associated with enhanced fenvalerate resistance in *H. armigera*. In order to determine the role of *CYP6B7* in the detoxification of this insecticide, RNAi-mediated knockdown of this gene alone or along with cytochrome b5, a component of P450 enzyme system, was performed in larvae of fenvalerate-resistant strain (HDFR) (Tang et al., 2012). Exposure to fenvalerate after knockdown of *CYP6B7* or cytochrome b5led to increased susceptibility to this insecticide, indicating the involvement of *CYP6B7* and cytochrome b5 in the detoxification of fenvalerate.

The yellow fever mosquito (*Aedes aegypti*) and cytochrome P450

Aedes aegypti (Linnaeus), commonly known as the yellow fever mosquito belongs to the subfamily Culicinae in the family Culicidae. It is believed to have originated from central Africa, where it is found in greatest abundance (Smith, 1956; Cheong, 1986). Currently, this mosquito is globally present in all tropical and subtropical regions. Its presence in the Americas was probably through the European exploration and colonization (Nelson, 1986). *Ae. aegypti* is an efficient vector of arboviruses, causing diseases (dengue fever, yellow fever and chikungunya) to human. Dengue fever is the most serious threat to public health with over 2.5 billion people at risk in more than 100 countries (WHO, 2008). While there is a protective vaccine for yellow fever, the fight against dengue fever and chikungunya relies heavily on the use of insecticides to control the mosquitoes. However, these control efforts are hampered by the development of resistance in *Ae. aegypti* to nearly all classes of insecticides used. Among many different

resistance mechanisms, P450s enzymes play important roles in detoxification of insecticides in *Ae. aegypti* (Strode et al., 2008; Vontas et al., 2012).

Biology of Aedes aegypti

As a holometabolous insect, *Ae. aegypti* has a complete metamorphosis with four life stages, including egg, larva, pupa and adult (Goma, 1966). The egg, larvae and pupae are aquatic, whereas adults are terrestrial. The adults usually live from two weeks to a month depending on the environmental conditions. Females are larger than males, and they both feed on nectar of plants; although female need to feed on blood in order to produce eggs. After a blood meal, females produce on average 100 to 200 eggs per batch depending on the size of the blood meal. Usually they are laid in several places (Clements, 1999) on damp surfaces in areas likely to be temporarily flooded, such as tree holes and man-made containers. The eggs are white in color and soft when they are newly laid. Within minutes, the eggs turn to shiny black and become hardened (Schlaeger and Fuchs, 1974; Christophers, 1960). They can survive desiccation for long periods of time and easily hatch once submerged in water. They usually hatch in instalments and may require repeated immersions in water followed by short periods of desiccation (Gillett, 1951).

The larvae in general undergo four larval sub-stages called instars that require 10 days to complete, although variations occur depending on the diet, temperature and relative humidity. Each instar is terminated by shedding the older cuticle. Larvae feed on organic matter in the water, such as algae and other microscopic organisms. They breathe oxygen by means of a siphon, which is held above the water surface while the rest of the body hangs vertically. The pupal stage is usually short, lasting 1 to 3 days depending on the environmental conditions. The

pupae do not feed but are active, swimming in the water, in contrast to the pupae of other insect species that are typically inactive.

Current research in Aedes aegypti cytochrome P450

Current research on *Ae. aegypti* P450s virtually focused on their roles in insecticide resistance. The molecular basis of insecticide resistance in mosquitoes has been established (Hemingway et al., 2004), and P450 have been described as major enzymes contributing to rapid detoxification of insecticides. Studies have shown the overexpression of P450 genes and their elevated activities in insecticide resistant strains of *Ae. aegypti* in various parts of the world (Poupardin et al., 2008, 2010; Strode et al. 2008; Marcombe et al., 2009, 2012).

The expression of many P450 genes in insects is highly inducible. They have been found to be induced not only by insecticides but also by heavy metals (Marcombe et al., 2009). However, the up-regulation of P450 genes is often mistaken as the proof of detoxification of insecticides although it is known that P450s cannot detoxify heavy metals. As a result, none of the *Ae. aegypti* P450 genes has been ascertain to detoxify insecticides to date. Rather, speculations are based on the general view that P450 are involved in detoxification of xenobiotics.

Research needed in Aedes aegypti cytochrome P450

Despite the annotation of *Ae. aegypti* P450s, little research has been done to understand their structures and biological functions as compared with research on other insect species. There is limited knowledge about which P450s are responsible for insecticide resistance in *Ae. aegypti* and in insects in general. There is an urgent need to functionally characterize P450 genes in *Ae. aegypti*. Nevertheless, in vitro analysis of insect P450s is generally difficult because of their membrane-bounded nature, and the enzymes require electrons from P450 reductase and

sometimes cytochrome b5 for catalysis. Therefore, development and optimization of a proper expression system is needed to allow research to directly investigate the role of a given P450 in metabolizing a specific insecticide. There is also a need to look at other P450 functions beyond detoxification of xenobiotics.

The objectives of this study include:

1. To perform a genome–wide analysis of P450 superfamily in *Ae. aegypti*;
2. To analyze the expression level of a set of selected P450 genes in different developmental stages and tissues and the effect of insecticides on the expression as well as the role of nuclear receptor *HR96* in the up-regulation of P450 genes by insecticides in larvae and adults of *Ae. aegypti*.
3. To performed functional studies of selected P450 genes for their role in the metabolism of pyrethroid insecticides.

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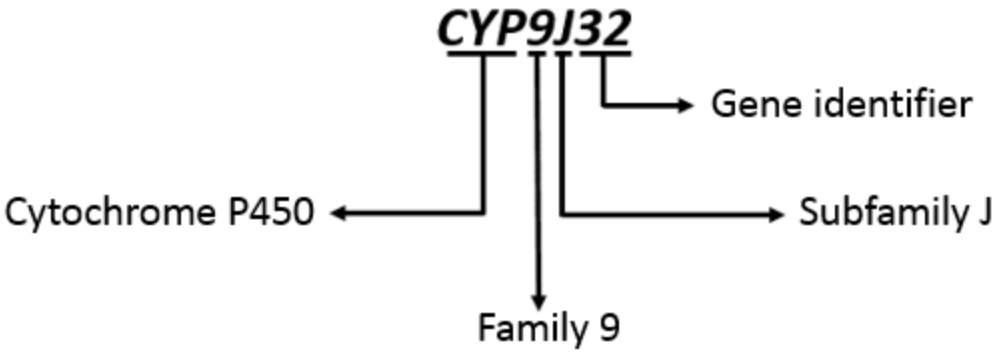

Figure 1.1 Scheme of the P450 nomenclature

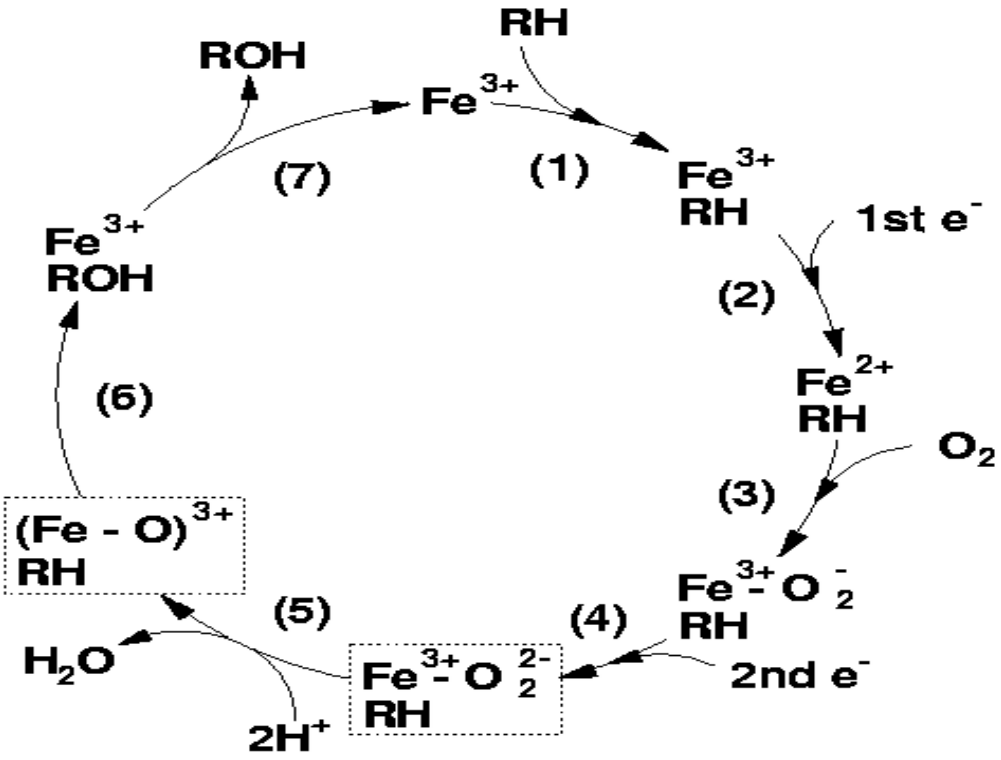

Figure 1.2 The catalytic cycle of cytochrome P450

Chapter 2 - Genome-wide Structural and Phylogenetic Analyses of Cytochrome P450 Superfamily in the Yellow Fever Mosquito *AedesAegypti* (Diptera: Culicidae)

Abstract

Cytochrome P450 monooxygenases (P450s) form a large family of enzymes are known to play important roles in the metabolism of xenobiotics and endogenous compounds in various organisms. Analysis of *Aedes aegypti* genome revealed 159 P450 genes, among which five are predicted to be pseudogenes probably devoid of functional activity. These genes are classified into 18 families and 63 subfamilies, which are distributed in four clans, including CYP2, CYP3, CYP4 and mitochondrial CYP clans with 11, 80, 58 and 10 genes respectively. The largest families included CYP6, CYP9, CYP4 and CYP325. The intron-exon organization of the genes was very diverse among the gene families. The highest conservation of gene structure was observed in the CYP6 and CYP9 families predominantly with single-intron genes. The P450 superfamily members are scattered across the genome with several large clusters. Phylogenetic analyses indicate that the clan level organization is conserved except for the CYP2 and the mitochondrial CYP clans that cluster together, suggesting their common ancestry. The CYP6 and CYP9 families appear to be derived from a common ancestor. The involvement of these two families in detoxification pathway seems to support this notion. This study highlighted the characteristics and diversity of *Ae. aegypti* P450 superfamily, which can be used as ground work for further study of this supergene family in the mosquito.

Keywords: *Aedes aegypti*, cytochrome P450, intron-exon organization, phylogeny.

Introduction

Cytochrome P450 monooxygenases (P450s), representing a large superfamily of enzymes widely involved in metabolic pathways, are found in almost all living organisms from bacteria to protists, plants, fungi, animals (Werck-Reichhart and Feyereisen, 2000; Nelson, 2009) and even in some virus (Lamb et al., 2009). Insect P450 enzymes mediate the biosynthesis of many biologically active compounds such as ecdysteroid hormones (ecdysone, juvenile hormone) involved in the regulation of metamorphosis, development and reproduction (Helvig et al., 2004a; Rewitz et al., 2007). They are also involved in the metabolism of fatty acids (Helvig et al., 2004b), pheromones and many other signaling molecules that are critical for insect communication (Maibeche-Coisne et al., 2004) and defense (Kumar et al. 2013; Vlachou et al., 2005). These enzymes are mostly known in insects for their role in the metabolism of xenobiotics such as insecticides and plant allelochemicals (Feyereisen, 2012). In mosquitoes, P450s in conjunction with others enzyme systems such esterases and glutathione *S*-transferases are responsible for the metabolic detoxification of insecticides (Hemingway et al., 2004) leading to resistance.

The classification of P450s is based on amino acid sequence identity.Those sequences having more than 40% identity are classified as a family and those having more than 55% identity as a subfamily (Nebert et al., 1987; Nelson, 2004). The genes encoding cytochrome P450 enzymes and the enzymes themselves are designated by the abbreviation CYP (stands for cytochrome P450) followed by a numeral number corresponding to the gene family, a capital letter that designates the subfamily and another numeral number to designate the individual gene or enzyme. By convention the gene name is italicized as opposed to the enzyme of the same name.

With the growing number of the P450 sequences from different insects, it has been proposed that P450 families be classified into clans (Nelson, 1998).This scheme places all P450 families with a monophyletic origin into a single clan. P450s from vertebrates, plants, arthropods, bacteria and fungi have been classified into clans based on this scheme (Nelson, 1998; Nelson and Werck-Reichhart, 2011; Feyereisen, 2011). Insect P450s are classified into four clans, including CYP2, CYP3, CYP4 and mitochondrial CYP clans (Feyereisen, 2012).

In general there are five conserved motifs in P450 enzymes, although they vary depending on the genes and the species to which they belong. These motifs include the C-helix motif (WxxxR) located at the C-terminal, followed by the Helix I motif (GxE/DTT/S), the motif (ExLR) in the K-helix, the PERF motif (PxxFxPE/DRF) also located in the K helix, and the heme-binding motif (PFxxGxRxCxG/A), which contain a cysteine residue involved in the binding of heme iron in the fifth coordination site (Feyereisen, 2005).

The sequencing of *Ae. aegypti* genome has revealed that its size is five-fold larger than that of *Anopheles gambiae,* and this expansion is principally prominent in the gene superfamilies encoding odorant binding, cuticle domains and cytochrome P450 (Nene et al., 2007). The availability of the genomic data offers the opportunity to carry out a genome-wide study on a variety of aspects of interest related to this species. The purpose of this study was to perform genome-wide, comprehensive analyses of cytochrome P450 genes in the yellow fever mosquito. This study highlights the characteristics and diversity of cytochrome P450 genes in *Ae. aegypti*.

Materials and Methods

Sequence retrieval and analysis of cytochrome P450 genes

The cytochrome P450 sequences used in this study were downloaded from two databases, including VectorBase (https://www.vectorbase.org/), which has the most updated annotation of genes and the Cytochrome P450 Homepage (http://drnelson.uthsc.edu/CytochromeP450.html) which is the repository database for all named P450 protein sequences. In some cases the sequences of novel P450 genes from VectorBase were downloaded and used as queries to perform BLASTP and/or BLASTX searches against *Ae. aegypti* named P450 sequences available on the P450 blast server (http://blast.uthsc.edu/). This allowed the determination of the correct name of gene with its more updated annotation. The putative amino acid sequences were characterized using the PROSITE database (http://prosite.expasy.org/) for P450 signature motifs. Additionally, the Multiple Expectation Maximization for Motif Elicitation (MEME) server (http://meme.nbcr.net/meme/) was used to analyze the conserved motifs. The amino acid frequencies of each motif were generated in individual profile, and the sequence logos depicting the conservation of amino acids in each motif was obtained.

Intron mapping

In order to determine the intron-exon position and phases, the predicted amino acid sequences of P450s from *Ae. aegypti* were aligned with their corresponding genomic DNA sequences using the Wise2 program (http://www.ebi.ac.uk/Tools/psa/genewise/) and default parameters. The P450 gene structures displaying the intron/exon organization along with the intron phases were generated with the gene structure display server (http://gsds.cbi.pku.edu.cn/), using coding sequences and genomic sequences.

Phylogenetic analysis of cytochrome P450 enzymes

In order to infer the relationship among different P450 genes, phylogenetic analysis of the deduced amino acid sequences of all P450 genes from *Ae. aegypti* was carried out. Pseudogenes were excluded from the analysis. Multiple sequence alignments were performed using ClustalW through Molecular Evolutionary Genetic Analysis version 5 software (MEGA 5) (http://www.megasoftware.net/). Pairwise and multiple alignments were both performed using default parameters. The multiple sequence alignment was manually edited to remove the gaps. The neighbor-joining tree methods by P-distance (Saitou and Nei, 1987) was used to construct the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkand and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The missing data were treated by pairwise deletions of the gaps. The significance of the trees was tested using 3000 bootstrap replicates in each case.

Results and Discussion

Diversity of Aedes aegypti cytochrome P450 genes

The recent update of *Ae.aegypti* genome annotation has revealed the presence of 159 P450 genes, representing over 1% of the total number (15,419) of predicted protein coding genes (Nene et al., 2007). These P450 genes were classified into 18 families and 63 subfamilies. These gene families showed high diversities in both the number and the genomic organization. Many families consist of just a few genes, while others involved a large number of closely related genes. There are four majors P450 families, including CYP4, CYP325, CYP6, and CYP9 with 25, 35, 44 and 35 genes, respectively.

Five genes (*CYP6CA1P*, *CYP325X3P*, *CYP325E2P*, *CYP9J10-de2b* and *CYP9J10-delc*) are likely to be pseudogenes (Table 2.1), meaning that they do not encode functional proteins, although their significances were not investigated in this study. These pseudogenes were annotated based on the absence of a complete open reading frame due to the truncation of the genes and/or the presence of several stop codons in their open reading frames. Two genes (*CYP4J16* and *CYP325Y2*) are predicted to have two alternative splicing variants each. Alternative splicing is a regulated biological process resulting in one gene coding for multiple enzymes. This process is not very common in insect P450 enzymes (Feyereisen, 2012), albeit 35 P450 genes were found to have alternative splicing variants in *Drosophila* (Graveley et al., 2011).

CYP2 clan

The CYP2 clan of *Ae. aegypti* is composed of 11 enzymes grouped in seven families (CYP15, 18, 303, 304, 305, 306 and 307) and nine subfamilies. The CYP304 family has three subfamilies while the families 305 and 307 have two subfamilies in each . The remaining families has only one subfamily each. The families 15, 18, 306, 307 are involved in essential physiological functions (Feyereisen, 2012). The genes *CYP306A1* (phantom), *CYP307A1* (spook), *CYP307A2* (spookier), *CYP307B1* (spookiest) in this clan belong to a set genes called Halloween genes involved in the early steps of biosynthesis of the insect molting hormone 20-hydroxyecdysone (Feyereisen, 2006). Studies conducted by Rewitz et al. (2007) indicate that these genes are conserved in many holometabolous insects, suggesting their common ancestry. Similar number of P450 genes was also found in the CYP2 clans (Table 2.2) of *A.gambiae* (Raghavendra et al., 2012) and *C. quinquefasciatus* (Yang and Liu, 2011).The orthologous gene *CYP18A1, a* key enzyme responsible for steroid hormone inactivation in *Drosophila* (Guittard et

al., 2011), is present in *Ae.aegypti* and *C. quinquefasciatus* but not in *A.gambiae* (Feyereisen, 2006).

CYP3 clan

The CYP3 clan emerged as the largest CYP clan in three mosquito species with complete genome sequences available, namely *A. gambiae*, *C. quinquefasciatus* and *Ae*. *aegypti* (Table 2.2). It consisted of 80 genes scattered in the families CYP6, CYP9 and CYP329 in *Ae. aegypti*. This clan is mostly related to vertebrates CYP3 and CYP5 families (Feyereisen, 2012). The families CYP6 and CYP9 are the largest and account for half of the total number of P450 genes in the genome, while the CYP329 family has only one gene as observed in others dipteran insects.

Members of the CYP6 and CYP9 families are shown to be involved in the detoxification of xenobiotic and endogenous compounds as they were frequently found to be overexpressed in insecticide resistant insects (Berenbaum, 2002; Strode et al., 2008). In *Drosophila*, some members of the CYP3 clan have been found to be inducible by ecdysone (Le Goff et al., 2006). Although up-regulation of enzymes does not necessarily indicate their involvement in a particular process, it is possible that some members of the CYP3 clan play other physiological function beside xenobiotic metabolism.

The CYP6 family is the most expanded P450 family in *Ae. aegypti*. It consists of 44 genes, of which one gene (*CYP6CA1*) is predicted to be pseudogene. The members of the family are distributed into 19 subfamilies (F, M, N, P, S, Y, Z, AA, Ag, AH, AK, AL, BB, BY, BZ, CA, CB, CC and CD), with the subfamily N being the largest with 10 different isoforms. It represents the largest P450 family from the entire superfamily. This predominance of CYP6 family over

others was also observed in the P450 superfamily of *A. gambiae* (Raghavendra et al., 2012) and *C. quinquefasciatus* (Yang and Liu, 2011).

The CYP9 family contains 35 genes classified into three subfamilies (J, M and AE). In general P450 families have only few member; however in *Ae. aegypti* CYP9 family, the subfamily J contains 28 isoforms. It is not clear why such an expansion has occurred in this particular subfamily. Two pseudogenes (*CYP9J10-de2b* and *CYP9J10-delc*) are present in the CYP9 family, and are both detritus exons, upstream and downstream, respectively, of *CYP9J10*. They are believed to arise from the duplication of one or more exons of the gene *CYP9J10* (Nelson, 2004). The CYP9 family in *Ae. aegypti* is significantly expanded as compared to *A. gambiae* and *C. quinquefasciatus* for which this family contains 9 and 24 isoforms, respectively (Table 2.2). The CYP329 family has a single member, with 19 possible orthologs in *C. quinquefasciatus* and several species of the genus *Anopheles.*

CYP4 clan

The CYP 4 clan of *Ae. aegypti* is composed of two families, CYP4 and CYP325. It comprises 58 genes, encoding 60 different proteins; two of the genes being alternatively spliced. The genes in this clan are classified in 25 subfamilies, making it the second largest *Ae. aegypti* CYP clan after the CYP 3 clan. This observation is also similar to those of *A. gambiae* and *C. quinquefasciatus* (Table 2.2). The CYP4 family consists of a total of 24 genes among which one (*CYP4J16*) had two alternative splicing variants (*CYP4J16A* and *CYP4J16B*), raising up a total number of putative proteins to 25 in this family (Figure 2.1). Members of the family have known orthologs in others mosquito species but also in the orders Phthiraptera, Hemiptera and Chelicerata.

The CYP4 family is common to both insects and vertebrates, and some members of the family are clearly inducible by xenobiotics (Sutherland et al., 1998; Bradfield et al., 1991). In insects, other physiological functions besides xenobiotics metabolism have been recognized to some members of this family. In cockroach, a CYP4C member has been found to be involved in the biosynthesis of juvenile hormone (Bradfield et al., 1991). The CYP325 family encompassed 34 genes, with one gene (*CYP325Y2*) being alternatively spliced into two variants (*CYP325Y2A* and *CYP325Y2B*). The genes in this family have appaerant orthologs in others dipteran species and in the subphylum chelicerata.

Mitochondrial CYP clan

The mitochondrial P450 clan is about the same size as the CYP2 clan. It comprises 10 genes belonging to six families (CYP12, 49, 301, 302, 314 and 315) with one subfamily each. The CYP12 family tops the list with 4 genes all in the subfamily F. Three of the genes, highly conserved Halloween gene among holometabolous insects, are known to be involved in the biosynthesis of insect molting hormone ecdyson (Rewitz et al 2006). These genes named *disembodied* (*CYP302A1), shade (CYP314A1*) and *shadow (CYP315A1*) are all involved in the last three steps of 20-hydroxyecdyson synthesis (Rewitz et al., 2006, 2008). In contrast to vertebrates mitochondrial clan P450s, which are all involved in essential physiological processes, arthropod mitochondrial clan P450 are both involved in physiological processes and xenobiotic metabolism. The CYP12 family of P450 is similar to the CYP6 and CYP9 families of microsomal P450 in that they are inducible by xenobiotics, and involved in xenobiotic metabolism. For instance, Guzov et al. (1998) have shown that heterogously expressed CYP12A1 from house fly can metabolize pesticides such as diazinon, but not ecdysteroids.

The high diversity of P450 genes present in *Ae. aegypti* may be in relation to the wide number of xenobiotics encountered by this mosquito in its environment, and it is not particularly clear why such bloom occurred in the CYP6, CYP9 CYP4 and CYP325 families. Gene family evolution theory suggests that gene duplication may be essential for the formation of major new evolutionary novelties, and thereby to the expansion of such a gene family (Ranson et al., 2002; Walsh and Stephan, 2001). Although expansions have been observed in xenobiotic-metabolizing families of P450 (CYP4, CYP6, CYP9), it is very unlikely that synthetic chemicals like insecticides play a major role in driving this evolution since synthetic insecticides have only been used for less than 100 years by human. Hence synthetic insecticides should have little influence on the evolution of P450 and other detoxification enzymes. In herbivorous insects it has been suggested that expansion of P450 genes evolved from the need to provide more adaptive response to plants allelochemichals.

Genomic distribution of cytochrome *P450 genes*

The P450 genes of *Ae. aegypti* are scattered across the entire genome. There are 42 P450 genes on chromosome 1, 14 on chromosome 2, and 26 on chromosome 3. The location of 78 P450 genes remains unknown. Analysis indicated the occurrence of more genes in the families CYP6, CYP9, CYP4 and CYP325, and the genes of these families clearly showed a distinct clustering, suggesting their relatedness. Several clusters of CYP6 genes were found, with the largest one consisting of 17 CYP6 genes located on scaffold 1.371 of chromosome 1. Similarly, two large clusters of CYP9 genes were present on scaffolds 1.1188 and 1.221 of chromosome 3 with 17 and 11 genes, respectively. The clustering of these functionally related genes reflects to some extent their common ancestry and subsequent duplication and divergence (Feyereisen, 2005; Walsh and Stephan, 2001).

Intron-exon organization of cytochrome P450 genes

The P450 genes of *Ae. aegypti* are highly diverse in terms of the number of introns and the intron-exon organization. The superfamily is characterized by the predominance of single intron-containing genes, found mostly in the CYP6 and CYP9 families, which account for nearly half of the entire superfamily. The number of exons per gene ranged from 1to 10 (Figure 2.2), and the density of these exons was estimated to be 2.35. Most of the genes (61out of 159) have two exons. The length of predicted amino acid sequences is similar in nearly all the P450s and is around 500 residues in average per gene. The shortest P450 protein (CYP9J25) has only 256 amino acid residues, which is about half of the size of other members. Similarly, the number of intron per gene ranged from 1to 9 with the majority of genes (61%) having a single intron (Figure 2.3). The smallest intron was found in *CYP314A1*, with 34 bp, whereas the longest intron was found in*CYP325Y2* with the length of gene sequence of 61,830 bp.

The genes in the mitochondrial CYP clan have the greatest number of introns (5 to 9). *CYP49A1* in this clan had nine introns of various lengths, and this same number of intron is also found in its orthologous genes in *A. gambiae* and *C. quinquefasciatus*. This conservation of structure in *CYP49A1* across these mosquito species suggest its involvement in conserved biological process in these taxa. Indeed, gene ontology analysis indicates that this gene may be involved in electron transfer and steroid biosynthesis, which are known to be crucial for many developmental processes in insects.

The CYP6 and CYP9 families showed the highest conservation of intron-exon organization as well as the position of introns (Figures 2.5, and 2.6), and many genes in the two families are clustered. Noteworthy, there is a predominance of single intron genes in both

families. The length of these short introns ranged from 50 to 70 bp. Both the CYP325 and CYP4 families belong to the CYP4 clan.

In the CYP4 family, the gene presented a heterogeneous organization, with the number of intron per gene ranging from two to seven (Figure 2.7). Most of the genes are very long (up to 43 kb), and the majority of introns in this family are long (up to 22,971 bp), and were phase 1. The phase of introns was named 0 when the intron splits two consecutive codons; 1 if an intron is located between the first and second codon nucleotides; and 3 if an intron is located between the second and third codon nucleotides.

The CYP325 family contains also multi-intronic genes; only one gene (*CYP325L1*) hasone intron whereas the others have 2 to 5 introns. Analysis of the distribution of the lengths of introns showed a skewed distribution to the right with the predominance of short introns of length ranging from 50 to 100 bp (Figure 2.3). This observation was consistent with the distribution of intron size observed in *Drosophila* P450 genes (Tijet et al., 2001). The total number of introns in the superfamily was 373 and among them, 87 were phase 0 (23.3%), 169 were phase 1 (45.3%) and 117 were phase 2 (31.4%). Interestingly, the canonical GT/AG splice sites is conserved in all intron-exon junctions. Among the 159 P450 genes, 10 genes (*CYP6AL1*, *CYL6AL3*, *CYP6CA1*, *CYP9J25*, *CYP9AE1*, *CYP9H32*, *CYP9J10*-de2b, *CYP9J10-delc*, *CYP325E2P* and *CYP325X3P*) contained no introns. Two genes (*CYP4J16,* and *CYP325Y2)* have been predicted to have alternative splicing variants. Analysis of conserved motifs showed that they are well conserved among the sequence analyzed (Figure 2.4). The conserved P450 motifs including the heme-binding region, the helix-I and helix-K showed high sequence conservation among the different P450 proteins.

In general, the mechanisms and rate of P450 superfamily evolution are poorly understood. Introns are lost and gained through evolution, and accordingly, intron position and phase are fossils of them evolutionary events that may have contributed to the present organization of these genes (Long et al., 1995; Stoltzfus et al., 1997).

Phylogenetic analysis

Phylogenetic tree using neighbor-joining method was conducted in order to determine the phylogenetic relationship among the entire P450 superfamily. In general, the tree showed seven monophyletic clusters, supported by high bootstrap values (Figure 2.8). It is noticeable that the organization of P450 families to clans was generally conserved, except for the mitochondrial CYP and the CYP2 clans, which formed a very distinctive monophyletic cluster on the tree. Some members of the two clans are conserved in insects, and have been implicated in key physiological processes (Rewitz et al., 2007; Feyereisen, 2012). This suggests that the two clans may have evolved from a common ancestor.

The CYP3 and CYP4 clans are all conserved in terms of the families assigned to them. In the CYP3 clan, there are four clusters of P450 genes on the tree. The CYP9 family forms a single cluster, well supported by high bootstrap value. Nonetheless, the gene *CYP329B1*, a member of the CYP3 clan, is clustered with the CYP9 family on the tree, suggesting that error may have been made in assigning this gene to that family. However, amino acid comparison provided no evidence that this gene belong to CYP9 family. The CYP6 family forms three clusters with relatively high bootstrap values. The genes in CYP6 and CYP9 families show similar gene organizations predominantely with the predominance of single introns of similar lengths. Similarly, the CYP4 and CYP325 families appear to have descended from a common ancestor.

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Table 2.1 Summaryof *Aedes aegypti* P450 enzymes based on genome-wide analysis

No.	Enzyme name	CYP clan	Accession number	Scaffold	Chromosome	Amino acid	Intron number (size)
1	CYP15B1	2	AAEL002067	1.48	2	498	4 (2,248) (61) (5,943) (55)
2	CYP18A1	2	AAEL004870	1.133	1	533	4 (8,546) (105) (90) (1,316)
3	CYP303A1	2	AAEL012144	1.66	1	497	3 (16,430) (117) (3,904)
4	CYP304B2	2	AAEL014412	1.1104	Unkn	517	2 (12,176) (56)
5	CYP304B3	2	AAEL014411	1.1104	Unkn	515	2 (14,199) (202)
6	CYP304C1	2	AAEL014413	1.1104	Unkn	521	3 (20,390) (63) (57)
7	CYP305A5	2	AAEL002043	1.48	2	497	2 (12,808) (60)
8	CYP305A6	2	AAEL002071	1.48	2	498	2 (10,527) (57)
9	CYP306A1	2	AAEL004888	1.133	1	499	4 (2,072) (177) (519) (28,819)
10	CYP307A1	2	AAEL009762	1.427	1	530	2 (31,032) (105)
11	CYP307B1	2	AAEL006875	1.224	Unkn	536	2 (67) (62)
12	CYP6F2	3	AAEL014678	1.1219	2	505	1 (62)
13	CYP6F3	3	AAEL014684	1.1219	2	505	1 (71)
14	CYP6M5	3	AAEL009117	1.371	1	493	1 (62)
15	CYP6M6	3	AAEL009128	1.371	1	492	1 (59)
16	CYP6M9	3	AAEL017297	1.371	1	493	1 (62)
17	CYP6M10	3	AAEL009125	1.371	1	493	1 (64)
18	CYP6M11	3	AAEL009127	1.371	1	498	1 (64)
19	CYP6N6	3	AAEL009126	1.371	1	498	1 (57)
20	CYP6N9	3	AAEL009121	1.371	1	499	1 (63)
21	CYP6N10P	3	AAEL017556	1.371	1	NA	4 (59) (275) (67) (81)
22	CYP6N11	3	AAEL009138	1.371	1	497	1 (60)

23	**CYP6N12**	**3**	**AAEL009124**	**1.371**	**1**	**493**	**1 (69)**
24	**CYP6N13**	**3**	**AAEL009137**	**1.371**	**1**	**499**	**1 (54)**
25	**CYP6N14**	**3**	**AAEL009133**	**1.371**	**1**	**522**	**1 (58)**
26	**CYP6N15**	**3**	**AAEL009122**	**1.371**	**1**	**497**	**1 (53)**
27	**CYP6N16**	**3**	**AAEL010151**	**1.457**	**2**	**498**	**1 (69)**
28	**CYP6N17**	**3**	**AAEL010158**	**1.457**	**2**	**499**	**1 (60)**
29	**CYP6P12v1**	**3**	**AAEL012491**	**1.702**	**Unkn**	**508**	**2 (2,488) (58)**
30	**CYP6P12v2**	**3**	**AAEL014891**	**1.327**	**Unkn**	**527**	**1(58)**
31	**CYP6S3**	**3**	**AAEL009120**	**1.371**	**1**	**511**	**1 (56)**
32	**CYP6Y3**	**3**	**AAEL009132**	**1.371**	**1**	**504**	**1 (60)**
33	**CYP6Z6**	**3**	**AAEL009123**	**1.371**	**1**	**490**	**1 (65)**
34	**CYP6Z7**	**3**	**AAEL009130**	**1.371**	**1**	**358**	**1 (65)**
35	**CYP6Z8**	**3**	**AAEL009131**	**1.371**	**1**	**490**	**1 (59)**
36	**CYP6Z9**	**3**	**AAEL009129**	**1.371**	**1**	**493**	**1 (55)**
37	**CYP6AA5**	**3**	**AAEL012492**	**1.702**	**Unkn**	**504**	**1 (59)**
38	**CYP6AG3**	**3**	**AAEL007024**	**1.231**	**Unkn**	**497**	**3 (4,834) (57) (58)**
39	**CYP6AG4**	**3**	**AAEL007010**	**1.231**	**Unkn**	**497**	**3 (6,414) (57) (59)**
40	**CYP6AG5**	**3**	**AAEL006984**	**1.231**	**Unkn**	**502**	**3 (52) (79) (55)**
41	**CYP6AG6**	**3**	**AAEL006992**	**1.231**	**Unkn**	**499**	**2 (75) (65)**
42	**CYP6AG7**	**3**	**AAEL006989**	**1.231**	**Unkn**	**499**	**2 (72) (61)**
43	**CYP6AG8**	**3**	**AAEL015654**	**1.4359**	**Unkn**	**499**	**2 (61) (61)**
44	**CYP6AH1**	**3**	**AAEL007473**	**1.257**	**Unkn**	**503**	**3 (58) (58) (23,537)**
45	**CYP6AK1**	**3**	**AAEL004941**	**1.136**	**Unkn**	**513**	**4 (85) (18,687) (72) (58)**
46	**CYP6AL1**	**3**	**AAEL008889**	**1.354**	**Unkn**	**508**	**0**
47	**CYP6AL3**	**3**	**AAEL009656**	**1.415**	**Unkn**	**511**	**0**

48	CYP6BB2	3	AAEL014893	1.327	1	507	1 (62)
49	CYP6BY1	3	AAEL017539	1.7	Unkn	490	1 (52)
50	CYP6BZ1	3	AAEL012494	1.702	Unkn	502	1 (60)
51	CYP6CA1P	3	AAEL014680	1.1219	Unkn	NA	0
52	CYP6CB1	3	AAEL002046	1.48	2	504	1 (55)
53	CYP6CB2	3	AAEL002872	1.1337	Unkn	499	1 (61)
54	CYP6CC1	3	AAEL014890	1.1327	Unkn	494	1 (62)
55	CYP6CD1	3	AAEL005006	1.138	Unkn	508	6 (64) (9,623) (252) (470) (59) (61)
56	CYP9J2	3	AAEL006805	1.221	3	536	1 (63)
57	CYP9J6	3	AAEL002638	1.62	2	531	1 (61)
58	CYP9J7	3	AAEL014606	1.118	Unkn	527	1 (57)
59	CYP9J8	3	AAEL006811	1.221	3	538	1 (58)
60	CYP9J9	3	AAEL006793	1.221	3	542	1 (59)
61	CYP9J10	3	AAEL006798	1.221	3	542	1 (61)
62	CYP9J10-de2b	3	AAGE02029680	1.221	3	NA	0
63	CYP9J10-delc	3	AAGE02011007	1.221	3	NA	0
64	CYP9J15	3	AAEL006795	1.221	3	537	2 (369) (66)
65	CYP9J16	3	AAEL006815	1.221	3	538	1 (62)
66	CYP9J17	3	AAEL006784	1.221	3	537	1 (61)
67	CYP9J18v1	3	AAEL018043	1.221	3	536	1 (54)
68	CYP9J18v2	3	AAEL014618	1.1188	Unkn	543	1 (54)
69	CYP9J19	3	AAEL018045	1.221	3	540	1 (68)
70	CYP9J20v1	3	AAEL006814	1.221	3	537	1 (62)

71	CYP9J20v2	3	AAEL014604	1.1188	Unkn	537	1 (62)
72	CYP9J21	3	AAEL014612	1.221	3	489	1 (60)
73	CYP9J22	3	AAEL006802	1.221	3	534	1 (62)
74	CYP9J23	3	AAEL014615	1.1188	Unkn	533	1 (60)
75	CYP9J24	3	AAEL014613	1.1188	Unkn	536	1 (63)
76	CYP9J25	3	AAEL017366	1.1188	Unkn	256	0
77	CYP9J26	3	AAEL014609	1.1188	Unkn	535	1 (60)
78	CYP9J27	3	AAEL014616	1.1188	Unkn	536	1 (62)
79	CYP9J28	3	AAEL014617	1.1188	Unkn	536	1 (63)
80	CYP9J29	3	AAEL014610	1.1188	Unkn	538	1 (60)
81	CYP9J30	3	AAEL014603	1.1188	Unkn	538	1 (58)
82	CYP9J31	3	AAEL002633	1.62	1	531	1 (62)
83	CYP9J32	3	AAEL008846	1.352	Unkn	540	1 (55)
84	CYP9M4	3	AAEL001320	1.29	1	531	1 (61)
85	CYP9M5	3	AAEL001288	1.29	1	517	2 (57) (47)
86	CYP9M6	3	AAEL001312	1.29	1	536	1 (62)
87	CYP9M7	3	AAEL001292	1.29	1	538	1 (56)
88	CYP9M8	3	AAEL009591	1.41	Unkn	536	1 (8,888)
89	CYP9M9	3	AAEL001807	1.43	2	529	1 (60)
90	CYP9AE1	3	AAEL003748	1.96	1	526	0
91	CYP329B1	3	AAEL003763	1.96	1	515	2 (52) (57)
92	CYP4C38	4	AAEL012266	1.673	1	509	7 (22,971) (140) (203) (56) (12,879) (2,018) (99)
93	CYP4C50	4	AAEL008017	1.295	Unkn	544	4 (26,265) (15,344) (56) (63)
94	CYP4C51	4	AAEL008018	1.295	Unkn	545	2 (16,819) (160)

95	CYP4C52	4	AAEL008023	1.295	Unkn	531	4 (19,859) (10964) (62) (60)
96	CYP4D23	4	AAEL007816	1.283	Unkn	499	3 (10,612) (62) (67)
97	CYP4D24	4	AAEL007815	1.283	Unkn	503	4 (73) (5063) (64) (64)
98	CYP4D37	4	AAEL007795	1.283	Unkn	502	4 (18,325) (69) (58) (6,710)
99	CYP4D38	4	AAEL007807	1.283	Unkn	502	4 (8,994) (69) (62) (2,932)
100	CYP4D39	4	AAEL007808	1.283	Unkn	504	5 (10,082) (12,309) (59) (60) (60)
101	CYP4G35	4	AAEL008345	1.318	1	554	2 (8,651) (73)
102	CYP4G36	4	AAEL004054	1.106	Unkn	566	4 (12,647) (3,526) (13,128) (70)
103	CYP4H28	4	AAEL003380	1.85	3	503	2 (11,454) (56)
104	CYP4H29	4	AAEL007830	1.285	1	512	1 (4626)
105	CYP4H30	4	AAEL003399	1.85	3	496	5 (17,238) (61) (55) (535) (62)
106	CYP4H31	4	AAEL002085	1.48	2	505	4 (140) (59) (54) (64)
107	CYP4H32	4	AAEL007812	1.283	Unkn	501	0
108	CYP4H33	4	AAEL013798	1.923	Unkn	506	3 (65) (62) (62)
109	CYP4J13	4	AAEL013555	1.869	Unkn	511	3 (59) (56) (57)
110	CYP4J14	4	AAEL013554	1.869	Unkn	510	3 (59) (55) (59)
111	CYP4J15v1	4	AAEL013556	1.869	Unkn	510	3 (61) (55) (59)
112	CYP4J15v2	4	AAEL014829	1.869	Unkn	510	3 (61) (55) (59)
113	CYP4J16A	4	AAEL014019-PA	1.985	Unkn	502	2 (59) (12,546)
114	CYP4J16B	4	AAEL014019-PB	1.985	Unkn	505	2 (58) (29,042)

115	CYP4K3	4	AAEL007798	1.283	Unkn	497	5 (162) (10,103) (63) (61) (61)
116	CYP4AR2	4	AAEL010154	1.457	2	500	2 (64) (61)
117	CYP325E2P	4	AAGE02000570	1.6	1	NA	0
118	CYP325E3	4	AAEL000338	1.6	1	519	4 (41,979) (63) (51) (60)
119	CYP325G2	4	AAEL012766	1.738	Unkn	501	3 (61) (59) (59)
120	CYP325G3	4	AAEL012772	1.738	Unkn	500	3 (62) (60) (64)
121	CYP325K2	4	AAEL005771	1.174	3	511	3 (61) (56) (643)
122	CYP325K3	4	AAEL005788	1.174	3	511	4 (56) (10,372) (95) (69)
123	CYP325L1	4	AAEL011770	1.61	Unkn	490	1 (70)
124	CYP325M1	4	AAEL012773	1.738	Unkn	503	4 (15,757) (68) (69) (59)
125	CYP325M2	4	AAEL012769	1.738	Unkn	495	4 (90) (62) (58) (8,132)
126	CYP325M3	4	AAEL012765	1.738	Unkn	497	4 (955) (65) (823) (62)
127	CYP325M4	4	AAEL011769	1.61	Unkn	504	4 (153) (57) (469) (57)
128	CYP325M5	4	AAEL011761	1.61	Unkn	505	4 (5,262) (69) (58) (55)
129	CYP325N1	4	AAEL012770	1.738	Unkn	506	4 (8,172) (68) (52) (6)
130	CYP325N2	4	AAEL012762	1.738	Unkn	502	4 (7,375) (575) (65) (3,660)
131	CYP325P1	4	AAEL000340	1.6	3	492	3(65) (14698) (5253)
132	CYP325Q1	4	AAEL006044	1.187	Unkn	496	4 (61) (15,888) (55) (62)
133	CYP325Q2	4	AAEL006058	1.187	Unkn	490	4 (8,771) (75) (695) (88)
134	CYP325R1	4	AAEL005775	1.174	3	502	4 (54) (58) (11,503) (64)
135	CYP325S1	4	AAEL000326	1.6	1	505	4 (61) (55) (439) (67)
136	CYP325S2	4	AAEL000325	1.6	1	498	4 (532) (54) (14023) (59)
137	CYP325S3	4	AAEL000357	1.6	1	501	5 (65) (63) (7,930) (61) (68)
138	CYP325T1	4	AAEL000320	1.6	1	499	4 (9,041) (56) (390) (59)
139	CYP325T2	4	AAEL012761	1.738	Unkn	488	4 (81) (7,971) (73) (1,021)

140	CYP325U1	4	AAEL017215	1.6	1	515	3 (65) (53) (6,861)
141	CYP325V1	4	AAEL017136	1.6	1	461	4 (64) (12,635) (66) (60)
142	CYP325X1	4	AAEL005695	1.17	3	506	4 (67) (65) (10,551) (58)
143	CYP325X2	4	AAEL005696	1.17	3	513	5 (18,583) (60) (532) (149) (60)
144	CYP325X3P	4	AAGE02009114	1.17	3	NA	0
145	CYP325X4	4	AAEL005700	1.170	3	519	5 (28,023) (64) (72) (60) (58)
146	CYP325Y1	4	AAEL006257	1.197	3	498	3 (13,518) (56) (59)
147	CYP325Y2A	4	AAEL900030-PA	1.197	3	498	3 (12,729) (61,830) (61)
148	CYP325Y2B	4	AAEL900030-PB	1.197	3	498	4 (12,729) (61) (55) (58)
149	CYP325Y3	4	AAEL015361	1.197	3	498	3 (4,970) (57) (61)
150	CYP325Z1	4	AAEL010273	1.467	Unkn	518	3 (83) (57) (8854
151	CYP325AA1	4	AAEL004012	1.105	Unkn	498	4 (61) (6,817) (94) (69)
152	CYP12F5	4	AAEL001960	1.47	3	526	6 (57) (60) (57) (61) (56) (62)
153	CYP12F6	4	AAEL002005	1.47	3	523	5 (61) (64) (67) (56) (65)
154	CYP12F7	4	AAEL002031	1.47	3	519	6 (7,631) (57) (56) (62) (72) (58)
155	CYP12F8	4	AAEL006827	1.222	1	510	6 (25,025) (65) (57) (59) (3414) (68)
156	CYP49A1	4	AAEL008638	1.338	2	534	9 (20,293) (2,328) (11,359) (91) (91) (8,904) (804) (109) (86)
157	CYP301A1	4	AAEL014594	1.1181	Unkn	518	5 (13,714) (10,830) (28,030) (60) (64)
158	CYP302A1V1	4	AAEL011463	1.581	2	515	6 (67) (62) (62) (58) (60) (62)
159	CYP302A1V2	4	AAEL015655	1.4395	Unkn	515	6 (67) (62) (62) (58) (60) (62)
160	CYP314A1	4	AAEL010946	1.524	Unkn	555	6 (402) (62) (34) (7,904) (63) (53)
161	CYP315A1	4	AAEL011850	1.622	Unkn	472	8 (662) (55) (5233) (60) (64) (57) (18,523)

Table 2.2 Comparative distribution of cytochrome P450 genes from three mosquito species

Name of P450 clan	P450 families	Number of P450 genes in three mosquito species		
		Aedes aegypti	*Anopheles gambiae*	*Culex quinquefasciatus*
CYP2 clan	CYP15	1	1	1
	CYP18	1	0	1
	CYP303	1	1	1
	CYP304	3	2	5
	CYP305	2	3	5
	CYP306	1	1	1
	CYP307	2	2	2
CYP3 clan	CYP6	44	30	64
	CYP9	35	9	24
	CYP329	1	1	1
CYP4 clan	CYP4	25	30	34
	CYP325	35	16	47
	CYP326	0	0	1
Mitochondrial CYP clan	CYP12	4	4	7
	CYP49	1	1	1
	CYP301	1	1	1
	CYP302	2	1	1
	CYP314	1	1	1
	CYP315	1	1	1
Total		161	105	199

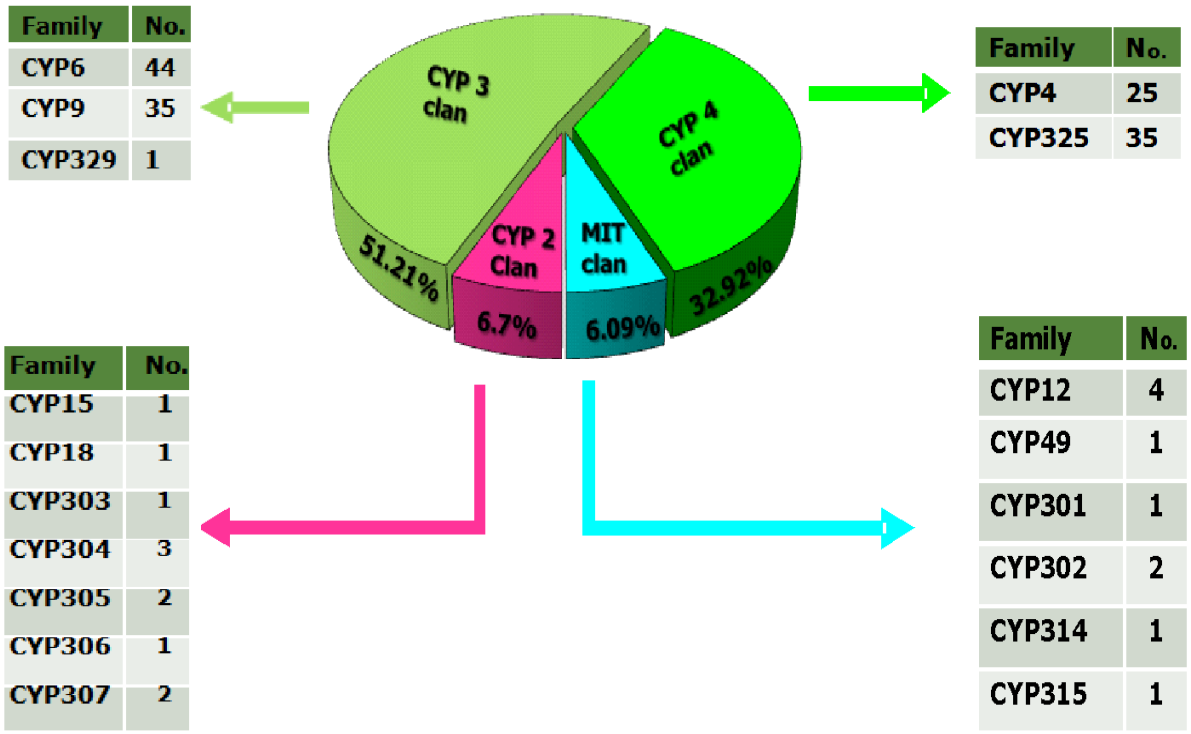

Figure 2.1 Distribution of *Aedes aegypti* P450 per clans and families. Data were retrieved and analyzed from the VectorBase website (http://vectorbase.org) and the Cytochrome P450 homepage (http://drnelson.utmem.edu/cytochromeP450.html).

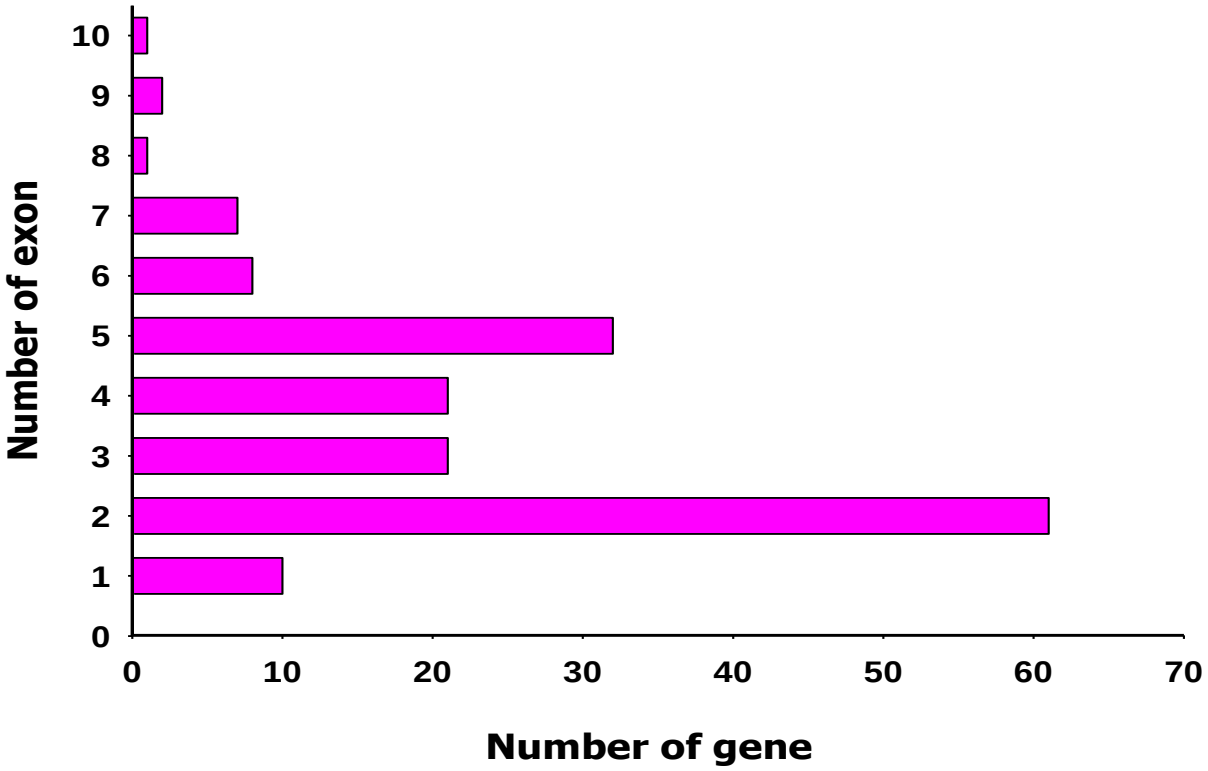

Figure 2.2 Distribution of the number of exon per gene in *Aedes aegypti* P450 superfamily

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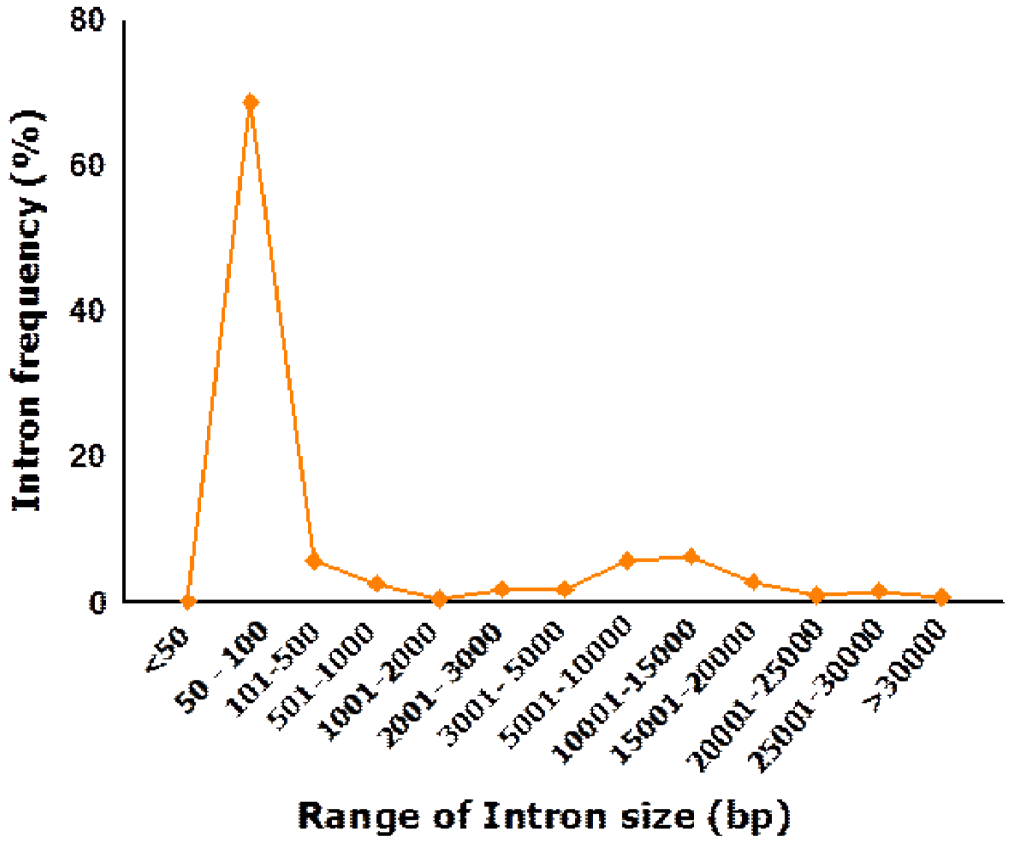

Figure 2.3 Distribution of the size of introns in *Aedes aegypti* P450 superfamily

Heme-binding motif

PERF motif

C- Helix motif

I-Helix motif

Figure 2.4 Sequence logos depicting the conservation of amino acids in each P450 motif of *Aedes aegypti* P450ome. The letter size is proportional to the degree of amino acid conservation. In the heme-binding motif, the cysteine (C) residue is highly conserved in P450 sequences in various taxa.

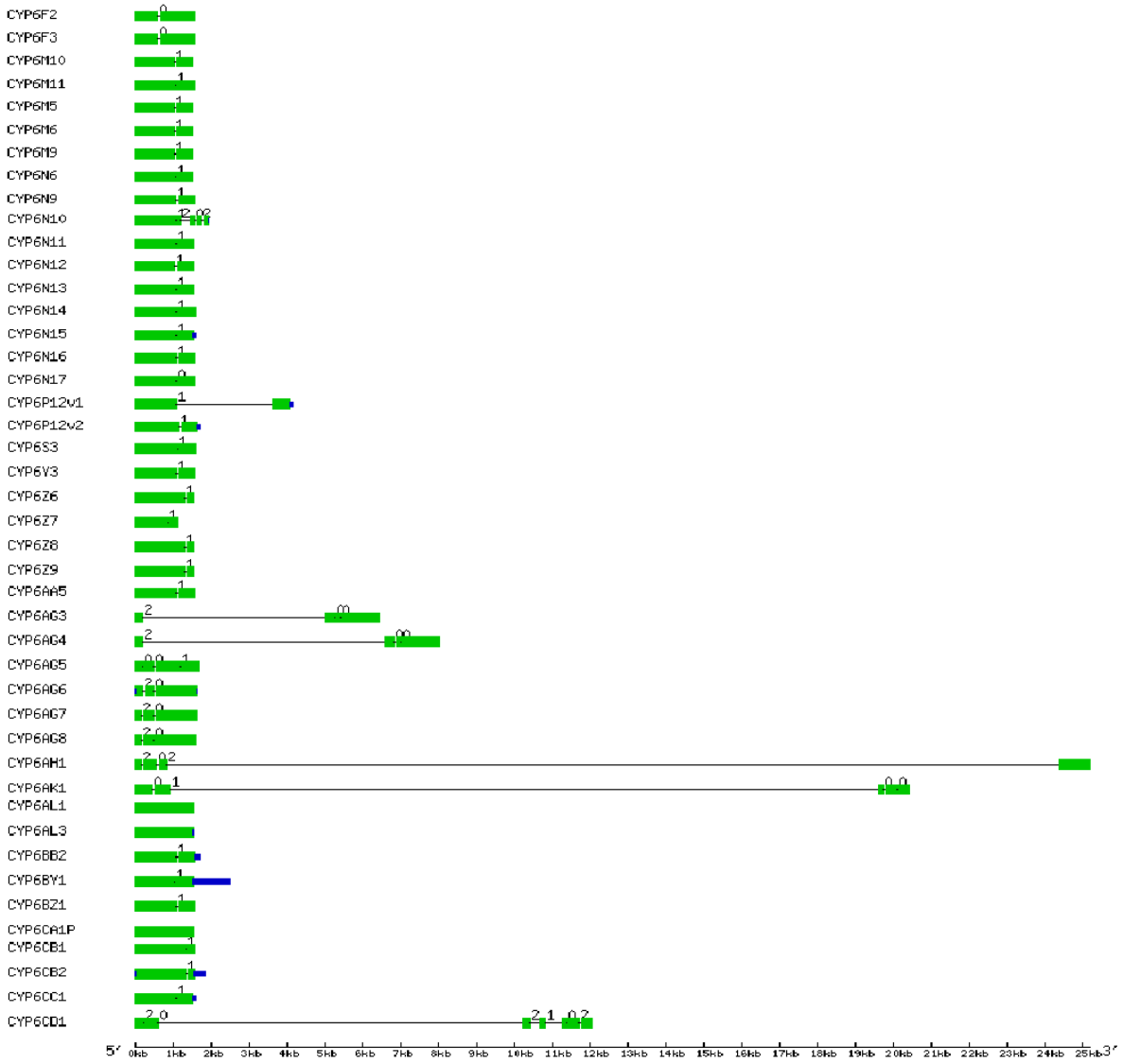

Figure 2.5 Exon-intron organization of the genes in CYP6 family. The gene structures displaying the intron/exon organization along with the intron phases were generated with the gene structure display server (http://gsds.cbi.pku.edu.cn/) using coding sequences and genomic sequences.

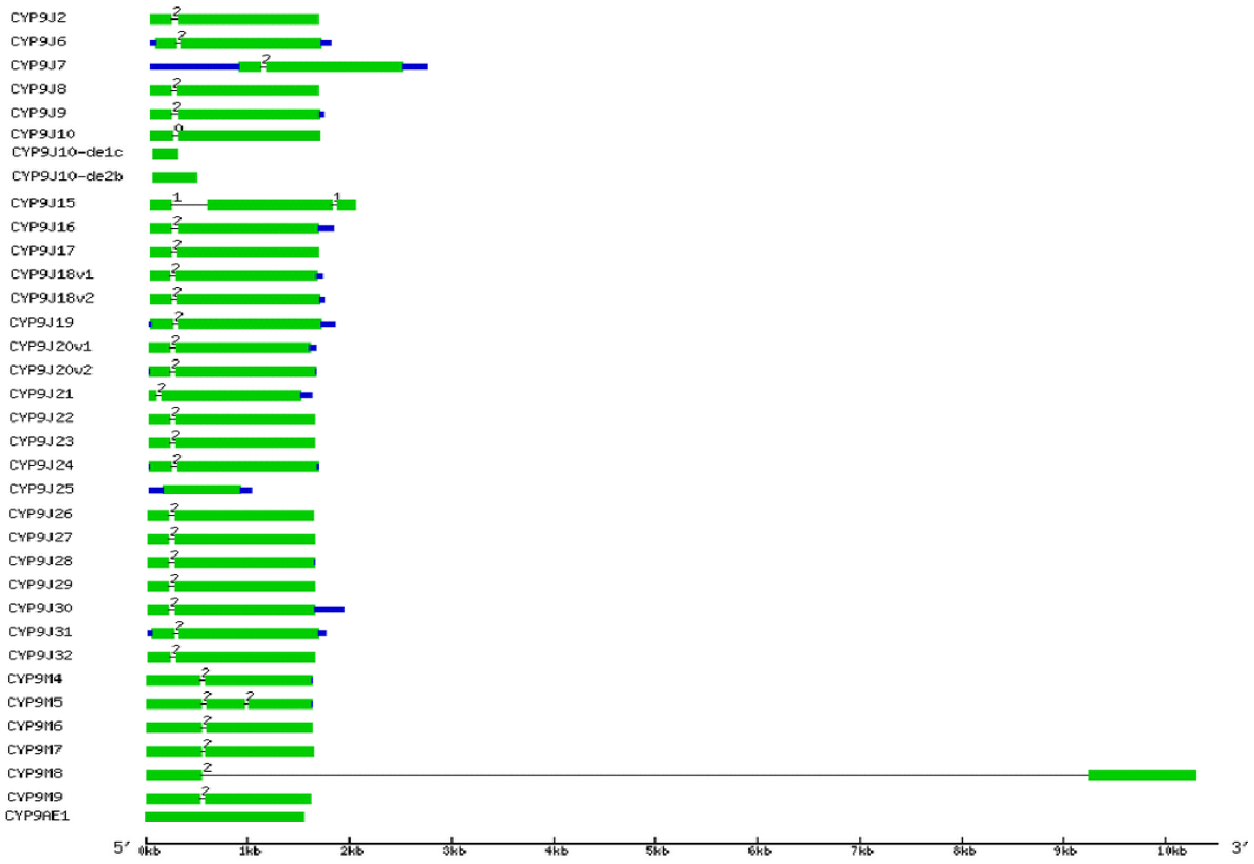

Figure 2.6 Exon-intron organization of the genes in CYP9 family. The gene structures displaying the intron/exon organization along with the intron phases were generated with the gene structure display server (http://gsds.cbi.pku.edu.cn/) using coding sequences and genomic sequences.

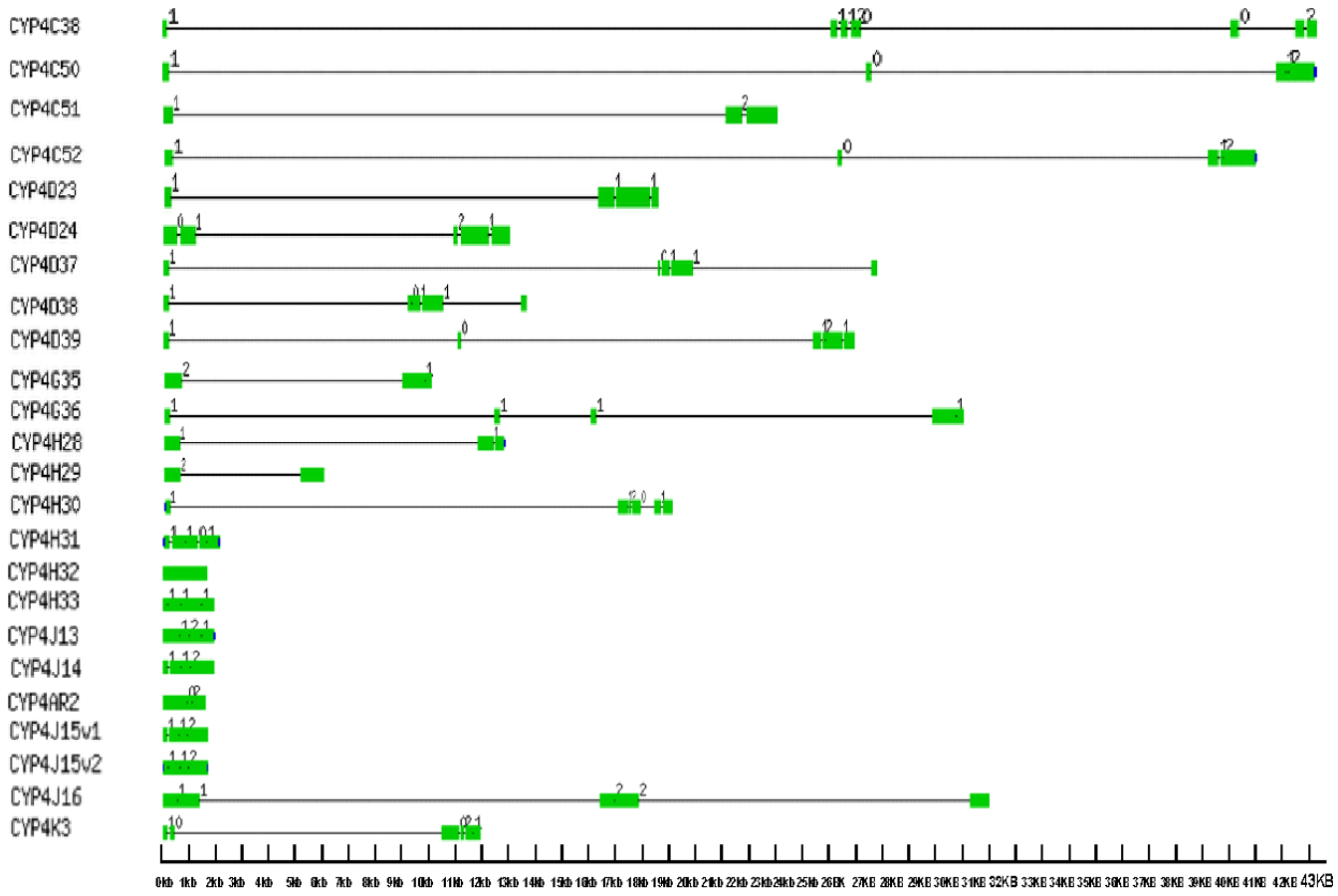

Figure 2.7 Intron-exon organization of the genes in CYP4 family. The gene structures displaying the intron/exon organization along with the intron phases were generated with the gene structure display server (http://gsds.cbi.pku.edu.cn/) using coding sequences and genomic sequences.

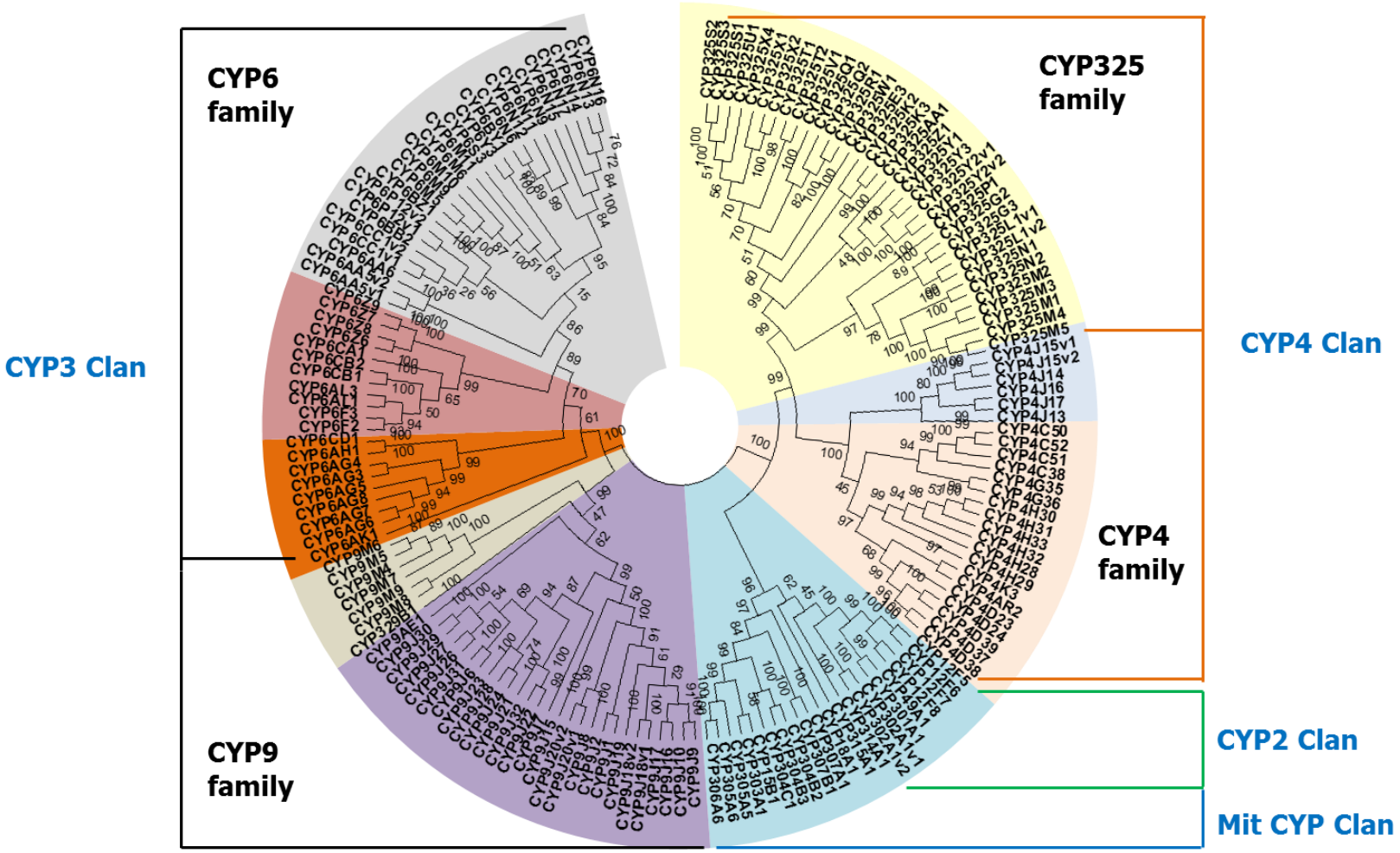

Figure 2.8 Phylogenetic tree of deduced amino acids sequences of putative functional P450 genes from *Aedes aegypti*. The tree was generated using neighbor joining algorithm in MEGA5 software. The significance of the trees was tested using 3000 bootstrap replicates.

Chapter 3 - Molecular Characterization of Selected Cytochrome P450 Genes in the Yellow Fever Mosquito *Aedes Aegypti*

Abstract

Cytochrome P450 monooxygenases (P450s) are known to be involved in the metabolism of xenobiotics and endogenous compounds in nearly all living organisms. *Aedes aegypti,* vector of many arboviral diseases has become resistant to several insecticides in various parts of the world, and P450 enzymes are known to contribute to insecticide resistance. In this study, we observed the differential expression of five transcripts including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* in various tissues and at different developmental stages. The transcripts of *CYP6AA5, CYP6AL1* and *CYP9J32* were highly expressed in larvae compared to other stages. *CYPJ16A* was predominantly expressed in eggs and adults, whereas the highest expression of *CYP4J16B* was observed in pupae. The tissue-specific expressions of the transcripts were higher in the midgut, Malpighian tubules of both adults and larvae in general. Interestingly, the two alternative splicing variants of *CYP4J16* showed differential expression profiles. *CYP4J16A* was predominantly expressed in the midgut while *CYP4J16B* showed the highest expression in the fat bodies. Exposure of third instar larvae to permethrin, cypermethrin and deltamethrin led to significant down-regulations of *CYP6AL1* and *CYP4J16A*. *CYP6AA5* was moderately up-regulated by cypermethrin but not by other insecticides. Exposure of adult mosquitoes to permethrin led to up-regulations of *CYP6AL1* and *CYP4J16B* by 2.1 and 2.5 fold ($P < 0.05$) respectively, whereas deltamethrin treatment caused the up-regulation of *CYP6AA5*, *CYP4J16A* and *CYP4J16B* by 2.2, 2.1 and 3.6 fold ($P < 0.05$), respectively. Similarly, cypermethrin up-

regulated *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* by 2.4, 3.5, 2.4 and 23.0 fold ($P < 0.05$), respectively. The possible role of the nuclear receptor *HR96* in the up-regulation of these P450 genes was also studied. RNAi was performed via injection of *dsHR96* to silence this gene and expose injected mosquitoes to each of the insecticides. Our results showed 81.7% ($P < 0.05$) reduction of the transcript of *HR96* 96 h after dsRNA injection. Exposure of the injected mosquitoes to cypermethrin led to a 10.1 fold reduction in the up-regulation of *CYP4J16B*, but did not significantly affect the up-regulation of other P450 genes. There were no significant effects of the RNAi on the up-regulation of all the P450 genes by permethrin and deltamethrin. These findings indicate that *HR96* is involved in the up-regulation of *CYP4J16B* by cypermethrin in *Ae. aegypti* but not in other P450 genes by other pyrethroids.

Keywords: Cytochrome P450, *Aedes aegypti*, up-regulation, insecticide, RNA interference.

Introduction

Cytochrome P450 monooxygenases (P450s) are heme-containing proteins involved in the metabolism of xenobiotics and endogenous compounds in nearly all kingdoms of life (Nelson, 1998; Werck-Reichhart and Feyereisen, 2000; Feyereisen 1999, 2005, 2012). In insects, P450 enzymes contribute to the metabolism of various compounds such as juvenile hormones and ecdysteroids, which are very important for insect growth, development, reproduction and communication (Feyereisen, 1999; Wen and Scott, 2001), but also in the detoxification of insecticides and plant allelochemicals leading to resistance and adaptation, respectively.

The role of P450 enzymes in insecticide resistance was first reported by Eldefrawi et al. (1960) when they noted that the carbaryl resistance in house flies was reduced by a P450 enzyme inhibitor sesamex. P450-mediated detoxification is an important resistance mechanism because it can cause a significant level of resistance (Brattsten et al., 1977; Scott and Georghiou, 1986, Feyereisen, 2012) and cross-resistance sometimes to unrelated insecticides (Scott, 1991; 1993).

Aedes aegypti, vector of many arboviral diseases (dengue fever, chikungunya and yellow fever) has become resistant to nearly all classes of insecticides, leading to control failures and thus put the life of millions of people at risk. Analysis of the genome sequencing data (Nene et al., 2007) of this mosquito has revealed the presence of 158 P450 genes. This high number of P450s may be driving the high resistance observed in *Ae*. *aegypti*. Previous studies have shown that some of these genes are highly expressed in resistant strains of this mosquito as compared to susceptible ones (Strode et al., 2008; Poupardin et al., 2008, 2010; Marcombe et al., 2009, 2012). In many cases, increased levels of P450 gene expression are known to result in increased levels of P450 activities and thus higher rate of detoxification (Shen et al., 2003). At a biochemical level, both basal and up-regulations of P450 genes have been found to be responsible for

increased detoxification of insecticides leading to resistance (Liu, 2011; Scharf et al., 2001; Terriere, 1983). Enzyme up-regulation is a process whereby the expression of a gene is triggered or increased in response to environmental substances to which an organism is exposed (Goldberg, 1980). The up-regulation of P450 genes by insecticides and plant toxins have been reported in many insect species (Feyereisen, 2005; Liu, 2011; Scott et al., 1996; Scharf et al., 2001; Bautista et al., 2007; Stevens et al., 2000; Poupardin et al., 2008; Guo et al., 2012) and often associated with insecticides resistance (Karunker et al., 2008; Liu, 2011; Feyereisen, 2012; Gong et al, 2013). Despite this wealth of information, neither the regulatory factors nor their molecular mechanisms are understood in insects.

In mammals, several nuclear receptors have been shown to act as transcription factor to mediate the up-regulation of P450 genes by a variety of xenobiotics (Pascussi et al., 1999; Honkakoski et al., 1998; Gonzalez and Fernandez-Salguero, 1998; Blumberg, 1998). In human, for instance, the up-regulation of the genes in *CYP1A, CYP2B, CYP3A* and *CYP4A* subfamilies are mediated by the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the peroxisome proliferator-activated receptor alpha (PPARα) (Graham and Lake, 2008), respectively. They all belong to the superfamily of transcription factors that bind to specific DNA sequences and to regulate the expression of the genes (Blumberg, 1998).

In insects, however, very little is known about the transcription factors involved in the regulation of P450 genes by xenobiotics. In *Drosophila* S2 cells, the nuclear receptor *HR96* (*hormone receptor-like in 96*), orthologs of vertebrate CAR and PXR (King-Jones and Thummel 2005; Laudet et al. 2005), has been shown to be involved in the up-regulation of *CYP6D1* by phenobarbital and in the control of other metabolic and stress-response genes (King-Jones et al.,

2006). Moreover, Abass et al. (2012) reported that some P450 genes (*CYP3A4, CYP2B6* and *CYP2A6*) in HepaRG cells could be induced by pyrethroids through both *PXR* and *CAR* mediation. Therefore, we hypothesized that *Ae. aegypti HR96*, orthologous to *CAR* and *PXR*, might also mediate the up-regulation of P450 genes.

In this study, we selected five representative P450 transcripts including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* and analyzed their expression patterns in various tissues and at different developmental stages. We also examined the effect of insecticide exposure on the expression of these transcripts by each of three pyrethroid insecticides. Finally, we evaluated the role of the nuclear receptor HR96 in the up-regulation of these P450 genes by RNA interference.

Materials and Methods

Mosquito strain and rearing

The *Ae. aegypti* Liverpool-IB12 strain (MRA-735), previously used for the genome sequencing project, was used in this study. This strain was originally from West Africa, and has been maintained at the Liverpool School of Tropical Medicine since 1936 in the United Kingdom (https://www.vectorbase.org). Eggs were obtained through BEI Resources (NIAID, NIH, Manassas, VA, USA) and reared under standard insectary conditions [27^{o}C, 80% humidity with a photoperiod of 12/12 (light/dark)].

A slice of filter paper with attached eggs was dipped into a 23 by 33 cm glass tray containing 500 ml of distilled water to allow the eggs to hatch. After emergence, the larvae were daily fed with ground dog food (Braga et al., 2005). When the larvae started to pupate, the pupae were collected and transferred into a mosquito cage to prevent the emerging adults from escaping. The adults were fed with 10 % sucrose solution soaked into cotton balls. Three-day-old

females were fed with defibrinated sheep blood in a glass feeder sealed with parafilm. The glass feeder was placed on top of the cage containing the adult mosquito and connected to a pump, circulating water at 37^{o} C to keep the blood warm. The mosquitoes were allowed to feed *ad libitum*. After the blood feeding, egg cups were placed in each cage for the females to lay eggs.

Selection of candidate genes

To select the candidate genes, we performed a detailed literature review on the P450 genes with over-expression in different pyrethroid resistant strains of *Ae. aegypti* as well as other mosquito species (Table 3.1). The amino acid sequences of the P450 genes from other mosquito species were queried against *Ae. aegypti* P450 database (http://blast.uthsc.edu/) for their similarities. The *Ae. aegypti* P450 genes presenting at least 50% identities were retained (Table 3.2). The list of candidate genes was further narrowed down to three genes (*CY6AA5*, *CYP6AL1* and *CYP9J32*) which appeared to be orthologous to *CYP6AA7* (putatively involved in pyrethroid resistance in *Culex quinquefasciatus*), *CYP6F1* (over-expressed in pyrethroid resistant *Culex quinquefasciatus*) and *CYP9J5* (involved in pyrethroid resistance in *Anopheles gambiae)*, respectively. Analysis of genome sequencing data has shown that two P450 genes (*CYP4J16* and *CYP325Y2*) from *Ae. aegypti* may have alternative splice variants. *CYP4J16* has two alternative splicing variants (*CYP4J16A*, *CYP4J16B*). Because *CYP4J16* belongs to *CYP4* family, which has widely known to be associated with insecticide detoxification in many insects, and alternative splicing is not common in insect P450 genes, we decided to characterize these splices variants.

Total RNA extraction and cDNA synthesis

In order to determine the stage-specific expression patterns of candidate genes, total RNA was isolated from each of four different developmental stages, including eggs, third-instar larvae, pupae and adults, by using TRIzol Total RNA Isolation kit (Life Technologies, Carlsbad, CA,

USA). For tissue-specific expression profiles, four different tissues, including midgut, Malpighian tubules, fat bodies and hindgut were dissected from third-instar larvae, and 3-days-old adults in 1xPBS solution, and used for RNA extraction by using the same protocol. For up-regulation of P450 genes, total RNAs were extracted from third instar larvae exposed to LC_{20} concentration of insecticides, and from 3-days-old adults exposed to a time corresponding to LT_{20} for each insecticide. The isolated RNAs from all the samples were quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific).

Five micrograms of total RNA were treated with DNase I (Life Technologies) to remove potential genomic DNA, and used for reverse transcription reactions to generate single stranded cDNA by using the first strand cDNA synthesis kit (Thermo Scientific) with oligo (dT) primers in a 20 µl reaction. The first stranded cDNA was used as a template to generate the double stranded cDNA of each specific gene by PCR and using specific primers. The PCR was performed using the PCR Master Mix (Thermo Scientific) with thermal cycling conditions of an initial denaturation at 94^{o}C for 5 min followed by 29 cycles at 94^{o}C for 30 s, 53^{o}C for 30 s, 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min. Primers used for P450s are shown in Table 3.3 and those use for *HR96* in Table 3.5. The cDNAs corresponding to three candidate reference genes (*AeRps17*, *AeRps10* and *AeRps5*) were also generated to test their stability of expression across different life stages and tissues to identify an appropriate reference gene. PCR product of each sample was run on 1% agarose gel and visualized by staining with ethidium bromide for its specific amplification and size. Three replicates were performed for each stage or tissue.

Reverse transcription quantitative PCR analysis

For reverse transcription quantitative PCR (RT-qPCR) analysis, cDNA prepared from each above-mentioned sample was diluted 25 times and used as a template. For each gene, a

serial dilution ranging from 5 to 625 times was performed to assess the efficiency of PCR. The reaction volume consisted of 25µl containing 12 µl of 2x of Maxima SYBR Green/Fluorescin qPCR Master Mix (Thermo Scientific), 5 µl of 25 time-diluted cDNA, 0.5 µM of each primer, and 6 µl of nuclease free water. Primers used for P450s are shown in Table 3.3 and those use for *HR96* in Table 3.5. RT-qPCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The *Ae. aegypti* ribosomal protein 17 (*Rps17*) was used as a reference gene. Three biological replicates and two technical replicates were used for each gene. The results were analyzed according to ΔΔCt method (Livak and Schmittgen, 2001).

Subcloning and sequencing of selected P450 cDNA

In order to subclone the cDNA of each candidate P450 genes and the P450 regulator *HR96*, RNA was extracted from third instar larvae and used to generate first strand cDNA as described above. The first strand cDNA was used as template to generate double-stranded cDNA corresponding to each gene of interest by using pair of specific primers (Tables 3.4 and 3.5) that were designed based on the predicted sequence of P450 from the genome. PCR was performed in a 50-µl volume by using PCR Master Mix (Thermo Scientific) with thermal cycling conditions of an initial denaturation at 94^{o}C for 5 min followed by 30 cycles at 94^{o}C for 30 s, 57^{o}C for 30 s, 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min. The PCR products were run on agarose gel for its specific amplification and size, and subsequently purified using QIAEX II Agarose Gel Extraction kit (Quiagen, Valencia, CA, USA). The purified PCR product was ligated into a pCR^{TM} 2.1 TA cloning vector (Life Technologies). The ligation mixture was used to transform *E. coli* cells by using Z-Competent *E. coli* transformation kit (Zymo research, Orange, CA, USA). Plasmids were isolated from the bacterial culture and used for sequencing at KSU Sequencing and Genotyping Facility (Kansas State University, Manhattan, KS, USA). The

sequences obtained for each gene were assembled using DNA BAser Assembler v3.x (Heracle BioSoft, SRL, Romania) and analyzed.

RNAi mediated knockdown of putative P450 regulator HR96 in adult mosquitoes

RNAi was carried out to evaluate the role of putative P450 regulator *HR96* in the up-regulation of selected P450 genes. Specific primers with T7 promoter sequences (Figure 3.5) were used to synthesize double-stranded RNA (dsRNA) targeting *HR96* and GFP using MEGAscript RNAi kit, (Life Technologies) according to the manufacturer's protocol. The dsRNA was dissolved in nuclease-free water, quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific), and stored at -80°C.

dsRNA was injected on the base of the adult wings by using a glass needle mounted on a microinjector Nanoject II (Drummond, Broomall, PA, USA). Each of 250 adult mosquitoes aged 3 days, was injected with 300 ng of HR96 dsRNA. The same numbers of mosquitoes were injected with GFP dsRNA (300 ng per adult) as negative controls. After the injection, the mosquitoes were fed with 10% sucrose as described previously. To confirm the depletion of each target transcript, total RNA was isolated at 24, 48, 72 and 96 h after the dsRNA injections to monitor the suppression of gene expression by RT-qPCR as previously described.

Insecticide exposures

Larval bioassay

Technical grade of three insecticides, including permethrin, cypermethrin and deltamethrin, were purchased from Chem Service (Chester, PA, USA). Stock solution and serial dilutions were prepared with 100% acetone and stored at -20^{o}C. The bioassays were carried out according to WHO larval bioassay procedure (WHO, 1981). Each bioassay consisted of four replicates of 20 to 25 third-instar larvae randomly picked and introduced into a disposable paper

cup containing 200 ml of water and an appropriate amount of insecticide. Six different insecticide concentrations with larval mortality ranging from 0 to 100% were used for each insecticide (WHO, 1981, 2005). The concentrations ranges were predetermined based on a separate diagnostic exposure (data not shown). For cypermethrin and deltamethrin, the concentrations used were 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ppm. In the case of permethrin, the concentrations were 1, 2, 3, 4, 5 and 6 ppm. Each bioassay included a control group for which acetone was alone in place of the insecticides. The experimental conditions included a temperature of 27^{o}C and 80% relative humidity. Mortality data were recorded 24 h after exposure. The larvae were considered dead if they could not swim properly or could not move when probed (Kasai et al., 2007, Hardstone et al., 2007, 2009). Data were subjected to probit analysis using SAS software version 9.2 (SAS Institute, Cary, NC, USA). The analysis used a likelihood method to fit a linear regression between log insecticide concentrations and probit mortalities. The goodness of the fit of the analysis was estimated with the Pearson chi-square.

For P450 genes up-regulation experiments, 20 to 25 third-instar larvae were exposed to the LC_{20} concentration of each insecticide for 24 h. After the exposure, the surviving larvae were collected and used of total RNA extraction and RT-qPCR analysis as described previously.

Adult bioassay

Bioassays in adult mosquitoes were performed according to the Center for Disease Control and Prevention bottle bioassay procedure (CDC, 2010). This technique uses time-mortality data to determine the insecticidal effect on adult survivals over a short period of time. The insecticides used were the same as those used for larval bioassay. Stock solutions and serial dilutions were prepared with 100% acetone. For treatment of bottles, 1 ml of diluted insecticide solution at the desired concentration was introduced into a 200-ml Kimax glass-sampling bottle

(Fisher scientific, Waltham, MA). The bottle was capped and rolled several times to allow all surfaces to be exposed to the insecticide solution. Once the liquid was evenly distributed, the bottle was opened and exposed at room temperature for 1 h to allow the evaporation of the remaining liquid.

To determine the suitable concentration of each insecticide to be used in subsequent experiments, a preliminary exposure was performed using six different concentrations for each insecticide. The concentrations were 0.25, 1, 2, 3, 4, and 5 µg/bottle for permethrin and 0.25, 0.5, 1, 1.5, 2 and 2.5 µg/bottle for both cypermethrin and deltamethrin. A batch of 20 to 25 3- to 7-days-old adults that had not been fed with blood was introduced into each of the insecticide-coated bottles and exposed to each insecticide for 1 h. Control mosquitoes were exposed to bottles treated only with acetone. Four biological replicates were used for each insecticide concentration. The bioassays were carried out at 27^{o}C and 80% RH. Cumulative mortality counts were recorded every 10 min until the mortality reached 100%. Mosquitoes were considered dead if they could not fly. Mortality data were analyzed to determine a suitable concentration of insecticide for the rest of the experiments (Figure 3.5). For all three insecticides, 0.25 µg/bottle was chosen for subsequent experiments.

In order to determine the time required to kill a given percentage of the mosquitoes by fixed concentration of each insecticide, batches of 20 to 25 non-blood fed adults mosquitoes aged 3 to 7 days old were exposed to each insecticide at the fixed concentration of 0.25µg/bottle for six different time points (10, 20, 30, 40, 50, and 60 mn). The exposures were repeated four times. Control mosquitoes were exposed to only acetone in the same way. After such exposures, the mosquitoes were transferred into clean cages without insecticides and provided with 10%

sucrose solution. Mortality data were recorded after 24 h, and subjected to probit analysis using SAS software version 9.2 (SAS Institute, Cary, NC, USA).

To evaluate potential up-regulation of P450 genes by each of the three pyrethroids (permethrin, cypermethrin and deltamethrin), 20 to 25 non blood-fed adult mosquitoes aged 3 to 7 days were exposed to each insecticide at 0.25 µg/bottle for a specific time that killed 20% of the test population (LT_{20}). Thereafter, mosquitoes were transferred into clean cages and supplied with sucrose solution for 24 h. Mosquitoes were collected for RNA extractions and RT-qPCR analyses.

Statistical analysis

The gene expression data were subjected to ANOVA followed by Tukey's HSD multiple comparisons to separate the means among the stages, tissues. For the suppression of transcript by RNAi, a Student's t-test (two-tailed paired t-test) was performed to analyze the difference in expression among different treatment groups. All analyses were performed using PROSTAT statistical analysis software (Poly Software International, Pearl River, NY, USA).

Results

Developmental stage expression patterns

The stage-dependent expression patterns of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* were examined by RT-qPCR (Figure 3.1). For *CYP6AA5*, *CYP6AL1* and *CYP9J32*, the expression level was significantly higher in larvae ($P < 0.05$) as compared with those in eggs, pupae and adults. Moreover, larval expression of *CYPP9J32* was 21.7 ± 2.9 fold higher than that in eggs. The two alternative splicing variants of *CYP4J16* showed a differential expression patterns across all the life stages. For the transcript *CYP4J16A*, the expression level

was low in larvae as compared to others stages, but similar in eggs and larvae. However, the alternative splicing variant *CYP4J16B* showed the highest expression level in pupae.

Tissue-specific expression patterns in larvae

The relative expression profiles of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* were determined in different tissues of third-instar larvae (Figure 3.2). The gene *CYP6AA5* was highly expressed in the midgut, Malpighian tubule and fat bodies. However, the highest expression was observed in the fat bodies for *CYP6AL1*, and in the midgut for *CYP9J32*. The transcript levels of the two alternative splicing variants were different across the tissues. *CYP4J16A* was highly expressed in the midgut, while *CYP4J16B* showed the highest expression in the fat bodies. The expression levels were similar in others tissues for both transcripts.

Tissue specific expression patterns in adults

The tissue-specific expression patterns of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, and the two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* were determined in the midgut, hindgut, Malpighian tubules and fat bodies of adult *Ae*. *aegypti* by RT-qPCR (Figure 3.3). Our results indicated that all the five transcripts were expressed in all the tissues examined. There were no statistically significant differences ($P > 0.05$) in the relative expression levels of *CYP6AA5*, *CYP6AL1*, *CYP4J16A* and *CYP4J16B* among the tissues. In contrast, the expression of *CYP9J32* was 4.1 fold higher ($P < 0.05$) in the midgut than in the hindgut.

Effect of insecticides on expression of cytochrome P450 genes in larvae

Insecticide bioassay allowed the determination of different toxicological parameters. Table 3.6 shows the slopes and intercepts of the regression lines along with the lethal concentrations that kill 20, 50 and 90% of the tested population, representing LC_{20}, LC_{50} and LC_{90}, respectively, with their 95% confidence intervals. In order to investigate the up-regulation

of P450 genes by insecticides, larvae were exposed to each of the three pyrethroid insecticides at their LC_{20} concentrations. The results indicated that significant differences among the genes in terms of their response to insecticide exposure (Figure 3.4). *CYP6AA5* was induced by 2.3 ± 1.3 fold in the larvae exposed to cypermethrin compared to the control group. The expression level of this gene was not statistically different between mosquitoes exposed to permethrin and deltamethrin as compared with their respective controls. The expression levels of *CYP9J32* and *CYP4J16B* were not statistically different between treatments and controls for all three insecticides. However, the transcript levels of *CYP6AL1* and *CYP4J16A* were significantly lowered in the mosquitoes exposed to all three insecticides as compared with their respective controls.

Effect of insecticides on expression of cytochrome P450 genes in adults

The expression levels of the P450 genes were characterized following exposure of adult mosquitoes to each of the three insecticides permethrin, cypermethrin and deltamethrin. RT-qPCR analyses indicated that some of the transcripts were induced while other were inhibited (Figure 3.6). Exposures to permethrin led to the up-regulation of *CYP6AL1* and *CYP4J16B* by 2.1 and 2.5-fold ($P < 0.05$), respectively. In contrast, the expression level of *CYP9J32* was repressed by 44.3%, while the expression of *CYP6AA5* and *CYP4J16A* were not affected by permethrin. Similarly, cypermethrin increased the expressions of *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* by 2.4, 3.5, 2.4 and 23.0-fold ($P < 0.05$), respectively, whereas the expression of *CYP6AA5* was not affected. In deltamethrin treatment, the expression levels of *CYP6AA5*, *CYP4J16A*and*CYP4J16B* were increased by 2.2, 2.1 and 3.6-fold ($P < 0.05$), respectively, but there were no significant differences in the expressions of both *CYP6AL1* and *CYP9J32.*

Analysis of role of the nuclear receptor HR96 in up-regulation of cytochrome P450 genes in adult mosquitoes

Our earlier experiments have shown that some P450 genes can be up-regulated by permethrin (*CYP6AL1, CYP4J16B),* cypermethrin (*CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B*) and deltamethrin (*CYP6AA5*, *CYP4J16A, and CYP4J16B*) in adult mosquitoes. In order to evaluate the possible role of the nuclear receptor *HR96* in regulating P450 genes in response to insecticide exposure, RNAi was performed to silence *HR96* gene in adult mosquitoes followed by insecticide exposure and monitoring the transcript level of a P450 gene.

RT-qPCR analyses at different times after the injection of *dsHR96* showed significantly decreased transcript level of *HR96* (77.65% at 72 h) and (81.7% at 96 h) as compared with the controls, indicating effective gene silencing (Figure 3.7). The exposure of mosquitoes to permethrin 96 h after silencing of *HR96* did not show the effect on the expressions levels of *CYP6AL1* and *CYP4J16B as* compared with the controls (Figure 3.8). Similar results were observed for *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* in *dsRH96*-injected mosquitoes that were subsequently exposed to deltamethrin (Figure 3.10). However, RNAi of *HR96* followed by the exposure to cypermethrin for 24 h led to a 10.1-fold decrease of the transcript level of *CYP4J16B* as compared with its expression in *dsGFP*-injected mosquitoes and exposed to the same insecticide ($P < 0.05$). The expression of *CYP6AA5* and *CYP4J16A* were not affected by RNAi (Figure 3.9). These results suggest that *HR96* play an important role in the up-regulation of *CYP4J16B* by permethrin, but does not appear to be involved in the up-regulation of other P450genes in adults of *Ae. aegypti*.

Sequence analysis of selected cytochrome P450 cDNA, deduced amino acid and genomic sequences

We sequenced the cDNAs of the five transcripts (*CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A*, and *CYP4J16B*) of the selected P450 genes. These cDNA sequences were consistent with those predicted based on the genome database. The deduced amino acid sequence (Figure 3.12 and 3.13) showed the structural features common to P450 genes such as the C-helix motif (WxxxR) located at the C-terminal, the Helix I motif (GxE/DTT/S), the motif (ExLR) in the K-helix, the PERF motif (PxxFxPE/DRF) also located in the K helix, and the heme-binding motif (PFxxGxRxCxG/A), which contain a cysteine residue involved in the binding of heme iron in the fifth coordination site (Feyereisen, 2005). Figure 3.11 shows the schematic exon-intron organization of the gene *CYP4J16* and its two alternative splicing variants (B), and the phylogenetic relationship among the candidate genes and their orthologous genes in other mosquito species (A).

Discussion

Insect P450 enzymes play important roles in the metabolism of xenobiotic compounds resulting in most cases in detoxification (Nelson, 1998; Feyereisen 1999, 2005, 2012; Werck-Reichhart and Feyereisen, 2000; Lamb et al., 2009). Although many studies have identified P450 genes over-expressed in pyrethroid resistant *Ae. aegypti*, identification of key resistance genes has proven difficult. One of the main reasons is the presence of up to 158 P450 genes in its genome, allowing this mosquito to cope with various xenobiotics. Moreover, the ability of P450 enzymes to be induced not only by insecticides but also by other chemicals such as heavy metals (e.g. copper) (Marcombe et al., 2009) lead to the development of resistance to insecticides not previously used. For these reasons, over-expression of a P450 gene cannot be accounted by itself for detoxification capability, but provides insight for candidate gene selection.

RT-qPCR analyses showed the differential expression of the selected P450 genes in four developmental stages and tissues of adults and larvae. Despite these differences, three of the genes (*CYP6AA5, CYP6AL1* and *CYP9J32*) were all highly expressed in larvae compared to other stages. The expression level of *CYPJ16A* was higher in eggs and adults while *CYP4J16B* was predominantly expressed in pupae. Larval exposure to insecticides led to the up-regulation and down-regulation of P450 transcripts depending of the gene and the insecticide used.

Larval tissue-specific expression profiles were higher in the midgut, fat bodies and Malpighian tubule. *CYP4J16A* was predominantly expressed in the midgut while *CYP4J16B* showed the highest expression in the fat bodies. Tissue-specific expression patterns of *CYP6AA5*, *CYP6AL1*, CYP9J32, *CYP4J16A* and *CYP4J16B* showed that these P450 were expressed in all adult tissues. The expression of *CYP9J32* was particularly higher in the midgut than others tissues.

The distinct expression patterns of selected P450 genes in larvae suggest that they may be involved in important biological processes during this life stage of the mosquito. In insects, the midgut and fat bodies are generally considered as the primary detoxification organs in which detoxification enzymes are mostly expressed (Hodgson 1985). The higher expression of these genes in these tissues supports this view, and may reflect the role of these genes in the detoxification of xenobiotics, although the expression level of a gene does not always reflect its functional importance.

The expression of insect P450 genes is clearly inducible by xenobiotics and the up-regulation of specific P450s have been observed after exposure of organisms to a variety of organic chemicals (Scott, 1999; Baldwin et al., 2006; Poupardin et al., 2008). This overexpression of P450 genes and their up-regulation by xenobiotics have often been associated

with enhanced detoxification of insecticides or plant secondary metabolites (Liu et al., 2011). The down-regulation of P450 genes has been also observed in some insect species in response to insecticide exposure (Riddick et al., 2003). In the oriental fruit fly, *Bactrocera dorsalis*, exposed to abamectin and beta-cypermethrin, the genes *CYP4AC4* and *CYP4D47* were down-regulated by 49 and 31%, respectively (Huang et al., 2013); however, the significance of this down-regulation remains unknown (Riddick et al., 2004). In mammals, it is known that exposure to some substrates can lead to the generation of reactive oxygen species (ROS) by P450 enzymes, which can cause lipid peroxidation and oxidative stress (Blanck et al., 1991; Gorsky et al., 1984; Zhukov and Archakov, 1982, 1985). In turn, oxidative stress down-regulates P450 expression levels by a variety of feedback mechanisms (Davydov, 2001; Zangar et al, 2004). In human, the down-regulation of *CYP1A1* is mediated by ROS inactivation of nuclear factor 1, thereby preventing the transcription of *CYP1A1* (Barouki and Morel, 2001).We speculated that larval exposure to permethrin, cypermethrin and deltamethrin also led to the formation of ROS that may have blocked the transcription factors regulating the expressions of *CYP6AL1* and *CYP4J16A.*

Knockdown of *HR96*, a putative transcription factor and ortholog of vertebrate CAR and PXR (King-Jones and Thummel 2005; Laudet et al. 2005) reduced the up-regulation of *CYP4J16B* by cypermethrin but no effects were found with the up-regulation of other P450 genes by cypermethrin. In addition, the up-regulation of all the investigated genes by permethrin and deltamethrin was not affected by silencing of *HR96*. These results indicate that *HR96* is indeed involved in the up-regulation of *CYP4J16B* by cypermethrin. In *Ae. aegypti*, there are 20 nuclear receptors (Cruz et al., 2009) probably acting as transcription factors for other P450 genes.

In conclusion, this study showed the differential expression patterns of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, and *CYP4J16A* and *CYP4J16B* in different tissues of larvae and adult mosquitoes. Furthermore, we demonstrated the role of *HR96* in mediating the up-regulation of *CYP4J16B*. Further studies are needed to understand the molecular mechanism of nuclear receptor-mediated up-regulation of P450 genes in insects.

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Table 3.1 Over-transcribed P450 genes in *Ae. aegypti* and others mosquito species resistant to insecticides

Species	Gene over-transcribed		Insecticides for resistance	Methods	References
	Larvae	adults			
Aedes aegypti	*CYP6M11, CYP9M8, CYP314A1, CYP6N12, CYP6AL1, CYP9M9, CYP314A1,*		Permethrin	Poupardin et al. (2008, 2010)	Microarray RT-qPCR
	CYP9J10, CYP9J27, CYP9J32		Permethrin	Strode et al. (2008)	RT-qPCR
	CYP6BB2, CYP6M6, CYP6Y3, CYP6Z6, CYP6M10, CYP6AA5, CYP9J23, CYP9J22, CYP9J9, CYP4J15, CYP4D23CYP6M11, CP9M9, CYP6Z6, CYP6Z8	*CYP6CB2, CYP6M11, CYP6Z6, CYP6M6, CYP9J22, CYP9M9, CYP9J6,CYP6Z8, CYP9J22, CYP9M9*	Temephos Deltamethrin	Marcombe et al(2009, 2012)	Microarray RT-qPCR
Culex quinquefasciatus	*CYP6AA7,CYP9J34, CYP9J40, CYP9M10*	*CYP6AA7*	Permethrin	Liu et al. (2011)	RT-qPCR
	CYP6F1		Permethrin	Kasai et al. (2000);Gong et al. (2005)	RT-qPCR
	CYP4H21, CYP4H22, CYP4H23, CYP4J4, CYP4J6		Deltamethrin	Shen et al. (2003)	RT-qPCR
Anopheles gambiae	*CYP6Z1*			RT-qPCR	Nikou et al. (2003)
	CYP4G16, CYP325C2, CYP6M2 CYP6AG2, CYP9J5	*CYP325C2, CYP4G16, CYP325C1, CYP12F2, CYP6Z1*		Strode et al. (2006)	Microarray
		CYP6P3	Permethrin	Stern et al. (2008)	RT-qPCR Microarray
Anopheles funestus	*CYP6P4, CYP6P9*		Pyrethroid	Amenya et al.(2008), Wondji et al.(2009)	RT-qPCR Microarray

Table 3.2 Amino acid comparison of *Ae. aegypti* P450s with those of other mosquito species

P450 gene from other species		*Aedes aegypti* P450 gene	Score	Expect value	% identity	Observations
Culex quinquefasciatus	*CYP6AA7*	*CYP6AA5*	626	0.0	58	orthologous
		CYP9J24	*756*	0.0	67	
		CYP9J28	753	0.0	67	
	CYP9J34	*CYP9J23*	684	0.0	58	
		CYP9J26	672	0.0	58	
		CYP9J31	646	0.0	57	
		CYP9J32	637	0.0	57	
		CYP9J32	764	0.0	68	
	CYP9J40	*CYP9J22*	733	0.0	65	
		CYP9J21	673	0.0	61	
	CYP6F1	*CYP6AL1*	575	e-166	56	orthologous
Anopheles gambiae	*CYP9J5*	*CYP9J26*	657	0.0	58	
		CYP9J32	649	0.0	59	orthologous
		CYP9J27	639	0.0	56	
		CYP9J22	637	0.0	59	
		CYP9J28	630	0.0	57	

Table 3.3 P450 genes primers used for RT-PCR and RT-qPCR and their relevant parameters

Gene Name	Sequence (5'-3')	Primer Length (base)	Tm (^{O}C)	Product size (bp)
AeRps17	F: CTAAGTCTGCTAAAGTAT	18	51.5	110
	R: CTTATACGATACACTCTC	18	51.9	
AeRps10	F: TCGTATGGAATTGTTGTAA	19	55.6	82
	R: GAAGAGGTACTCGTAGAT	18	55.7	
AeRps5	F: GCATTCCGTAACATTAAG	18	54.9	104
	R: AATTCGTCCTTCTTCTTG	18	55.9	
CYP6AA5	F: ACAGAGTCTTCAATCCAA	18	56.7	165
	R: TGTTGTTCTTCTCACGAT	18	57.5	
CYP6AL1	F: GATAGATAAGGCGACTGT	18	56.9	95
	R: TAGTGTAACGAGCGAATA	18	56.4	
CYP9J32	F: CCGTGATAATGAGTTCTAC	19	56.3	175
	R: TACTGCTTCCTTGATGAT	18	56.6	
CYP4J16	F_A: ATATCTTGCCGAAGTTCTTG	20	59.7	85
	R_A: TTGCCTGCCTTAACATCT	18	60.4	
	F_B: CAACAACGGATAATGAACC	19	58.2	92
	R_B: ATTGGACGGAGAATCTTG	18	58.1	

Table 3.4 Primers used for P450 sequencing and their relevant parameters

Gene name	Sequence (5'-3')	Primer Length (base)	Tm (^{O}C)	Product size (bp)
CYP6AA5	F1: ATGGTGTTCCTCTTCACAACACT	23	59.95	800
	R1: CGGGAAATGTTGTTCTTCTCA	21	60.10	
	F2: GAACATGGTGCGCAAGAC	18	58.73	766
	R2: CTAGATCCGTTCAACATCCAGC	22	61.01	
CYP6AL1	F1: ATGCTGTTCTTAGCTTTCGCTATT	24	59.97	780
	R1: AATTCCATTACGTTCCCGATG	21	60.92	
	F2: AACCATTGAACATCGGGAAC	20	59.65	778
	R2: TCATCGCTTCTTAACAACTAGCTG	24	60.10	
CYP9J32	F1: ATGGAGGTGAACCTGCTTTTATT	23	60.24	850
	R1: TGTCAGGACGAACGATCC	18	58.01	
	F2: TCAAGGAAGCAGTACGAGCTC	21	59.77	824
	R2: TCACTTCCTCTTCTTAAATCTCAAATG	27	60.48	
CYP4J16	F1: ATGTACGTTTTTACCACCGTTG	22	58.86	800
	R1: TCCACGTTTTTCATCGTTTC	20	58.50	
	F2: CATCATCCGACAGCGAAGA	19	60.94	760
	R2: TTAGCGTCGTTCGAATTTGAC	21	60.26	
	F3: ATGGATTGGCTTACGATCGTAC	22	60.23	700
	R3: GTCGGAAACCTAACAGGCG	19	60.64	
	F4: ACCGCCTGTTAGGTTTCCG	19	62.32	681
	R4: CAATACAGTTCCTCGGCCC	19	60.47	

Table 3.5 Primers and their relevant parameters used for *HR 96* dsRNA synthesis, RT-qPCR analysis and sequencing

Application of Primers	Sequence (5'-3')	Primer Length (base)	Tm (^{O}C)	Product Size (bp)
RT-PCR and RT-qPCR	F: TTTAGTGAGAGTTGCGAAAT	20	59	92
	R: TCCTTCTTCATGCCTATCT	19	59	
dsRNA synthesis	F: TAATACGACTCACTATAGGGCCCAGGTTGAACCACTGTTG	40	61.3	434
	R: TAATACGACTCACTATAGGGTTTCCGTGTTCATTGATTGC	39	59.5	
cDNA cloning	F1: ATGAGCACCGAAAGTTCGG	19	61.19	800
	R1: AGAGTCAGAGCCGGTAGCAA	20	60.16	
	F2: CTCTTATCAGCAGCGATGTCTG	22	60.18	830
	R2: CAAAGTGCTCGAGAAAAATGTG	22	59.92	
	F3: CAGACCCCGCCACTAAATC	19	60.47	830
	R3: GCAGCTCGGAGATCTTCTGTAT	22	60	
	F4: TACAGAAGATCTCCGAGCTGC	21	59.73	538
	R4: GAAGTATTGATAACGTGACGATTTATT	27	58.22	

Table 3.6 Susceptibility of *Ae. aegypti* larvae to three pyrethroid insecticides

Insecticide	n	LC_{20} (ppm) (95% CI)	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)	Slope ±SE	Intercept ±SE	χ^2	$P > \chi^2$
Permethrin	377	1.60 (1.39 -1.77)	2.24 (2.04 - 2.42)	3.73 (3.41- 4.16)	5.77 ± 0.527	15.3 ± 1.36	10.8	0.977
Cypermethrin	360	0.21 (0.18 - 0.20)	0.28 (0.26 - 0.3)	0.44 (0.41 - 0.49)	6.53 ± 0.613	23.16 ± 2.14	14.18	0.894
Deltamethrin	361	0.22 (0.20 - 0.24)	0.28 (0.27 - 0.3)	0.42 (0.39 - 0.47)	7.52 ± 0.701	26.62 ± 2.45	16.92	0.767

Table 3.7 Susceptibility of *Ae. aegypti* adults to three pyrethroid insecticides

Insecticide	n	LT_{20} (min) (95% CI)	LT_{50} (min) (95% CI)	LT_{90} (min) (95% CI)	Slope ±SE	Intercept ±SE	χ^2	$P > \chi^2$
Permethrin	475	14.33 (12.33-16.11)	22.87 (20.86-24.82)	46.61 (42.17-52.78)	4.14 ± 0.32	-5.63 ± 0.47	15.84	0.823
Cypermethrin	496	8.95 (7.20-10.54)	15.59 (13.68-17.36)	36.24 (32.41-41.59)	3.49 ± 0.29	-4.17 ± 0.41	8.58	0.99
Deltamethrin	482	9.45 (7.63-11.10)	16.24 (14.27-18.1)	37.02 (33.15-42.38)	3.58 ± 0.3	-4.33 ± 0.42	6.39	0.99

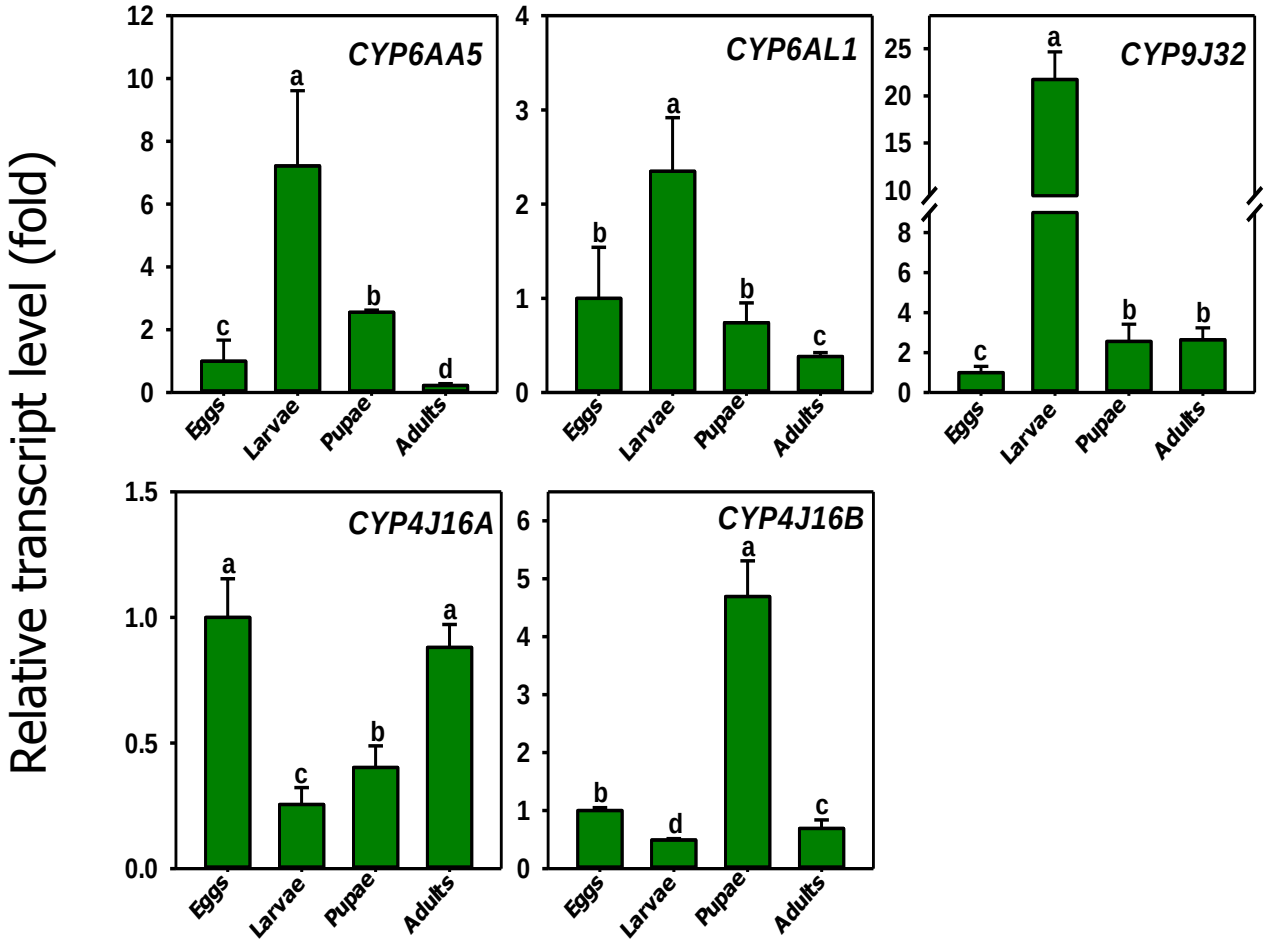


Figure 3.1 Developmental stage expression patterns. ***Ae. aegypti Rps17*** **was used as reference gene. The mean expression in each treatment is shown as fold change compared to the expression in eggs, which has been arbitrary set as 1. Different letters on the bars indicate statistically significant difference among the different life stages.**

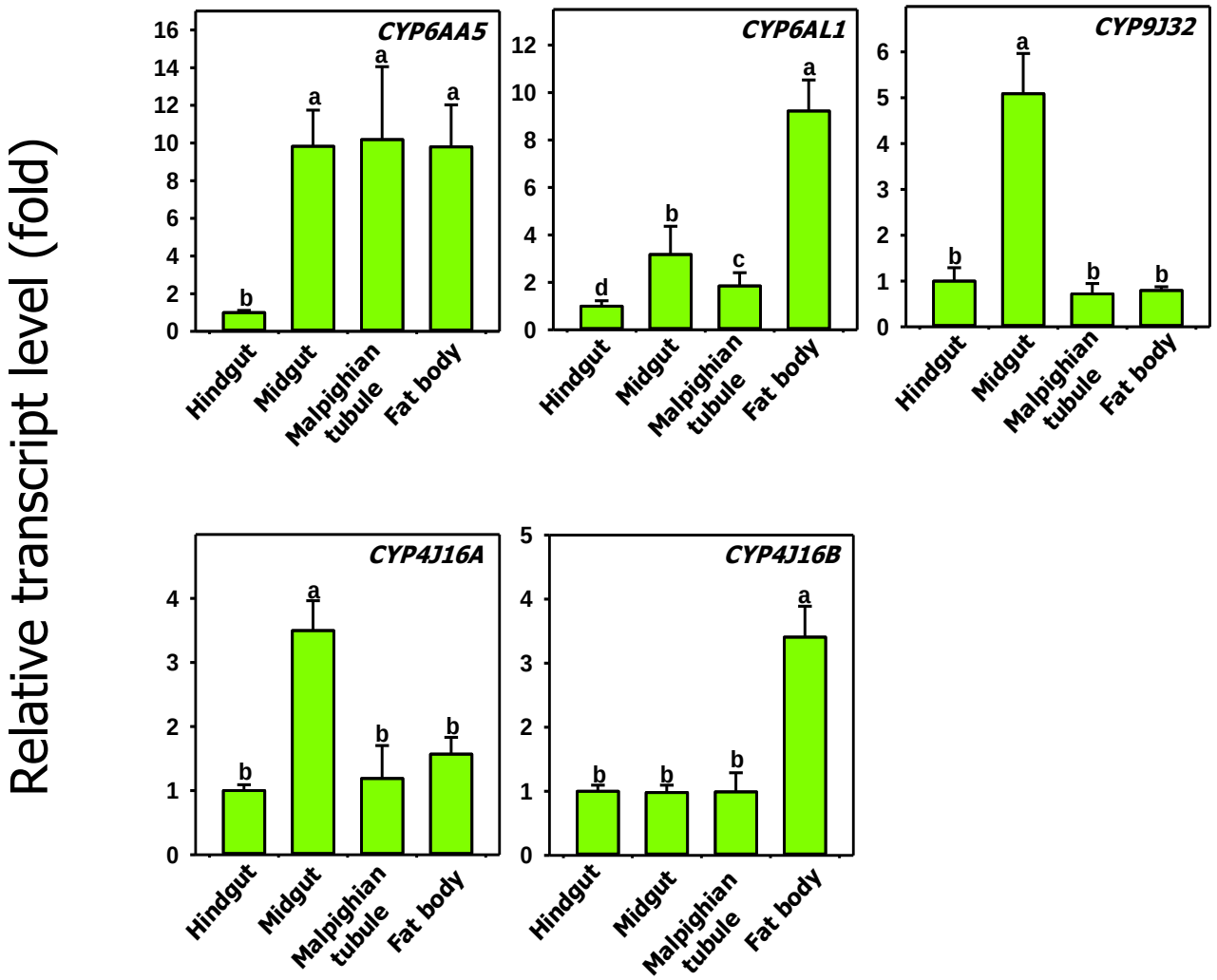


Figure 3.2 Tissue-specific expression patterns in larvae. The relative expression of genes was normalized using *AeRps17* as reference gene. The mean expression for each tissue is shown as fold change compared with the mean expression in hindgut, which has been arbitrary set as 1. Different letters on the bars indicate significant difference of the expression levels among the tissues.

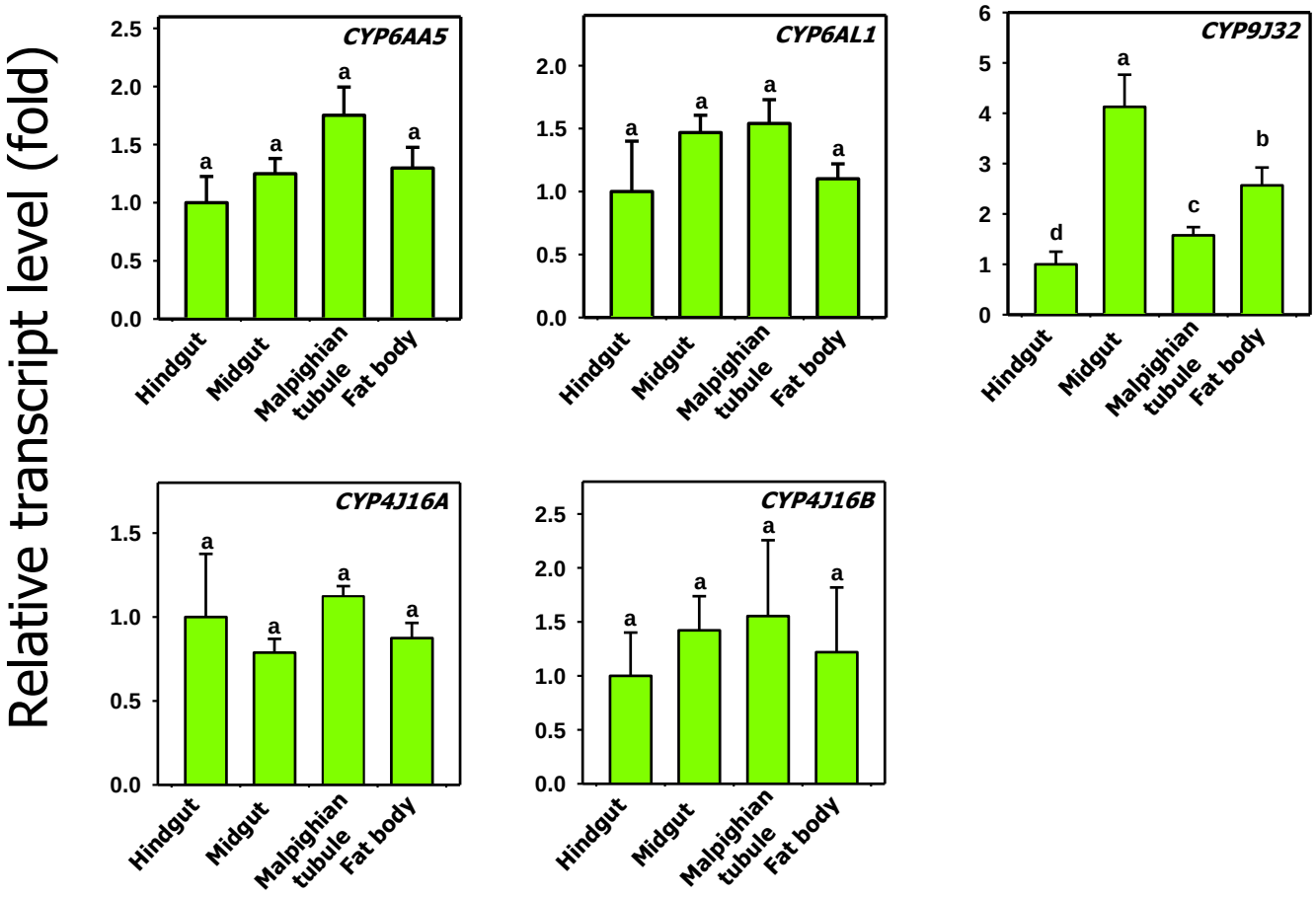


Figure 3.3 Tissue-specific expression patterns in adults. The relative expression of genes was normalized using *AeRps17* as reference gene. The mean expression for each tissue is shown as fold change compared with the mean expression in hindgut, which has been arbitrary set as 1. Different letters on the bars indicate significant difference of the expression levels among the tissues.

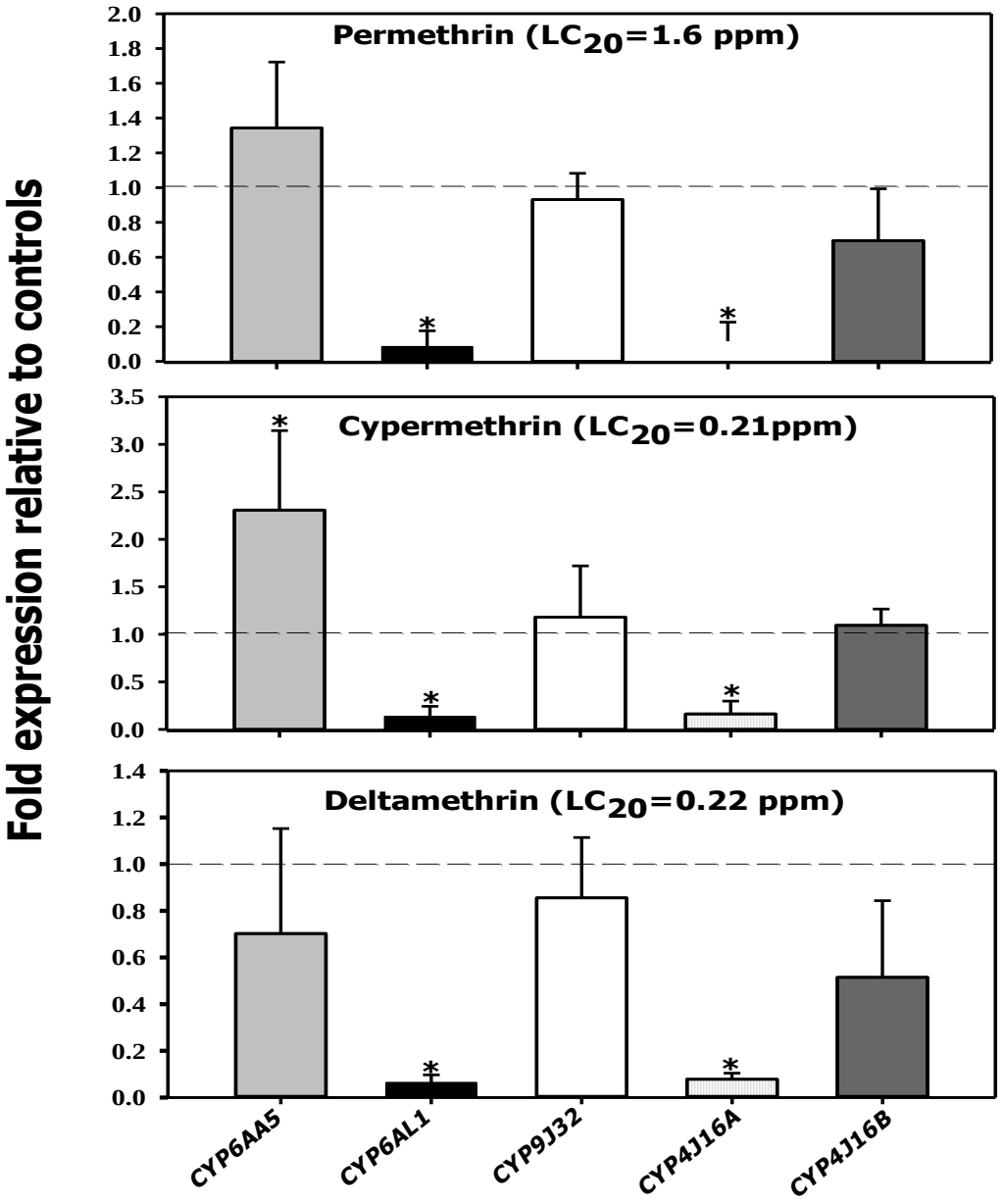


Figure 3.4 Effects of three insecticides(permethrin, cypermethrin and deltamethrin) on the expression levels of P450 genes in larvae. Dashed lines indicate the controls arbitrary

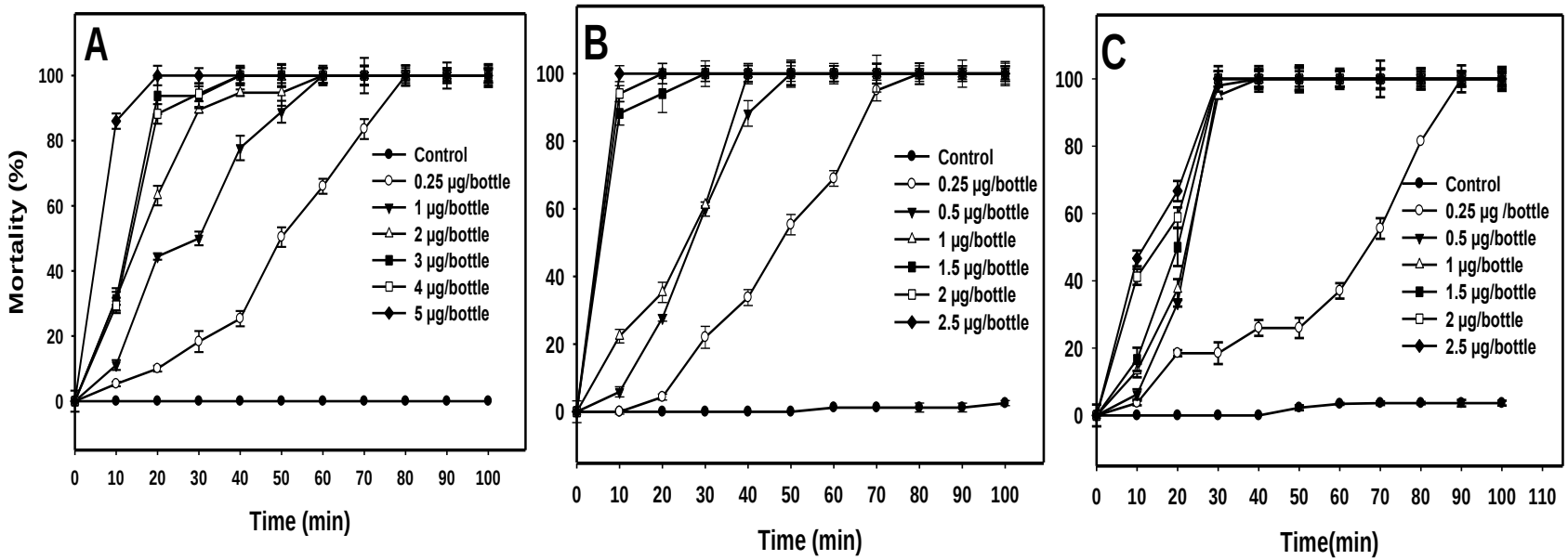


Figure 3.5 Pre-exposure of *Ae. aegypti* adults to different concentrations of insecticides for a period of 100 min. A: permethrin, B: cypermethrin, C: deltamethrin.

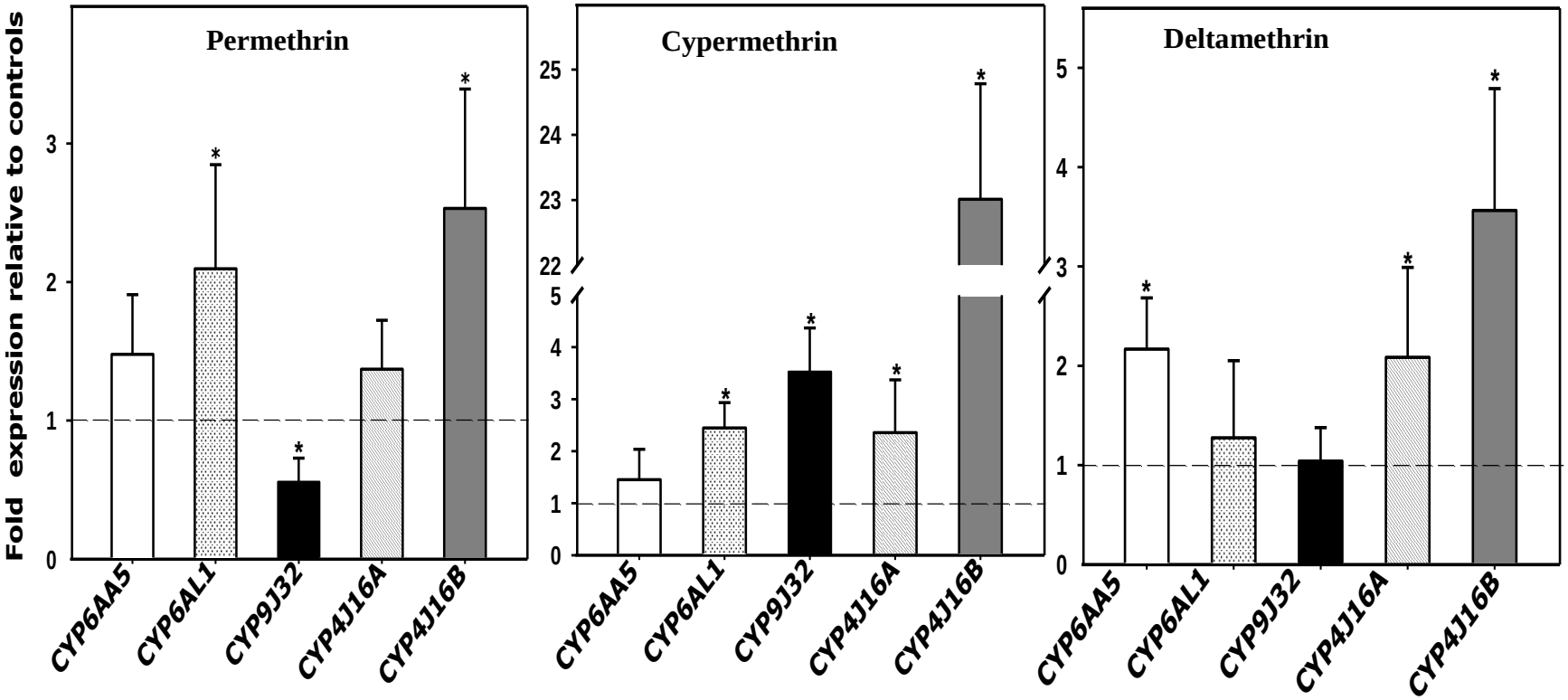


Figure 3.6 Effect of insecticide treatment on the transcript levels of P450 genes in *Ae. aegypti* adults. Mosquitoes were exposed to each insecticide at the concentration of 0.25 g/bottle. Data are presented as mean ± SE of three biological replicates and two technical replicates. Dashed lines indicate controls which have been arbitrarily set as 1. An asterisk on the bars indicate significant differences between treatment and controls (P < 0.05 t-test).

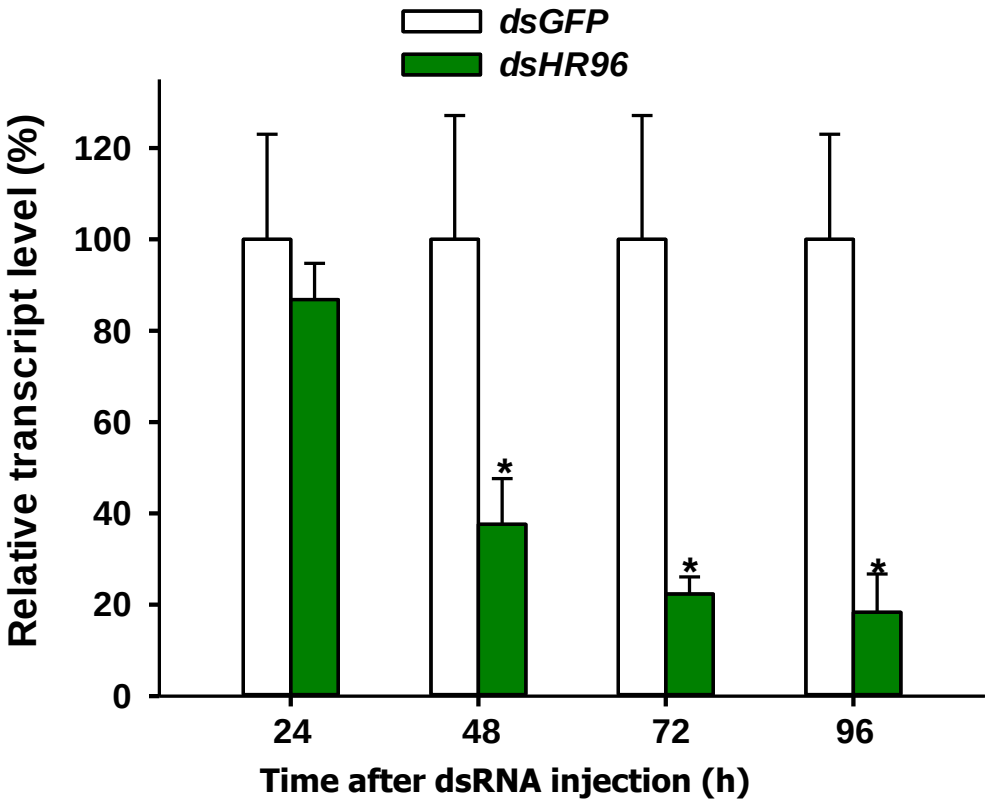


Figure 3.7 Effect of dsRNA treatment on the transcript level of *HR96* in adult of *Ae. aegypti.* The relative expression levels (%) are presented as the mean and standard errors of three biological replicates. An asterisk on the bar indicates significant difference between the mosquitoes injected with dsRNA of a P450 gene and dsRNA of GFP gene based on Student's t-test within the same time point (P < 0.05).

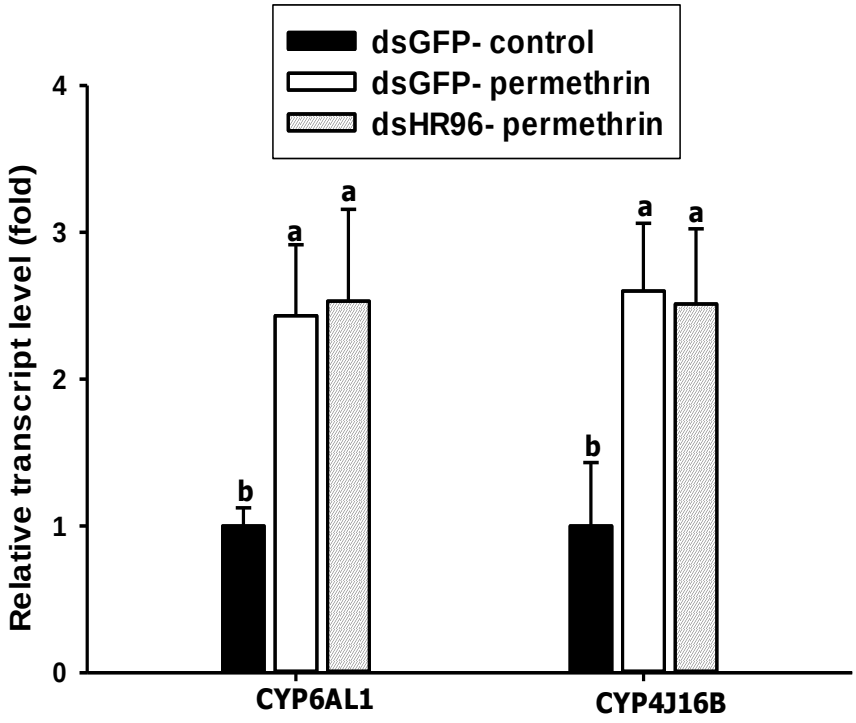


Figure 3.8 Effect of injection of dsHR96 on the induction of P450 genes by permethrin. Different letters on the standard error bars indicate significant differences based on ANOVA followed by Tukey's honestly significant difference (HSD) multiple comparison ($P < 0.05$).

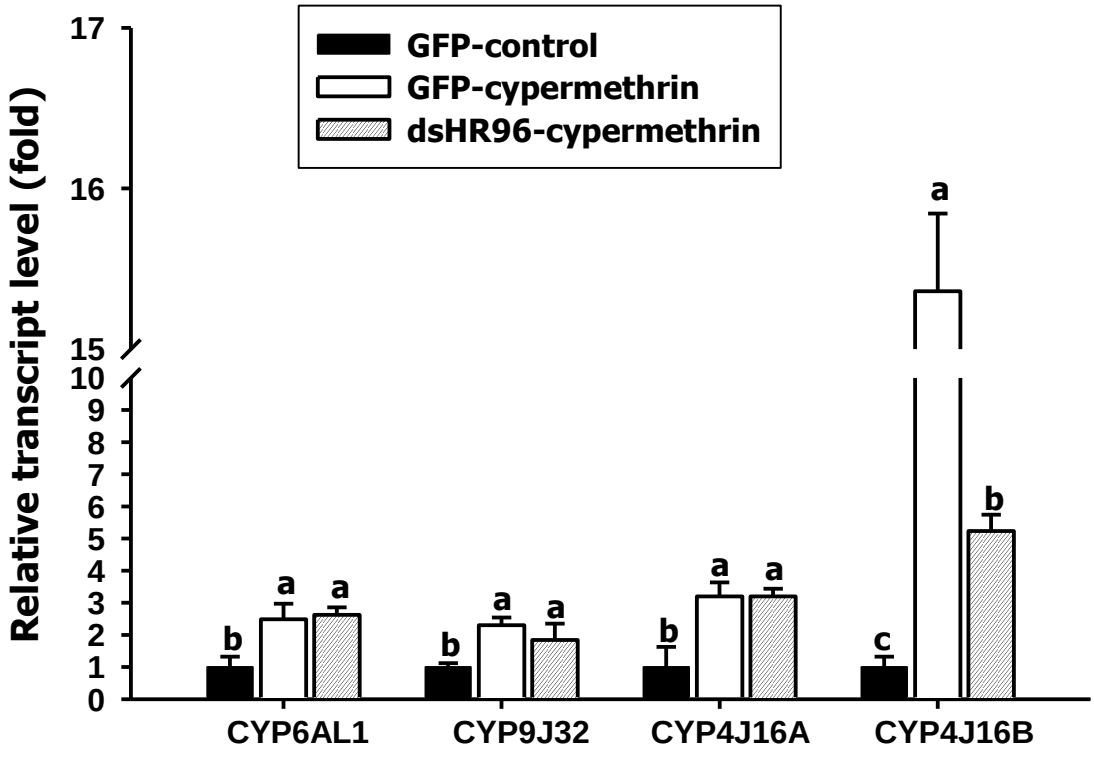


Figure 3.9 Effect of injection of dsHR96 on the induction of P450 genes by cypermethrin. Different letters on the standard error bars indicate significant differences based on ANOVA followed by Tukey's honestly significant difference (HSD) multiple comparison ($P < 0.05$).

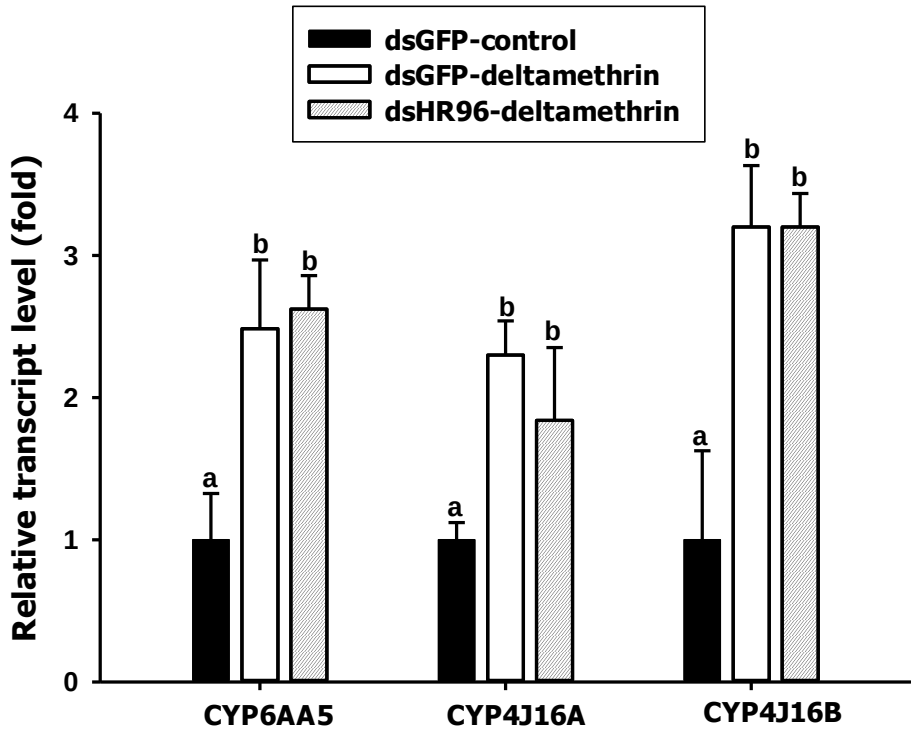


Figure 3.10 Effect of injection of dsHR96 on the induction of P450 genes by deltamethrin. Different letters on the standard error bars indicate significant differences based on ANOVA followed by Tukey's honestly significant difference (HSD) multiple comparison ($P < 0.05$).

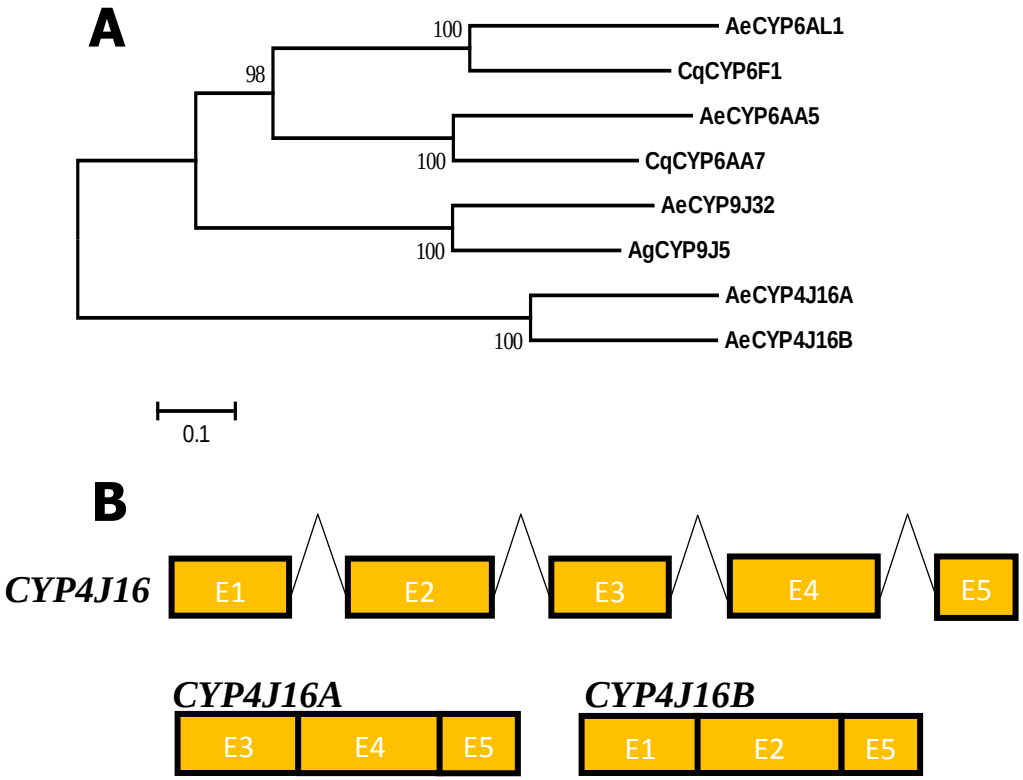
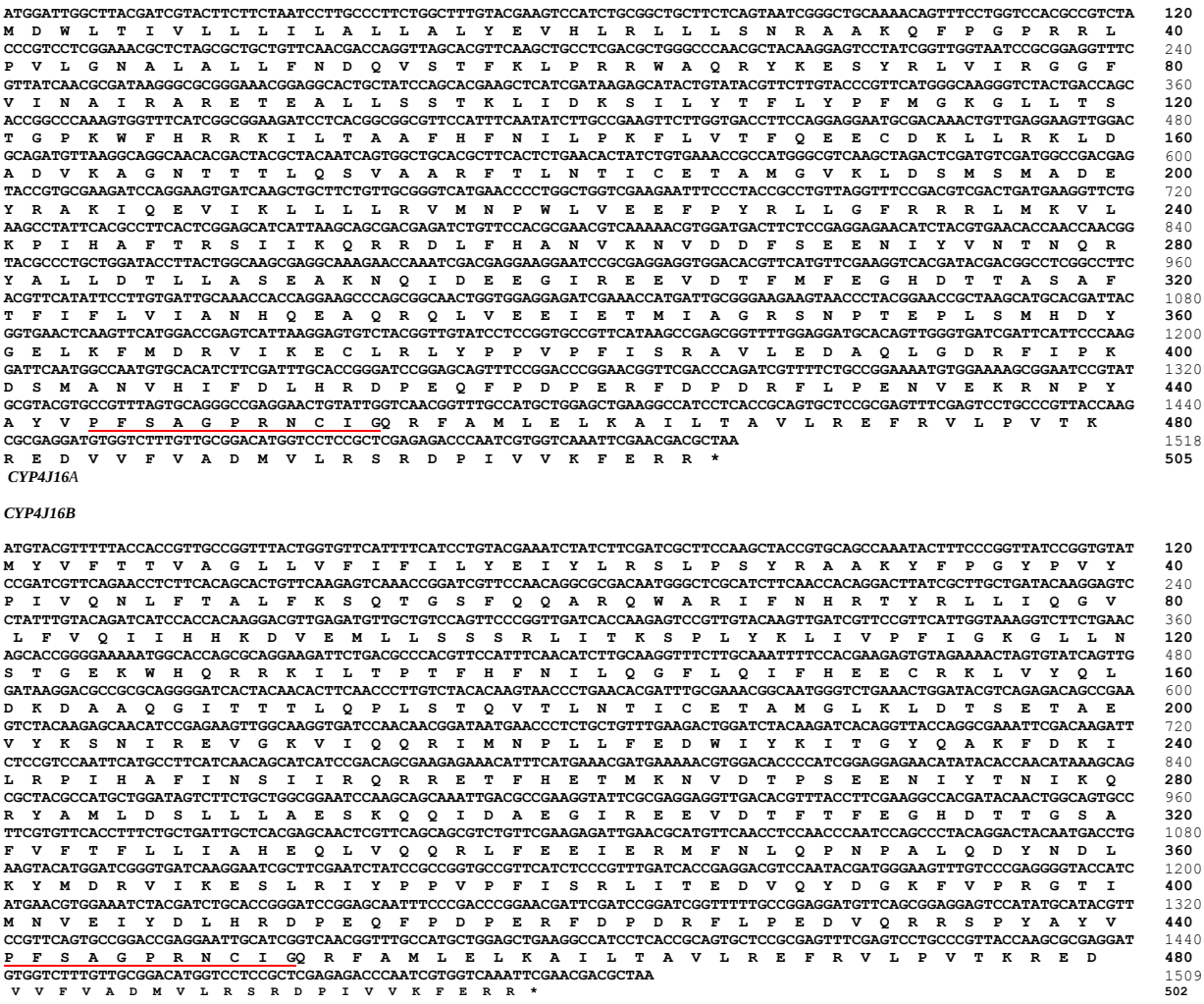


Figure 3.11 Phylogenetic relationship of deduced amino acid sequences of candidate genes from *Ae. aegypti* and their orthologs from other mosquito species: *Ae. aegypti* (*AeCYP6AA5*, *AeCYP6AL1*, *AeCYP9J32*, *AeCYP4J16A*, *AeCYP4J16B*); *Culex quinquefasciatus* (*CqCYP6F1*, *CqCYP6AA7*); *Anopheles gambiae* (*AgCYP9J5*). The tree was constructed using neighbor joining algorithm in MEGA5 software. The significance of the trees was tested using 3000 bootstrap replicates (A). Exon-intron organization of *CYP4J16* and its two alternative splices variants (B)

Figure 3.12 Nucleotide and deduced amino acid sequences of *CYP6AA5*, *CYP6AL1* and *CYP9J32*.The start and stop codons are highlighted in black. The heme signature regions are underlined in red, and the other motifs highlighted in cyan.



CYP4J16A

CYP4J16B

Figure 3.13 Nucleotide and deduced amino acid sequences of two alternative splicing variants of *CYP4J16* (*CYP4J16A* and *CYP4J16B*).The start and stop codons are highlighted in black. The heme signature regions are underlined in red, and the other motifs highlighted in cyan.

Chapter 4 - Functional Analysis of Cytochrome P450 Genes in *Aedes Aegypti*

Abstract

In this study, we evaluated the role of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16* (*CYP4J16A*, *CYP4J16B*) in the detoxification of permethrin, cypermethrin and deltamethrin by RNA interference (RNAi) followed by insecticide bioassays in both adults and larvae of *Aedes aegypti*. RNAi by feeding larvae with chitosan/dsRNA nanoparticles led to reduction of the transcripts *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* by 38.7, 46.0, 46.52, 44.0 and 41.0%, respectively. Analysis of the detoxification roles of these genes by RNAi followed by insecticide bioassay increased larval mortality by 41.2% to cypermethrin when *CYP6AA5* was silenced and by 46.0% to permethrin when *CYP9J32* was silenced. These results suggested that *CYP6AA5* and *CYP9J32* play important roles in the detoxification of cypermethrin and deltamethrin, respectively, in *Ae. aegypti* larvae. In adult mosquitoes, RNAi experiment showed the reductions of *CYP6AA5*, *CYP6AL1*, and *CY4J16A* transcripts by 77.9, 80.0 and 87.1%, respectively, 96 h after the injection of their corresponding double-stranded RNAs (dsRNAs). The expression level of *CYP9J32* was suppressed by 46.5% 24 h after injection, but slightly increased for the rest of the experiment. In contrast, *CYP4J16B* was repressed by 78.2% at 72 h following dsRNA treatment. Exposure of mosquitoes injected with *CYP6AA5* dsRNA to cypermethrin for 24 h led to 29.3% increase in mortality as compared with the control. Furthermore, regression analysis of the mosquito susceptibility to cypermethrin showed significant statistical differences between the slopes and intercepts ($P = 0.028$ and $P = 0.010$, respectively) of the regression lines corresponding to the mosquitoes injected with *CYP6AA5*

dsRNA and those injected with *GFP* dsRNA. These results indicate that *CYP6AA5* is involved in detoxification of cypermethrin in *Ae. aegypti* adults. Homology modeling of CYP6AA5 followed by ligand docking, have allowed us to examine the different binding conformation of the three pyrethroid insecticides used in this study. The distances between the heme iron and the putative aromatic hydroxylation site were 9.2, 9.4 and 7.2 Å for permethrin, cypermethrin and deltamethrin, respectively. For aliphatic hydroxylation site these distances were 5.3, 2.8 and 2.9 Å. These results supported that CYP6AA5 may be able to metabolize cypermethrin and deltamethrin preferentially by aliphatic hydroxylation as indicated by the close interaction with the heme iron.

Keys words: Cytochrome P450, *Aedes aegypti*, detoxification, RNA interference, homology modeling.

Introduction

Cytochrome P450 monooxygenases (P450s) constitute important enzymes because of their involvement in the metabolism of various compounds in many organisms (Werck-Reichhart and Feyereisen, 2000; Feyereisen, 2012). In insects, they play critical roles in the metabolism of ecdysteroids, fatty acids and xenobiotics such as insecticides and plant allelochemicals (Berenbaum, 1991; Schuler, 1996; Feyereisen, 2012; Hemingway et al., 2004; Scott, 1999). Most of the studies on insect P450s have focused on P450's involvement in the detoxification of insecticides leading to resistance.

The sequencing and annotation of several insect genomes have revealed the presence of large number of P450 genes. For instance, 90 genes in *Drosophila melanogaster* (Adams et al., 2000), 105 in *Anopheles gambiae* (Raghavendra et al., 2012), 84 in *Bombyx mori* (Ai et al., 2011), 48 in *Apis mellifera* (Claudianos et al., 2006), 159 in *Aedes aegypti* (Nene et al., 2007), 204 in *Culex quinquefasciatus* (Yang and Liu, 2011), 38 in *Pediculus humanus humanus* (Lee et al., 2010), and 143 in *Tribolium castaneum* (Richards et al., 2008) have been reported.

Ae. aegypti, a major vector of arboviral diseases (dengue fever, chikungunya and yellow fever), has become resistant to pyrethroid insecticides in many parts of the world (Chuaycharoensuk et al., 2011; Flores et al., 2013; Fonseca-Gonzalez et al., 2011; Hemingway et al., 1989; Koou et al., 2014; Thanispong et al., 2008; Ponlawat et al., 2005). Although *Ae. aegypti* P450s have been annotated, very little has been known for their roles in metabolism of insecticides. RNA interference (RNAi) is a powerful tool for studying the function of a gene by suppressing its mRNA thus preventing the formation of the gene product (usually a protein) (Fire et al., 1998) and, potentially, for developing novel strategies for vector control and pest management (Mao et al., 2007; Baum et al., 2007). RNAi experiments with different dsRNA

delivery methods have been widely used to investigate the functions of essential genes in insects (Belles, 2010; Guo et al., 2012). Currently there are many approaches of delivering dsRNA in insects, which include injection, ingestion, soaking and transfection (Yu et al., 2013). Injection through the body of insect has been the most commonly used method, and has proven to be efficient in many insect species including adult mosquitoes. In mosquito larvae, however, this technique has shown limited success mainly because of the high rate of mortality observed. Recent studies have demonstrated that in mosquito larvae RNAi can be achieved by ingestion of chitosan/dsRNA nanoparticles. The first report was by Zhang et al. (2010) when they developed chitosan/dsRNA nanoparticles to feed *Anopheles gambiae* larvae, and were able to knockdown two chitin synthase genes. The same method has been used to disrupt the axon guidance gene semaphoring involved in olfactory system development in *Ae. aegypti* (Mysore et al., 2013). These facts highlight the significance of RNAi by ingestion of chitosan/dsRNA nanoparticles. Chitosan is de-acetylated chitin polymers and has positively charged polysaccharides that can interact with macromolecules (DNA, RNA, and dsRNA) through electrostatic interactions (Zhang et al., 2010). The role of the chitosan/dsRNA nanoparticles is to stabilize the dsRNA molecules through these interactions and thus protect them from degradation during and after feeding.

In the present study, we evaluate the role of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16* (*CYP4J16A*, *CYP4J16B*) in the detoxification of permethrin, cypermethrin and deltamethrin in both adults and larvae of *Ae. aegypti* by using RNAi followed by insecticide bioassay. We further built a homology model of CYP6AA5 three-dimensional structure and docked the insecticides in its catalytic pocket to investigate whether these substrates were sterically accessible and potentially be metabolized by CYP6AA5.

Materials and Methods

Mosquito strain and rearing

Eggs of *Ae. aegypti* (Liverpool LVP-IB12 strain, cat. No MRA-735) were obtained from BEI Resources (NIAID, NIH, Manassas, VA, USA) and reared under standard insectary conditions [27^{o}C, 80% humidity with a photoperiod of 12/12 (light/dark)]. Larvae were fed with ground dog food, whereas adults were fed either on 10% sucrose or on defibrinated sheep blood.

Total RNA extraction and reverse transcription

Total RNA was extracted from third-instar larvae by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The total RNA sample was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, 5 µg of total RNA were treated with DNase I kit (Life Technologies) to remove potential genomic DNA, and used for reverse transcription to generate single strand cDNA with the first strand cDNA synthesis kit (Thermo Scientific) and oligo $(dT)_{18}$ primers in a 20-µl reaction. The first strand cDNA was then used as a template for downstream applications.

RNAi-mediated knockdown of cytochrome P450 genes

Synthesis of dsRNA

To examine the role of five P450 transcripts including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* in detoxification of pyrethroid insecticides (permethrin, cypermethrin and deltamethrin) in both larvae and adults of *Ae. aegypti*, RNAi-mediated knockdown of each of these genes was performed. Specific primers with T7 promoter sequences (Table 4.1) for

synthesizing dsRNA of each P450 or GFP gene were designed using E-RNAi web server (http://www.dkfz.de/signaling/e-rnai3/) (Horn and Boutros, 2010). The cDNA previously synthesized was used as template to generate the dsRNAs using MEGAscript RNAi kit (Life Technologies) following the manufacturers protocol. Similarly, to prepare GFP dsRNA for controls, specific primers with T7 promoter sequences were synthesized. The synthesized dsRNA was dissolved in nuclease-free water and quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific). If needed, the concentration was adjusted to 5µg/µl. Five microliters of each synthesized dsRNA were examined by 1% agarose gel to make sure the band of the correct size was obtained. The dsRNA samples were stored at -20°C if it they were to be used in less than one week, or at -80°C for longer period of time.

Preparation of chitosan/dsRNA nanoparticles and larval feeding

For mosquito larvae, RNAi was achieved via feeding of dsRNA using a technique developed by Zhang et al. (2010) who used it to knockdown chitin synthase genes in *Anopheles gambiae*. The technique involves the formation of dsRNA/chitosan nanoparticles, and the preparation of larval food containing the nanoparticles. Briefly, chitosan from crab shells (Cat. No. C3646-25G, ≥75 deacetylated; Sigma-Aldrich, Milwaukee, WI, USA) was dissolved in sodium acetate buffer (0.1M sodium acetate–0.1 M acetic acid, pH 4.5) to make a 0.02% wt/vol working solution. In order to form the dsRNA/chitosan complexes, 100 µl chitosan solution was mixed with 35 µg dsRNA. The mixture was then heated at 55^{o}C for one min and mixed by vortexing for 30 s. Once the dsRNA had been entrapped in nanoparticles, the preparation was centrifuged at 13,000 g for 10 min using a high speed vortex to form particles. These particles resulting from the centrifugation were mixed with 6 mg of grinded larval and subsequently coated with pre-melted agarose. The preparation was exposed at room temperature for

solidification. The gel was then cut into small pieces using a razor blade and used to feed the larvae.

A group of 20 third-instar larvae was daily fed with one third of the diet for three consecutive days. For each gene, the experiment was conducted with three replicates. Similarly, nanoparticles containing GFP dsRNA were also prepared and used to feed larvae for RNAi control. RNAs were extracted at 24, 48 and 72 h of feeding, and used to analyze the RNAi efficiency.

Injection of dsRNA into adults

dsRNA was injected on the base of the adult wings by using a glass needle mounted on a microinjector Nanoject II (Drummond, Broomall, PA, USA). For each gene, 250 to 300 adult mosquitoes aged 3 days, were injected with 300 ng of a specific dsRNA. The same numbers of mosquitoes were injected with 300 ng of GFP dsRNA as negative controls. After the injection, the mosquitoes were fed with 10% sucrose as described previously. To confirm the depletion of each target transcript, total RNA was isolated at 24, 48, 72 and 96 h after the dsRNA injections to monitor the suppression of gene expression by reverse transcription quantitative PCR (RT-qPCR) as previously described.

Reverse transcription quantitative PCR analysis

RT-qPCR was used to monitor the change of the P450 transcripts levels after the feeding or injection of dsRNA as described above. RNA was extracted from dsRNA-treated larvae and adult mosquitoes and cDNA was prepared as previously described. Each cDNA was diluted 25 folds, and used as template for RT-qPCR. The reaction volume of 25 µl contained 12 µl of 2x of Maxima SYBR Green/ Fluorescin qPCR Master Mix (Thermo Scientific), 5 µl of 25x-diluted cDNA, 1 µl of 0.5 µM of each primer, and 5 µl of nuclease free water. The primers used were

listed in Table 4.1. The *Ae. aegypti* ribosomal protein 17 (*Rps17*) was used as a reference gene. The experiment was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Three biological replicates, each with two technical replicates, were used for each sample. The results were analyzed using ΔΔCT method (Livak and Schmittgen, 2001).

Insecticide exposures after RNAi

Technical grade of three insecticides, including permethrin, cypermethrin and deltamethrin, were purchased from Chem Service (Chester, PA, USA) and used in this study. Stock solution and serial dilutions were prepared with 100% acetone and stored at -20^{o}C.

Larval bioassay

Larval bioassays were carried out according to WHO larval bioassay procedure (WHO, 1981). Larvae were exposed to LC_{20} concentration of each of the three insecticides previously determined. These concentrations were 1.6, 0.21 and 0.22 ppm for permethrin, cypermethrin and deltamethrin, respectively. In each experiment 20 to 25 third-instar larvae fed with specific dsRNA/chitosan nanoparticles for three consecutive days were exposed to each insecticide in a disposable paper cup containing 200 ml of water and an appropriate amount of insecticide. In control group, larvae fed with equal amount of GFP dsRNA/chitosan nanoparticles were also exposed to insecticides. The experiments were conducted in four replicates and mortality data were recorded 24 h after the insecticide exposure.

Adult bioassay

Bioassays in adult mosquitoes were performed according to the Center for Disease Control and Prevention bottle bioassay procedure (CDC, 2010). Adults were exposed to 0.25 µg of each insecticide for a time corresponding to LT_{20} for each of the three insecticides as

previously determined. These times were 15, 9 and 10 min for permethrin, cypermethrin and deltamethrin, respectively. For functional studies, 20 to 25 mosquitoes from each dsRNA-injected group or control group at the time point showing highest RNAi efficiency (72 h) were exposed to each of the three insecticides for a time corresponding to their respective LT_{20} followed by 24 h in an insecticide-free cage under the same conditions as previously stated. Control group included dsGFP-injected mosquitoes exposed to the same insecticide for the same length of time as in the treatment group. The experiments were conducted in four replicates. The mortality data were recorded after 24 h and transformed into probit units for regression analysis.

Probit regression analysis

To evaluate whether RNAi of a specific P450 gene affected mosquito susceptibility to an insecticide, we compared the probit regression lines between the P450 to dsRNA treated and the GFP dsRNA treated (control) groups, based on the following statistical model to compare the slopes and intercepts of the probit regression lines:

$$\text{Probit}_i = \beta_0 + \beta_1 \text{Log}(\text{Time}) + \beta_2\ \text{RNAi} + \beta_3 \text{Log}(\text{Time})\text{RNAi} + \varepsilon_i$$

Log (Time) represents the covariate, while RNAi is the dummy variable with the value 1 if mosquitoes were injected with P450 dsRNAs and 0 if GFP dsRNA was injected.

The tested hypotheses include: (1) the lines are parallel but not equal, (2) the lines are equal, and (3) the lines are neither parallel nor equal. Probit mortality data were subjected to analysis of covariance using SAS software version 9.2 (SAS Institute, Cary, NC, USA).

Homology modeling of CYP6AA5 and ligand docking

Homology modeling is an alternative method for obtaining the structure of a protein when the crystal structure of the protein under the study is not available (Szklarz et al., 2000). In order to determine whether each of the tree insecticides (permethrin, cypermethrin and

deltamethrin) were sterically accessible and potentially be metabolized by CYP6AA5, *in silico* homology modeling of this enzyme followed by ligand docking was conducted. Modeling of CYPAA5 was performed by submitted the translated amino acid sequence to the I-TASSER web server (Roy et al., 2010). Five three-dimensional models were generated by the I-TASSER web server and the model with the highest ranking was subjected to molecular dynamic refinement through the FG-MD web server (Zhang et al., 2011). Heme prosthetic group was attached to the refined model by creating a covalent bond between the heme's iron atom and the sulfur of cysteine residue 449 of CYP6AA5. The heme coordinates were duplicated from the crystal structure of CYP2E1 (pdb code: 3KOH), which was the closest structural homologue of CYP6AA5 model based on the I-TASSER output. No specific water molecules were added to the model. Ramachandran plot was constructed to assess the overall quality of the model using the RAMPAGE program (Lovell et al., 2003). Ligands (permethrin, cypermethrin and deltamethrin) structures were retrieved from ChEBI database (http://www.ebi.ac.uk/chebi/) and their three-dimensional structures in mol2 format were generated using Open Babel software (http://openbabel.org). All the ligands have rotatable bounds that can adopt multiple conformations within the active site of CYP6AA5. Polar hydrogen were added to both the CYP6AA5 model and ligands, and subsequently converted into PDBQT format required for docking using AutoDock Tools v1.5.6 (Morris et al., 2009). Partial charges of protein and ligands were generated using Gasteiger method. A grid box of 20 x 20 x 20 Å centered at the heme group and comprising the entire catalytic cavity was set. Docking simulations were performed with Autodock Vina v1.1.2 (Trott and Olson, 2010). The docking conformations were viewed with PyMol v 1.5.0.4 (Schrödinger, LLC).

Results

RNAi of P450 genes and its effect on mortality of mosquito larvae

To investigate the efficiency of RNAi through larval feeding on the chitosan/dsRNA nanoparticles, RT-qPCR analysis of the P450 genes was performed. Results showed that the transcript levels can be repressed by 38 to 46% and the suppressions appeared to be similar at different time points (Figure 4.1). For *CYP6AA5*, the highest suppression of the transcript observed was 38.7% at 48 h. For *CYP6AL1* and *CYP9J32*, their transcripts were repressed by 46.0 and 46.5%, respectively, after 24 h of feeding. The silencing of these two genes was slightly reversed after 48 and 72 h. Both the alternative splicing transcripts, *CYP4J16A1* and *CYP4J16B,* from *CYP4J16* were repressed by 44.0% after 24 h and by 41.0% after 48 h. Specific silencing of these two transcripts were confirmed at 72 h (Figure 4.2).The transcripts remained relatively constant across the three time points. Increase in the concentration of dsRNA targeting different transcripts did not improve the efficiency of RNAi (data not shown).

Based on the time-dependent suppression of these P450 genes by feeding the larvae with chitosan/dsRNA nanoparticles, we chose 72 h as feeding time to evaluate larval response to each of the three insecticides at LC_{20} concentration after RNAi. This low concentration allowed to evaluate the change in the susceptibility of the mosquitoes. Knockdown of *CYP6AA5* resulted in an increased susceptibility of larvae to cypermethrin from 20.3 to 61.5%. Exposure to permethrin and deltamethrin did not show a difference in susceptibility between the treatment and control groups. Similarly, silencing of *CYP9J32* showed an increase in mortality from 20.3 % to 66.3% in larvae exposed to permethrin, but not with other insecticides. The RNAi targeting *CYP6AL1* and the two transcripts (*CYP4J16B* and *CYP4J16B*) of *CYP4J16* did not affect the susceptibility

of larvae to any of the three insecticides. Moreover, the susceptibility of larvae exposed to deltamethrin was not significantly affected after each of these genes was silenced (Figure 4.3).

RNAi of P450 genes and its effect on mortality of mosquito adults

To assess the role of P450 genes in the detoxification of pyrethroids in adult mosquitoes, RNAi mediated knockdown of *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* was performed by the injection of specific dsRNA followed by the exposures of the mosquitoes to each of the three insecticides. RT-qPCR analysis at different time points (24, 48, 72 and 96 h) after the dsRNA injection showed a significant decrease of the target transcript (Figure 4.4). The expression levels of *CYP6AA5*, *CYP6AL1*, and *CY4J16A* were suppressed by 77.9, 80.0 and 87.1 % ($P < 0.05$), respectively, 96 h after the injections as compared with the controls. The expression level of *CYP9J32* was suppressed by 46.5% 24 h after injection, but slightly bounced back at 96 h. Similarly, *CYP4J16B* was repressed by 78.2% at 72 h, but slightly bounced back at 96 h. Specific silencing of the two alternative splicing variants were confirmed at 72 h (Figure 4.5).

As most of the transcripts were significantly repressed, we assessed the susceptibility of the dsRNA-injected mosquitoes to each of the insecticides at 72 h post-dsRNA treatments. Exposures of the mosquitoes injected with *CYP6AA5* dsRNA and GFP dsRNA to cypermethrin for 24 h resulted in 61.4 and 32.0% mortalities, respectively. This represents an increase of 29.3% ($P < 0.05$) in mortality as compared with the control group injected with *dsGFP* (Figure 4.6).We also observed a decrease in LT50 value from 12.46 to 8.54 min when mosquitoes were injected with *CYP6AA5* dsRNA and exposed to cypermethrin (Table 4.2). However, we did not observe any significant statistical difference in mortality in the mosquitoes exposed to permethrin and deltamethrin after *CYP6AA5* was silenced. Furthermore, the suppression of other

P450 transcripts did not significantly affect the susceptibility of the mosquitoes to any of the three insecticides as compared with the controls.

Regression analysis of the mosquito responses to cypermethrin showed significant differences both in the slopes and intercepts (P = 0.028; P = 0.010 respectively) between the mosquitoes injected with *CYP6AA5* dsRNA and those injected with *GFP* dsRNA (Figure 4.7). This result indicates that the two regression lines are statistically different and that the injection of *dsCYP6AA5* significantly increased the susceptibility of mosquitoes to cypermethrin. However, neither the slopes nor the intercepts were statistically different when the mosquitoes were exposed to either permethrin or deltamethrin after *CYP6AA5* was silenced.

There were no statistically significant differences in either the slopes or intercepts of the regression lines when the mosquitoes were exposed to each of the three insecticides after *CYP6AL1* or *CYP9J32* was silenced. Silencing of *CYP4J16B* and subsequent exposure of mosquitoes to permethrin and deltamethrin also resulted in no differences in either the slopes or intercepts of the regression lines from the controls and treated groups. In the case of mosquitoes injected with ds*CYP4J16A* and exposed to either permethrin or deltamethrin, the intercepts of the regression lines of control and treatment responses were statistically different but not the slopes (Figure 4.8). For cypermethrin treatment, neither of them was significant.

Homology modeling and docking of insecticides in the catalytic pocket of CYP6AA5

The predicted three-dimensional structure of CYP6AA5 (Figure 4.9) showed all the structurally conserved fold of P450 enzymes along with the alpha helices and beta sheets. The Ramachandran plot showed that 98% of all residues were within the allowed region and 2% of the residues were in the disallowed regions. Nine predicted binding conformations were generated for each of the three insecticides inside the protein, and ranked according to the sum of

the ligand's internal energy, van der Waals and electrostatic energy terms of the potential energy function. All three insecticides were fully enclosed into the catalytic cavity of CYP6AA5, suggesting the potential ability of metabolizing these substrates. For each insecticide the binding conformation with the lowest docking energy and closest interaction to heme iron were considered for further analysis. These docking energies were -11.2, -10.5 and -11.1 kcal mol^{-1} for permethrin, cypermethrin and deltamethrin, respectively. Two putative hydroxylation sites (aromatic and aliphatic) by CYP6AA5 were analyzed. The distances between the heme iron and the putative aromatic hydroxylation site were 9.2, 9.4 and 7.2 Å for permethrin, cypermethrin and deltamethrin, respectively. For aliphatic hydroxylation site these distances were 5.3, 2.8 and 2.9 Å.

Discussion

Cytochrome P450 are important enzymes involved in the metabolism of various compounds in all known forms of life (Nelson, 1998; Feyereisen 1999, 2005, 2012; Werck-Reichhart and Feyereisen, 2000; Lamb et al., 2009). Insect P450s are known to play important roles in the metabolism xenobiotics such as insecticides and plants allelochemicals (Hemingway et al., 2004; Feyereisen, 2012).

Overexpression of certain P450 genes is often associated with the detoxification of insecticides in many insects, although the expression level of specific P450 gene does not necessarily indicate its detoxification function. P450s are not only involved in insecticide detoxification, but also in other biological processes. Therefore, only functional studies can provide evidence of the involvement of a given P450 gene in the detoxification process.

Our RNAi by feeding the mosquito larvae with chitosan/dsRNA nanoparticles followed by insecticide bioassays showed that the repression of *CYP6AA5* and *CYP9J32* led to increased

susceptibility to cypermethrin, and permethrin, respectively, whereas the depletion of the transcripts of *CYP6AL1* and *CYP4J16* (*CYP4J16A* and *CYP4J16B*) did not affected the sensibility of mosquito larvae to all the insecticides tested. These observations suggest that *CYP6AA5* and *CYP9J32* played important roles in the detoxification of cypermethrin and permethrin, respectively. This nanoparticle-feeding based RNAi showed similar results to those observed by Zhang et al. (2010) in *Anopheles gambiae* and demonstrated that RNAi can be achieved through feeding of nanoparticles in mosquito larvae. Further studies are hence needed to enhance the efficacy of this technique.

RNAi results in adult mosquitoes showed increased susceptibility to cypermethrin when the transcript level of *CYP6AA5* was depleted by injection of dsRNA, whereas silencing *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B* did not affected the mortality of the mosquitoes. These results suggested that *CYP6AA5* was involved in the detoxification of cypermethrin. Furthermore, these results demonstrate that the detoxification of insecticides is both gene and insecticide specific. Our findings are consistent with other functional studies of P450 enzymes in relation to the detoxification of insecticides. In *Locusta migratoria*, injection of dsRNAs significantly reduced the transcripts of *CYP409A1* and *CYP408B1,* and led to increased susceptibility to deltamethrin (Guo et al., 2012), indicating the involvement of these genes in the detoxification of deltamethrin. Similarly, *CYP6BG1*was involved in the detoxification of permethrin as indicated by the increased susceptibility observed when this gene was silenced in *Plutella xylostella* (Bautista et al., 2009).

The model structure of CYP6AA5 generated in this study has allowed us to examine the different binding conformation of the three pyrethroid insecticides used in this study. Analysis of these conformations indicate that there were favorable and close orientation of the putative

aliphatic hydroxylation sites to the heme iron compared to the aromatic hydroxylation for all three insecticides as indicate by the interaction distances. These results indicate that in *Ae. aegypti* metabolism of pyrethroids might occur preferentially through aliphatic hydroxylation based on our model. The interactions distance between the heme iron and the aliphatic hydroxylation site of cypermethrin and deltamethrin were closer to the heme iron as compared that of permethrin. This observation indicates that CYP6AA5 may metabolize cypermethrin and deltamethrin but not permethrin. These docking results are consistent with our RNAi results both in larvae and adult mosquitoes.

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Table 4.1 Primers and their relevant parameters used for P450s dsRNA synthesis and RT-qPCR analysis.

Application	Gene Name	Sequence (5'-3')	Primer Length (base)	Tm (^{O}C)	Product Size (bp)
RT-qPCR	*Rps17*	F: CTAAGTCTGCTAAAGTAT R: CTTATACGATACACTCTC	18 18	51.5 51.9	110
	CYP6AA5	F: ACAGAGTCTTCAATCCAA R: TGTTGTTCTTCTCACGAT	18 18	56.7 57.5	165
	CYP6AL1	F: GATAGATAAGGCGACTGT R: TAGTGTAACGAGCGAATA	18 18	56.9 56.4	95
	CYP9J32	F: CCGTGATAATGAGTTCTAC R: TACTGCTTCCTTGATGAT	19 18	56.3 56.6	175
	CYP4J16	F_A: ATATCTTGCCGAAGTTCTTG R_A: TTGCCTGCCTTAACATCT F_B: CAACAACGGATAATGAACC R_B: ATTGGACGGAGAATCTTG	20 18 19 18	59.7 60.4 58.2 58.1	85 92
dsRNA synthesis	*CYP6AA5*	F: TAATACGACTCACTATAGGGTATCGGAATGCGATTTGCTT R:TAATACGACTCACTATAGGGTAGATCCGTTCAACATCCAGC	40 41	60.55 60.1	168
	CYP6AL1	F: TAATACGACTCACTATAGGGCAGGATGCCTGAAAGGAATG R:TAATACGACTCACTATAGGGCGATTCCATAAGCAACTGACG	40 41	60.59 60.64	159
	CYP9J32	F: TAATACGACTCACTATAGGGACTACTTCCACGACAAGCCG R:TAATACGACTCACTATAGGGAAACAGTCCAAAAATCGTCCC	40 41	60.31 60.21	161
	CYP4J16	F_A:TAATACGACTCACTATAGGGGTTCATGGGCAAGGGTCTAC R_A TAATACGACTCACTATAGGGTGCGTCCAACTTCCTCAAC F_B:TAATACGACTCACTATAGGGACATCCGAGAAGTTGGCAAG R_B:TAATACGACTCACTATAGGGATGGGGTGTCCACGTTTTT	40 39 40 39	59.41 59.82 60.25 60.1	151 195
	GFP	F: TAATACGACTCACTATAGGGGGACGTTGCAACACGGATTC R:TAATACGACTCACTATAGGGGTGTCATCGTAAAGCTTGCCA	40 41	60.11 40.40	385

Table 4.2 Toxicity of three pyrethroid insecticides to adults *Ae. aegypti* injected with different P450 dsRNAs.

	Permethrin				Cypermethrin				Deltamethrin			
	LT_{50} (Min)	95% CI	$\chi 2$	$P > \chi 2$	LT_{50} (Min)	95% CI	$\chi 2$	$P > \chi 2$	LT_{50} (Min)	95% CI	$\chi 2$	$P > \chi 2$
control	21.68	19.62-23.67	22.03	0.45	12.46	10.31-14.39	17.76	0.72	16.39	14.26-18.07	16.39	0.99
dsCYP6AA5	21.24	19.19-23.21	23.04	0.40	8.54	5.99-10.77	20.21	0.57	15.27	13.50-16.91	16.50	0.79
control	23.14	21.12-25.10	15.86	0.82	15.59	13.68-17.36	18.58	0.99	15.70	13.89-17.39	12.75	0.93
dsCYP6AL1	19.24	17.06-21.30	25.81	0.75	13.26	11.10-15.21	18.72	0.66	14.73	12.90-16.42	16.18	0.80
control	22.88	20.99-24.72	18.26	0.69	15.61	13.68-17.41	19.8	0.98	16.01	14.28-17.62	12.35	0.94
dsCYP9J32	22.71	20.86-24.54	23.63	0.86	15.82	13.80-17.70	14.62	0.87	15.53	13.53-17.38	11.43	0.96
control	23.14	21.12-25.11	18.92	0.65	15.33	13.34-17.16	8.67	0.99	15.94	14.10-17.66	8.94	0.99
dsCYP4J16A	20.19	17.44-22.81	31.28	0.87	14.01	11.84-15.97	14.37	0.88	12.73	10.72-14.54	13.74	0.91
control	22.87	20.86-24.82	15.84	0.82	15.25	13.26-17.10	12.33	0.95	15.23	13.33-16.99	12.57	0.94
dsCYP4J16B	21.66	19.01-24.23	25.40	0.83	13.41	11.18-15.41	14.72	0.87	12.29	10.24-14.13	14.27	0.89

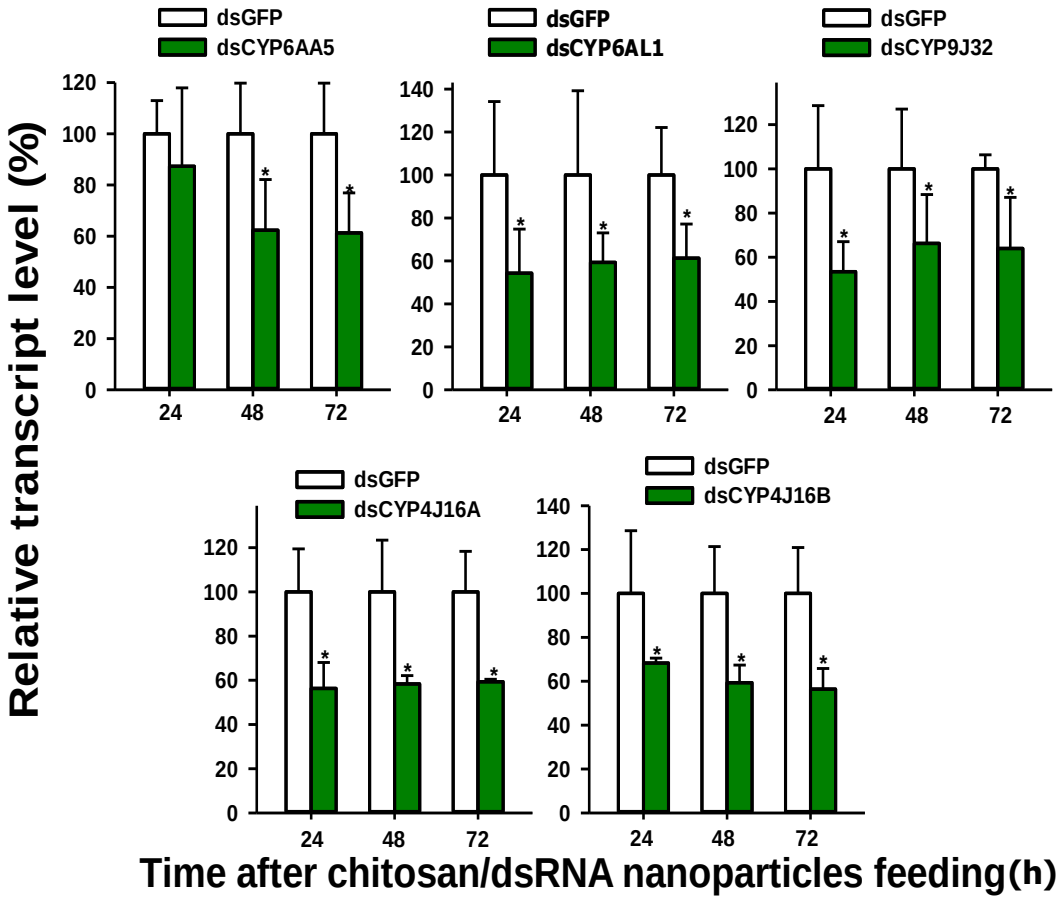

Figure 4.1 Effect of oral dsRNA/chitosan nanoparticles on the transcript level of selected P450 genes in *Ae. aegypti* larvae. For each P450 transcript, specific dsRNA was used in preparing the nanoparticles. GFP dsRNA was used to prepare nanoparticles for each control group. RNA was extracted at 24, 48 and 72 h after the feeding from both treatment and control groups and used for cDNA preparation and RT-qPCR analysis. Three biological replicates each with two technical replicates were used in each case. The transcript levels in larvae fed with nanoparticles of dsGFP were arbitrary ascribed 100%. The asterisk on the bars indicates significant difference between treatment and the control based on student's t-test ($P < 0.05$).

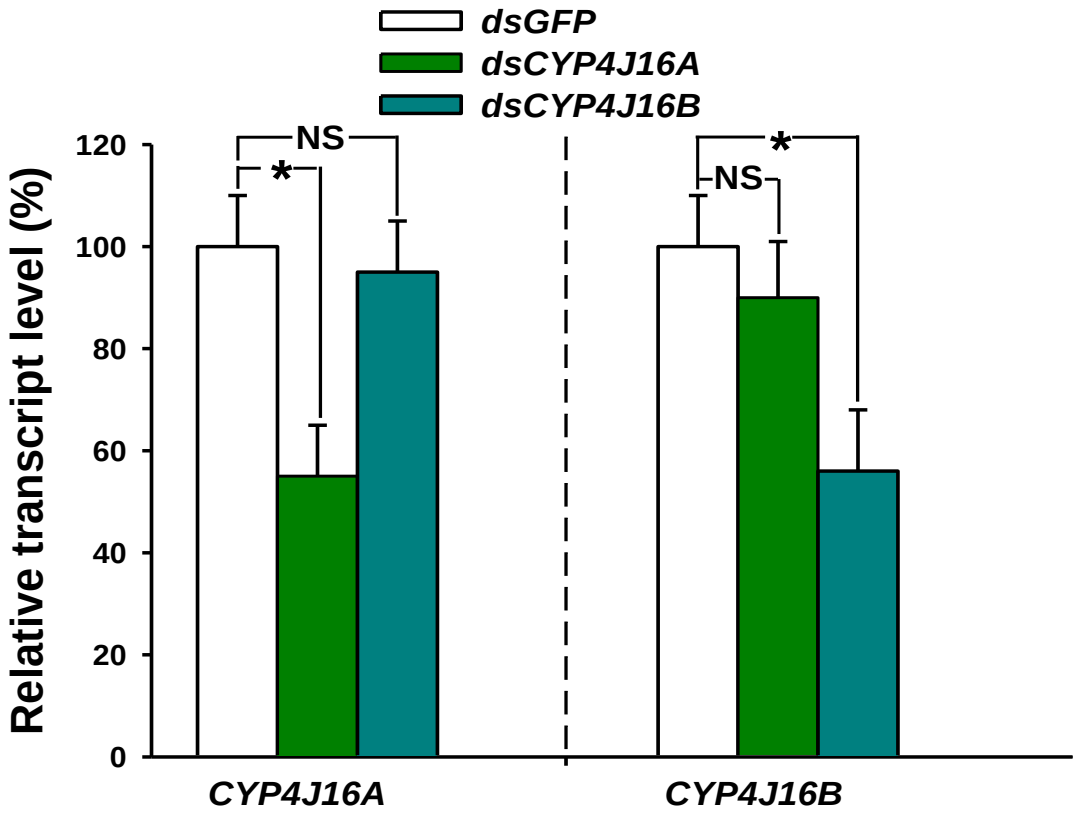

Figure 4.2 Effect of oral dsRNA/chitosan nanoparticles of *CYP4J16A* and *CYP4J16B* on the transcript levels of *CYP4J16* 72 h after feeding in *Ae. aegypti* larvae. Control groups were fed with GFP dsRNA. Three biological replicates each with two technical replicates were used in each case. The transcript levels in larvae fed with nanoparticles of dsGFP were arbitrary ascribed 100%. The asterisk shows significant differences between larvae fed with nanoparticles containing *dsCYP4J16A* or *dsCYP4J16B* and those fed with nanoparticles containing dsGFP based on student's t-test (P < 0.05). NS indicates no significant difference between the two groups.

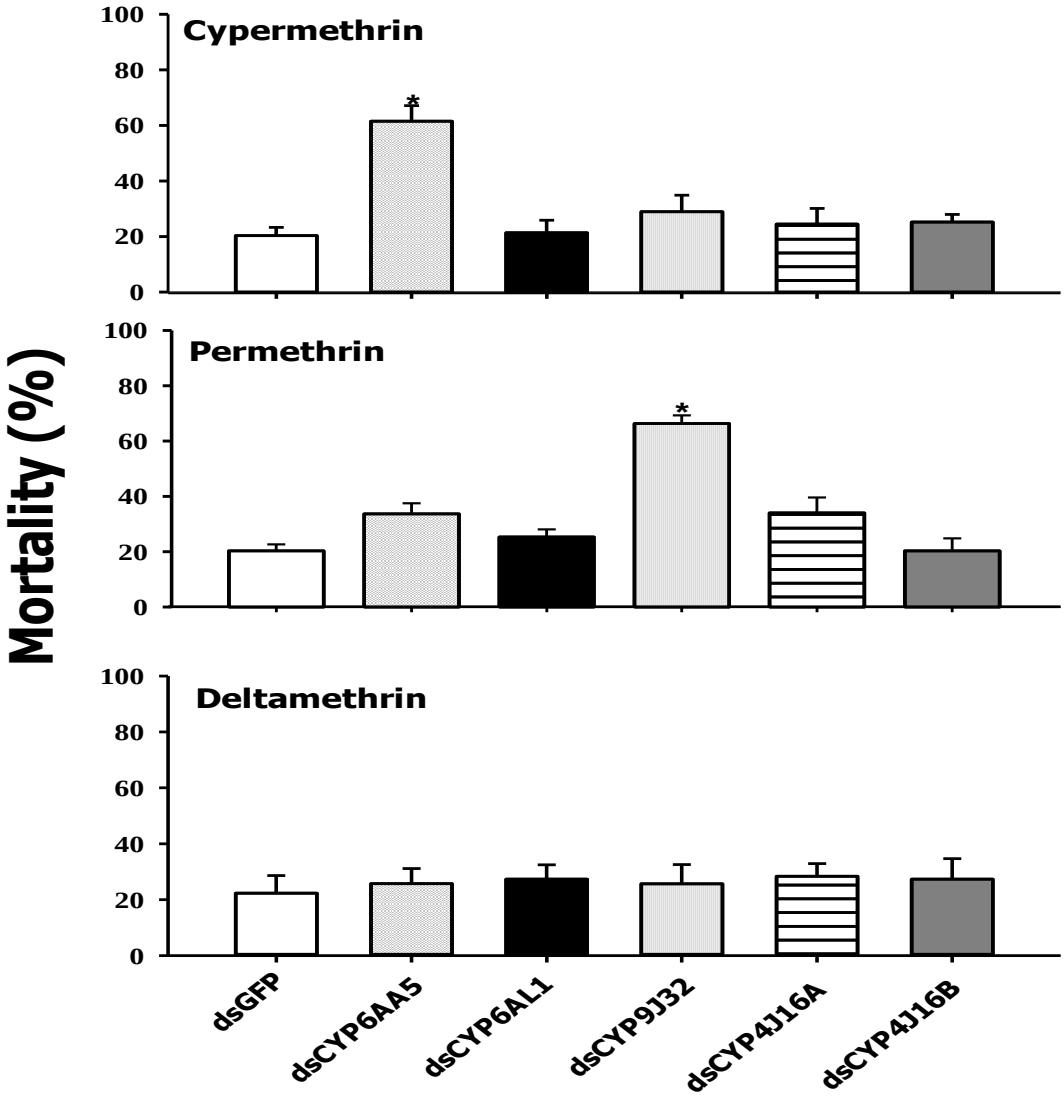

Figure 4.3 Susceptibility of *Ae. aegypti* larvae to three pyrethroid insecticides (permethrin, cypermethrin and deltamethrin) 72 h after larvae were fed on chitosan/dsRNA nanoparticles targeting each of the five transcripts including *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B*). Control group included the same number of larvae fed for 72 h with the equal amount of GFP dsRNA/chitosan nanoparticles and exposed to the same insecticides. The bars indicate the mean of three biological. Asterisks on the bars indicate that the means are significantly different among the control and treatments (student's t-test, $P < 0.05$).

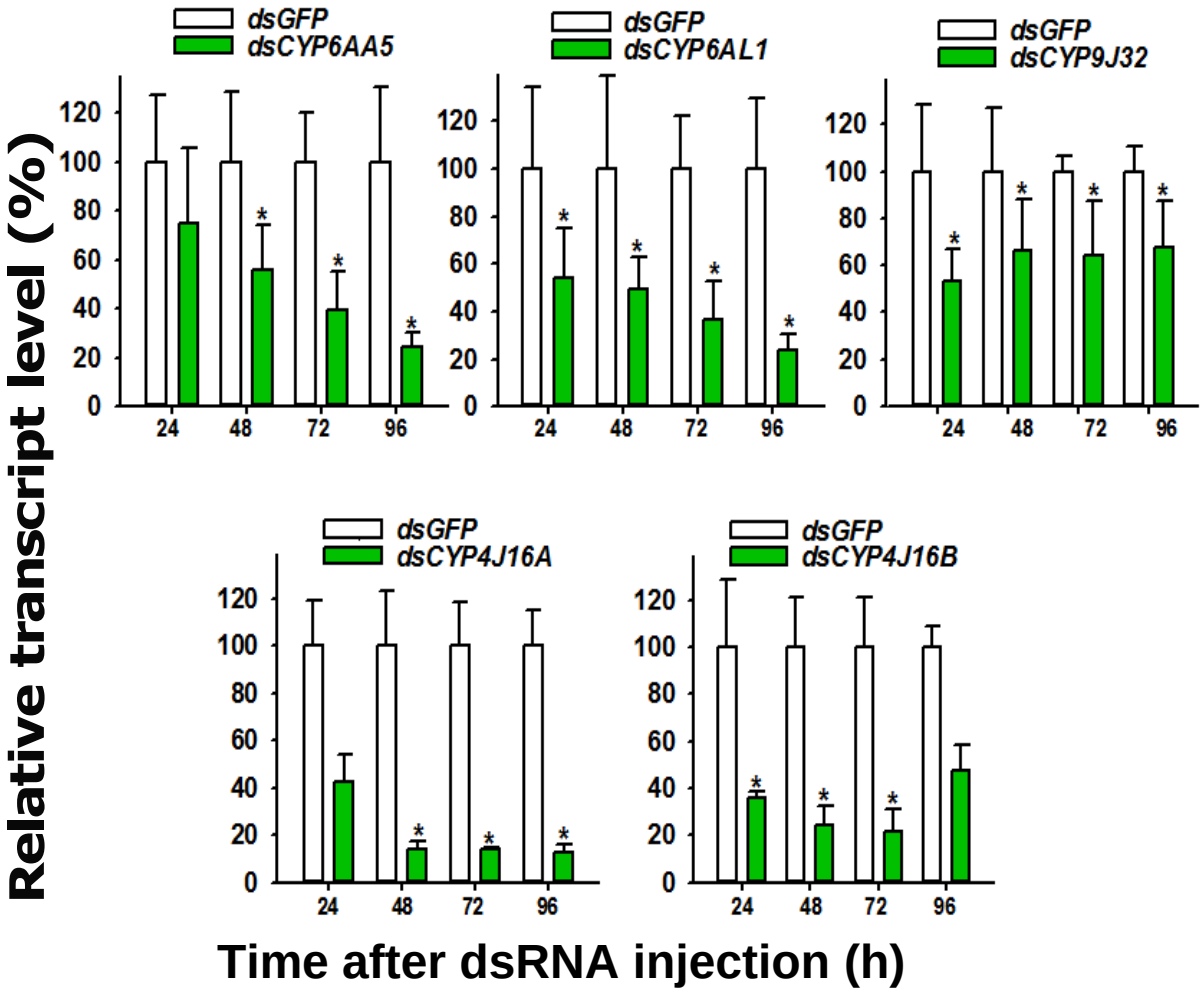

Figure 4.4 Effect of dsRNA treatment on the transcript levels of P450 genes in adult *Ae. aegypti.* The relative expression levels (%) are presented as the mean and standard errors of three biological replicates. An asterisk on the bar indicates significant difference between the mosquitoes injected with dsRNA of a P450 gene and dsRNA of GFP gene based on Student's t-test within the same time point ($P < 0.05$).

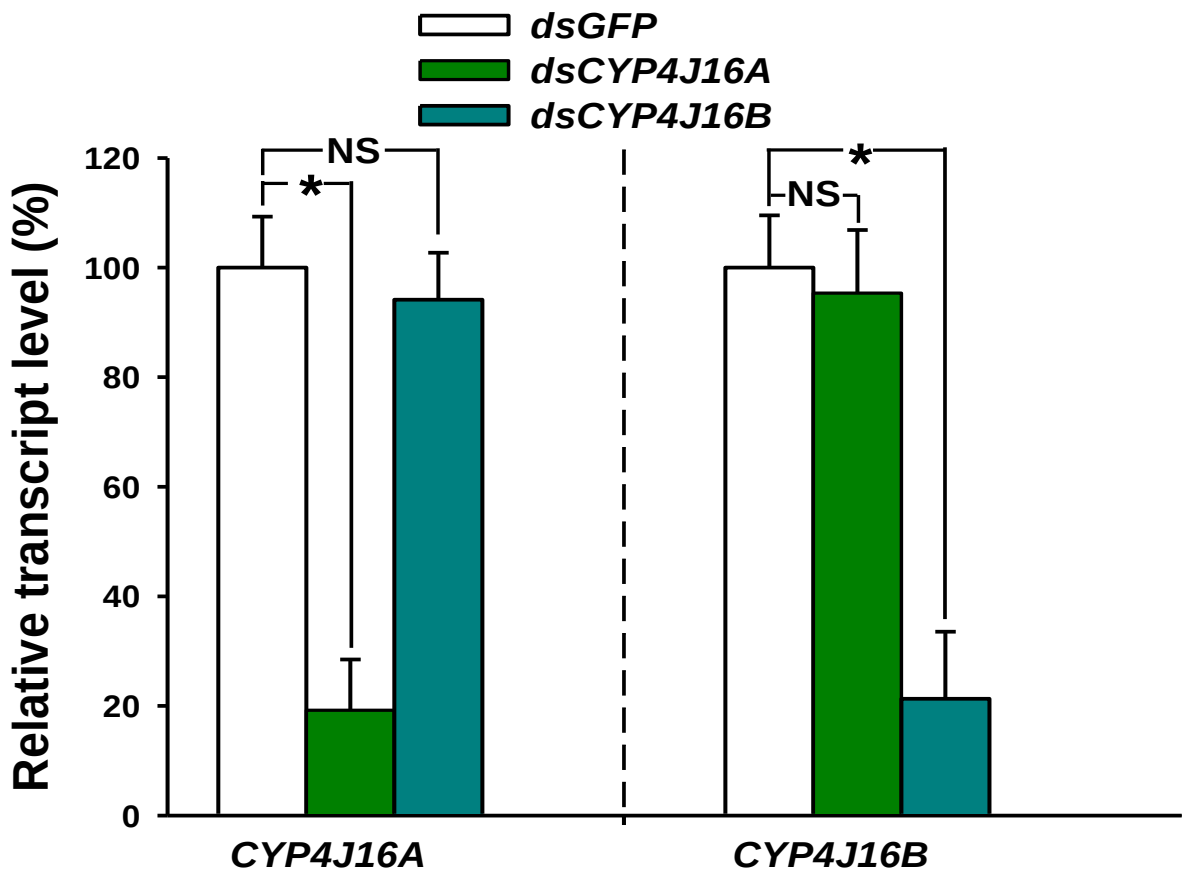

Figure 4.5 Effect of *CYP4J16A* and *CYP4J16B* dsRNA on the transcript levels of *CYP4J16* 72 h after dsRNA injection in *Ae. aegypti* adults. Control groups were injected with GFP dsRNA. Three biological replicates each with two technical replicates were used in each case. The transcript levels in larvae fed with nanoparticles of dsGFP were arbitrary ascribed 100%. The asterisk shows significant differences between adults injected with *dsCYP4J16A* or *dsCYP4J16B* and those injected with dsGFP based on student's t-test ($P < 0.05$). NS indicates no significant difference between the two groups.

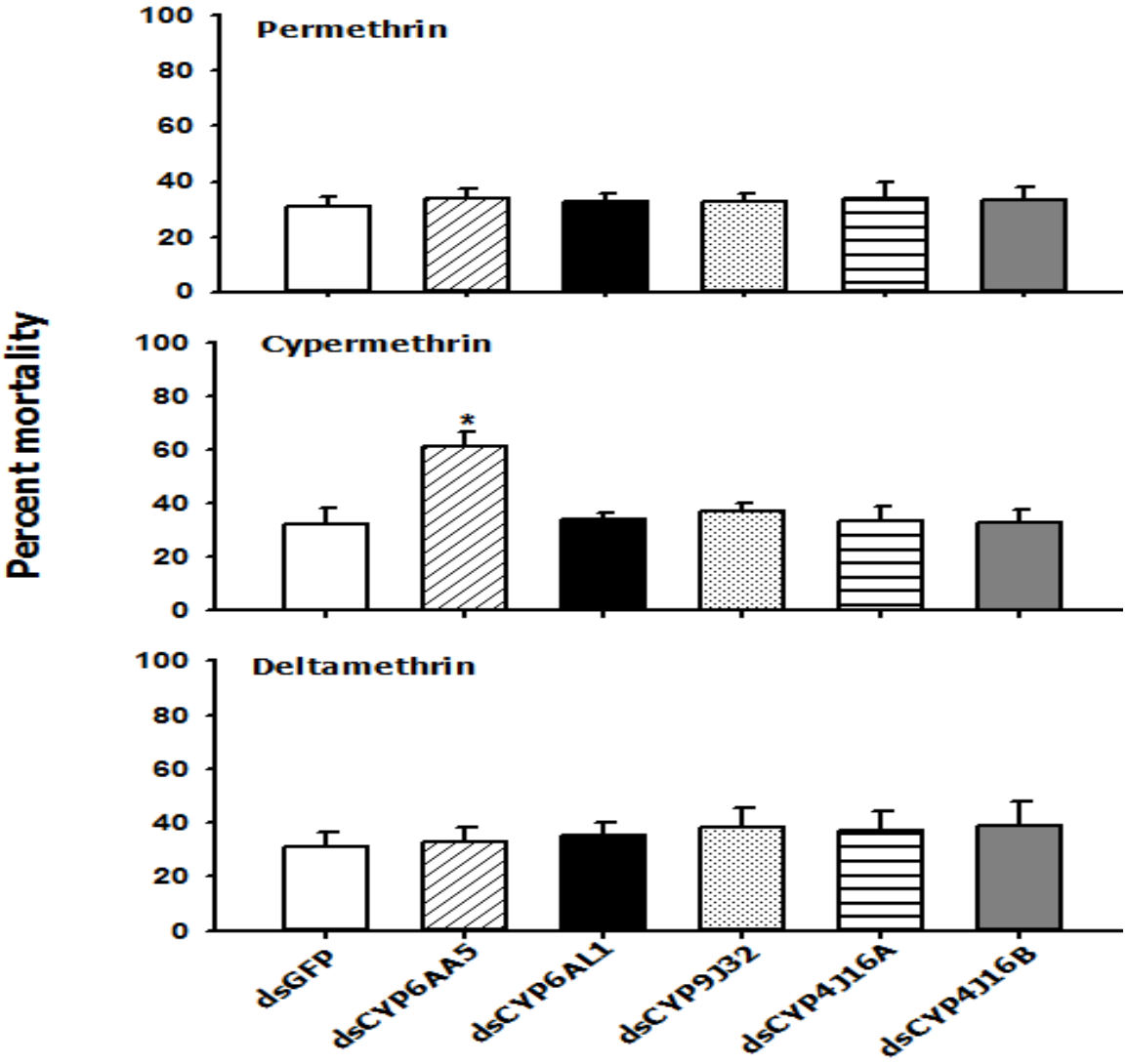

Figure 4.6 Susceptibility of *Ae. aegypti* adults to each of three pyrethroid insecticides (permethrin, cypermethrin and deltamethrin) 72 h after injection of dsRNA targeting each of five P450 transcripts including *CYP6AA5*, *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B*. Control group included the same number of mosquitoes injected with the equal amount of GFP dsRNA and exposed to the same insecticides. Asterisks on the bars indicate that the means are significantly different between the control and each treatment (student's t-test, $P < 0.05$).

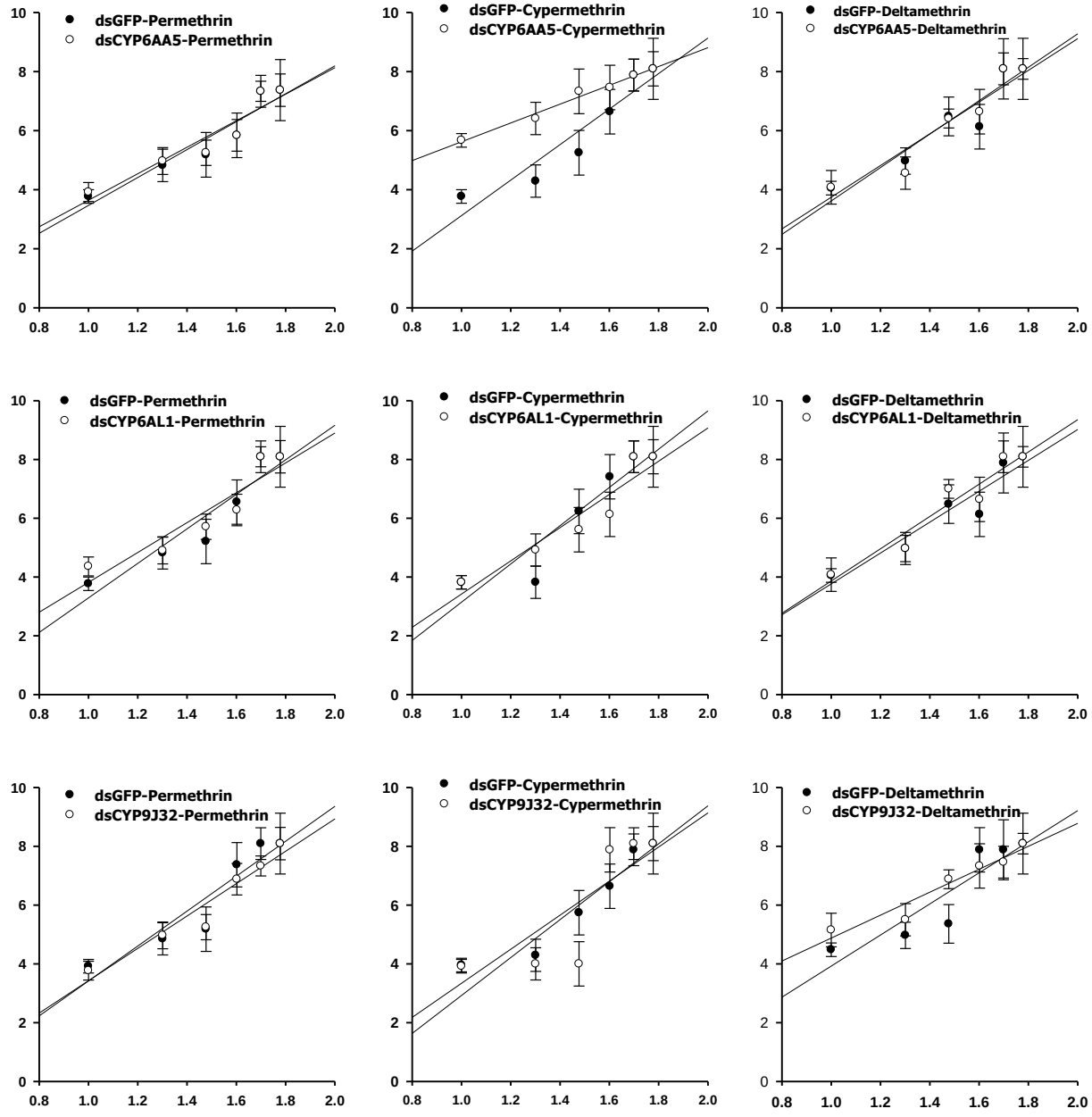

Figure 4.7 Regression analysis of the response of dsRNA-injected mosquitoes to each of the three insecticides. Each data point was generated from the average of three replicates (15-20 individuals for each replicate).

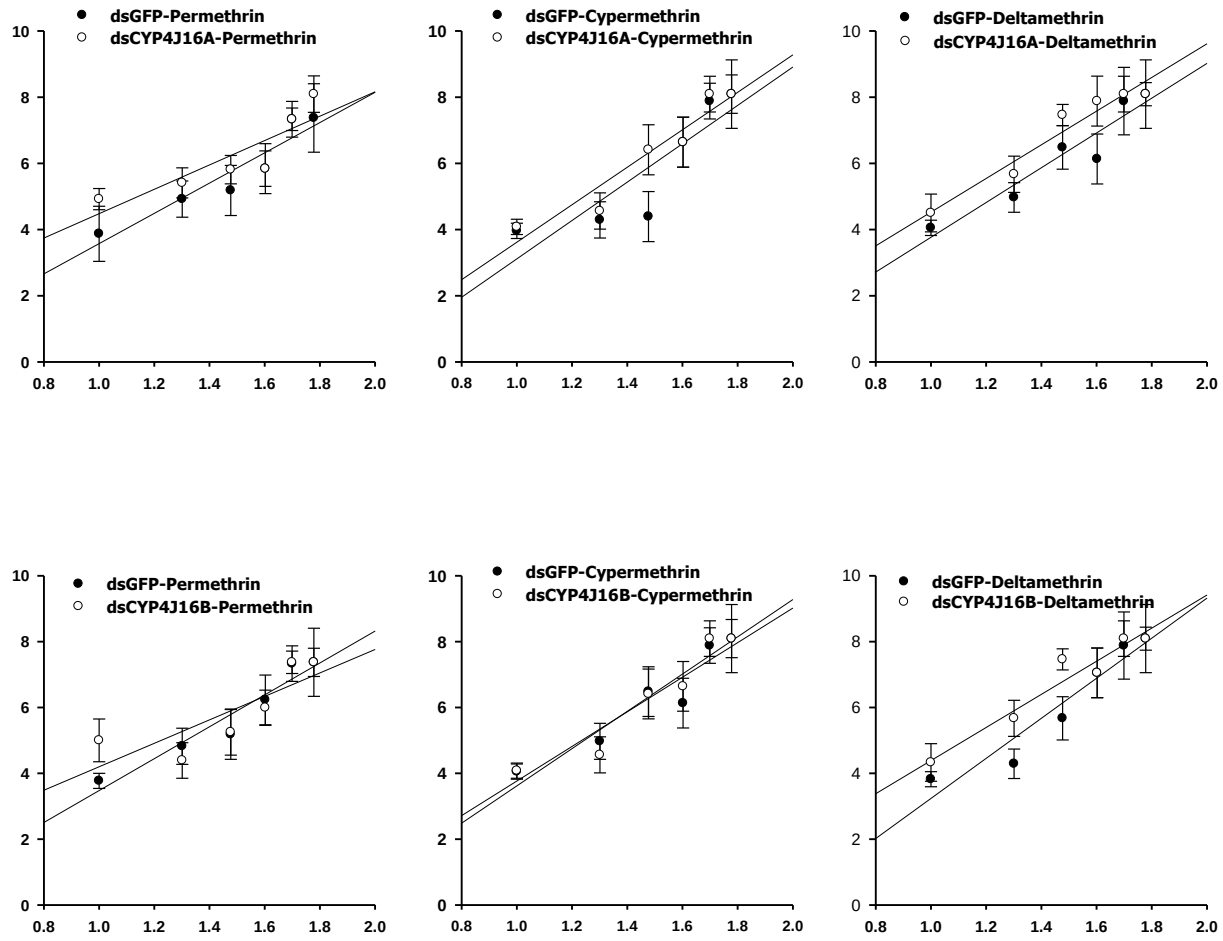

Figure 4.8 Regression analysis of the response of dsRNA-injected mosquitoes to each of the three insecticides. Each data point was generated from the average of three replicates (15-20 individuals for each replicate.

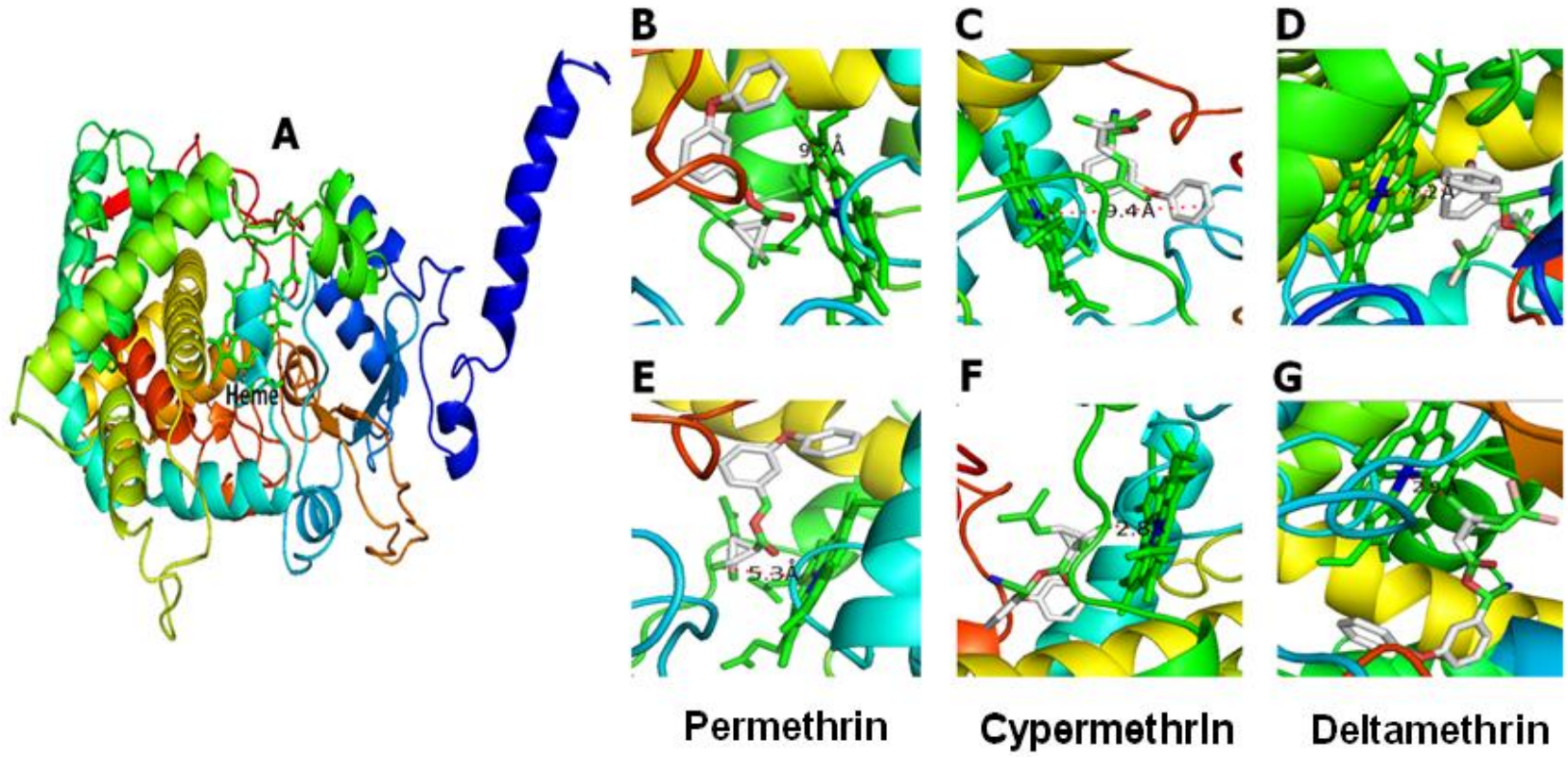

Figure 4.9 Homology modeling of CYP6AA5 and docking of permethrin, cypermethrin and deltamethrin in its predictive catalytic pocket. Three-dimensional model structure of CYP6AA5 is shown (A). Docked conformations with the putative aromatic hydroxylation site oriented towards the heme iron are shown are shown for permethrin (B), cypermethrin (C) and deltamethrin (D). The interactions with the putative aliphatic hydroxylation site are shown in E, F and G for the three insecticides respectively. Images were generated with PyMol .

Chapter 5 - Summary

The sequencing and annotation of *Aedes aegypti* genome have provided the opportunity to carry out genome-wide study of cytochrome P450 monooxygenases (P450s). This research has focused on the genome-wide analysis of *Ae. aegypti* P450 superfamily, characterization of five representative transcripts, including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* , and evaluation of their role in detoxification of three pyrethroid insecticides including permethrin, cypermethrin and deltamethrin. We also investigated the role of the nuclear receptor *HR96* in the up-regulation of P450 genes in response to exposure to each of the three insecticides both in mosquito larvae and adults.

The genome-wide analysis of *Ae. aegypti* P450 superfamily revealed the presence of 159 genes, which can be classified into 18 families and 63 subfamilies according to the nomenclature criterion. These genes are distributed in four clans, including CYP2, CYP3, CYP4 and mitochondrial CYP clans with 11, 80, 58 and 10 genes, respectively. The largest families included CYP6, CYP9, CYP4 and CYP325. The intron-exon organization of the genes was very diverse among the gene families, and the highest conservation of gene structure was observed in the CYP6 and CYP9 families with the predominance of single-intron containing genes. The phylogenetic analysis suggested that the CYP6 and CYP9 families were likely to be derived from a common ancestor. The clan level organization was not respected for the CYP2 and mitochondrial CYP clans, which cluster together, suggesting the common ancestry of their member genes. This study highlighted the characteristics and diversity of *Ae.aegypti* P450 superfamily, which can be used as ground work for further study of mosquito P450s.

We selected five P450 transcripts including three individual genes (*CYP6AA5*, *CYP6AL1* and *CYP9J32*) and two alternative splicing variants (*CYP4J16A* and *CYP4J16B*) of *CYP4J16* and analyzed their expression at different developmental stages and in various tissues of larvae and adults. The transcripts of *CYP6AA5, CYP6AL1* and *CYP9J32* were highly expressed in larvae compared to other stages.*CYPJ16A* was predominantly expressed in eggs and adults, while the highest expression of *CYP4J16B* was observed in pupae. The larval tissue-specific expression of the transcripts were higher in the midgut, Malpighian tubules in general. The two alternative splicing variants of *CYP4J16* were differentially expressed in the tissues. *CYP4J16A* was predominantly expressed in the midgut while *CYP4J16B* showed the highest expression in the fat bodies. In adults, all the transcripts were detectable in all tissue. Nevertheless, the expression of *CYP9J32* was particularly higher in the midgut than in other tissues.

Exposure of third-instar larvae to permethrin, cypermethrin and deltamethrin led to significant down-regulations of *CYP6AL1* and *CYP4J16A*. *CYP6AA5* was moderately induced by cypermethrin but not by other insecticides. Adult exposure to permethrin led to the up-regulation of *CYP6AL1* and *CYP4J16B* by 2.1 and 2.5-fold ($P < 0.05$), respectively. In contrast the expression level of *CYP9J32* was repressed by 44.3%, while the expression of *CYP6AA5* and *CYP4J16A* were not affected by permethrin treatment. Cypermethrin treatment resulted in the increase of the expressions of *CYP6AL1*, *CYP9J32*, *CYP4J16A* and *CYP4J16B*by 2.4, 3.5, 2.4 and 23.0-fold ($P < 0.05$), respectively whereas the expression of *CYP6AA5* was not affected. In deltamethrin treatment, the expression levels of *CYP6AA5*, *CYP4J16A*and*CYP4J16B* were increased by 2.2, 2.1 and 3.6-fold ($P < 0.05$), respectively.

RNA interference (RNAi) by feeding larvae with chitosan/dsRNA nanoparticles led to reduction of the transcripts of selected genes by 38.7, 46.0, 46.5, 44.0 and 41.0% for *CYP6AA5*,

CYP6AL1,CYP9J32, CYPJ16A and *CYP4J16B,* respectively. Analysis of the detoxification roles of these genes by RNAi followed by insecticide bioassay showed increased mortality by 41.2% to cypermethrin when *CYP6AA5* was silenced, and by 46.0% to permethrin when *CYP9J32* was silenced. Similarly, RNAi by injection of dsRNA in adults resulted in the reduction of *CYP6AA5*, *CYP6AL1*, and *CY4J16A* transcripts by 77.9, 80 and 87.1% ($P < 0.05$), respectively, 96 h post treatment comparatively to controls. The expression level of *CYP9J32* was suppressed by 46.51% 24 h after injection, but slightly increased for the rest of the experiment whereas *CYP4J16B* was highly repressed by 78.17% at 72 h following dsRNA treatment. Exposure of mosquitoes injected with *CYP6AA5* dsRNA to cypermethrin for 24 h led to 29.34% ($P < 0.05$) increase in mortality as compared to control. RNAi targeting *HR96* led to 81.7% ($P < 0.05$) reduction of its transcript, indicating significant gene silencing. Exposure of injected mosquitoes to cypermethrin led to a 10.13-fold reduction in the up-regulation of *CYP4J16B,* but did not affect the up-regulation of other P450 genes. There were no significant effects of the RNAi on the up-regulation of all the P450 genes by permethrin and deltamethrin. These findings indicate that *HR96* is involved in the regulation of *CYP4J16B* up-regulation by cypermethrin in *Ae. aegypti* adults.

Homology modeling of CYP6AA5 followed by ligand docking, have allowed us to examine the different binding conformation of the three pyrethroid insecticides used in this study. Analysis of these conformations showed CYP6AA5 may be able to metabolize cypermethrin and deltamethrin as indicated by their close interaction with the heme iron. Further enzymatic studies are needed to confirm these findings.