MOLECULAR MECHANISMS OF PYRETHROID RESISTANCE IN THE

MOSQUITO, *CULEX QUINQUEFASCIATUS* (SAY)

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MOLECULAR MECHANISMS OF PYRETHROID RESISTANCE IN THE MOSQUITO, *CULEX QUINQUEFASCIATUS* (SAY)

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MOLECULAR MECHANISMS OF PYRETHROID RESISTANCE IN THE MOSQUITO, *CULEX QUINQUEFASCIATUS* (SAY)

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VITA

Qiang Xu, son of Binglin Xu and Zhixiang Hu, was born in March, 1973, in Lanxi, Zhejiang province, People's Republic of China. He graduated with honor from Lanxi No. One High School in Lanxi in 1990. In September 1992, he entered Zhejiang University in Hangzhou and received a Bachelor of Science degree in Food Sciences in July 1996. After working as a Research Assistant in Zhejiang University's Department of Applied Biosciences, he went to the Graduate School of Zhejiang University in September 2000 and got his Master of Science degree in Biophysics in July 2003. In August 2003, he was enrolled as a Ph. D. student at the Cellular and Molecular Bioscience Program (CMB) of Auburn University. After one-year rotation training at CMB, he transferred to the Department of Entomology and Plant Pathology to complete his Ph. D degree. He married Qi Hang in April 1998.

DISSERTATION ABSTRACT

MOLECULAR MECHANISMS OF PYRETHROID RESISTANCE IN THE MOSQUITO, *CULEX QUINQUEFASCIATUS* (SAY)

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Mosquitoes have been the ideal transmitters of a wide variety of disease agents due to their repeated blood feedings over their life span. *Culex quinquefasciatus* (Say) is an important disease vector in the Southeastern U.S.A. Mechanisms of resistance in mosquitoes have attracted much attention since they help in the elucidation of pathways of resistance development and in the design of novel strategies to prevent or minimize the spread and evolution of resistance.

A mosquito strain of *Cx. quinquefasciatus*, $HAmCq^{G0}$, from Huntsville, Alabama, has been established and further selected with permethrin, a pyrethroid insecticide, in the laboratory. The level of resistance to permethrin in the $HAmCq^{G0}$ strain is 100-fold compared with an insecticide-susceptible mosquito strain S-Lab. Following permethrin

selection for 8 generations, the level of resistance to permethrin in HAmCq^{G8} strain is 3100-fold compared with the S-Lab strain. High levels of resistance to pyrethroid insecticides in HAmCq parental and selected mosquito strains are conferred by multiple mechanisms, including P450 monooxygenase-, hydrolase- and/or GST-mediated detoxification. Incomplete suppression of pyrethroid resistance by synergists, PBO, DEF and/or DEM, suggests that one or more additional mechanisms are involved in overall resistance of HAmCq. The L-to-F *kdr* mutation of the sodium channel, the target site of pyrethroid insecticides, plays a very important role in pyrethroid resistance. My study has, for the first time, revealed that the transcriptional regulation of the L-to-F *kdr* mutation of the sodium channel gene through RNA allelic variation and RNA editing is an important mechanism involved in the evolution of *kdr*-mediated pyrethroid resistance in mosquitoes. Both Southern Blot and DNA sequencing analyses indicate multiple sodium channel genes presented in mosquitoes. The first full-length mosquito sodium channel gene has been cloned and sequenced from mosquitoes. Sequence comparison of the sodium channel gene between resistant and susceptible mosquitoes has revealed several non-silent and silent mutations that correspond with the levels of pyrethroid resistance in mosquitoes. This result suggests the importance of these mutations in altering the sensitivity of the sodium channels to insecticides and in insecticide resistance. Taken together, my research indicates a complexity of the sodium channel gene expression and its role in pyrethroid resistance of insects and provides a new framework to study the sodium channel gene expression regulation in insecticide resistance of mosquitoes.

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
bp	base pair
cDNA	complementary DNA
DDT	dichloro-diphenyl-trichloroethane
DEF	S,S,S-tributylphosphorotrithioate
DNA	deoxyribose nucleic acid
EDTA	ethylene diamine tetracetic acid
GABA	gamma aminobutyric acid
GST	glutathione S-transferase
$HAmCq^{G0}$	a pyrethroid resistant mosquito strain
$HAmCq^{G8}$	a highly pyrethroid resistant mosquito strain
kdr	knockdown resistance
LC_{50}	lethal concentration necessary to kill 50% of a test population
LD_{50}	lethal sosage necessary to kill 50% of a test population
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
OP	organophosphate

PBO	piperonyl butoxide
PCR	polymerase chain reaction
RNA	ribose nucleic acid
RR	resistance ratio
RT-PCR	reverse transcription- polymerase chain reaction
SDS	sodium dodecyl sulphate
S_Lab	an insecticide susceptible mosquito strain
SNP	single nucleotide polymorphism
SSC	sodium chloride and sodium citrate
TAE	tris-acetate/EDTA

CHAPTER 1: LITERATURE REVIEW

1.1 Insecticide resistance

Insecticides are wildly used to control insect pests. Unfortunately, resistance has developed to all five major chemical classes of insecticides: cyclodienes, carbamates, dichloro-diphenyl-trichloroethane (DDT) and its analogues, organophosphates, and pyrethroids (Georghiou, 1986), and some relatively new insecticides, such as *Bacillus thuringiensis* (Bt) (Gerghiou et al., 1983; Goldman et al., 1986; Tabashnik et al., 1990), imidacloprid (Liu and Yue, 2000; Wei et al., 2001), spinosad (Moulton et al., 2000), and fipronil (Liu and Yue, 2000). Insecticide resistance has been defined as "the development by some insects in a population of an ability to survive doses of a toxicant, which would prove lethal to the majority of individuals in a normal population of the same species." (WHO, 1957).

Insecticide resistance is a major obstacle to the control of agriculturally and medically important insect pests. The development of resistance to insecticides happens quite fast. The discovery in 1939 that DDT killed insects won Paul Muller the 1948 Nobel Prize. But evolution of DDT resistance had already been reported in house flies (Georghiou, 1986) before the Nobel ceremony occurred. By the 1960s, mosquitoes resistant to DDT prevented the worldwide eradication of malaria (Desowitz, 1991), and by 1990, over 500 species of insects have developed resistance to one or more

insecticides (Georghiou, 1990). Insects usually evolve resistance within about a decade after introduction of a new insecticide (NRC, 2000). The price of developing a single new insecticide was about $80 million in 1999 (NRC, 2000). It is an ongoing cost of agricultural business to develop new insecticides. Insecticide resistance and the consequent losses of food and fiber caused by failure to control insect pests result in several billion dollars in economic losses worldwide each year (Elzen and Hardee, 2003). Furthermore, insecticide resistance may lead to vector-borne diseases when vectors cannot be controlled. Insecticide resistance was called "the biggest single obstacle in the struggle against vector-borne disease" (WHO, 1976). Insecticides resistance has appeared in the major insect vectors from every genus, including 56 species anopheline and 39 species culicine mosquitoes, 8 species of fleas, and 9 species of ticks (WHO, 1992). Other public health important insects, such as certain flies and cockroaches, show resistance in all genera (Brogdon and McAllister, 1998). Therefore, it is very important to preserve useful insecticides by slowing and preventing development of resistance in insect pests. To achieve this goal, it is urgent to understand and monitor the development of insecticide resistance, and to find ways of preventing resistance development.

1.2 Mechanisms of insecticide resistance

Most insecticide resistance mechanisms can be sorted into 2 major groups, the insensitivity of target site (i.e., insensitivity of the sodium channel, acetylcholinesterase and GABA receptor), or increased metabolic detoxification (alterations in the levels or activities of detoxification proteins). In addition, many insecticides also cause behavioral and physiological changes.

1.2.1 Resistance due to insensitivity of target sites

1.2.1.1 Insensitivity of sodium channel

Insect sodium channels have been broadly recognized by virtue of the wealth of physiological evidence as the primary target for DDT and synthetic pyrethroids, which are structural derivatives of naturally-occurring pyrethrins. Pyrethroids are grouped into two categories (i.e., type I and type II) based on their distinct poisoning symptoms, effects on nerve preparations, and their chemical structures (Narahashi, 1986). The molecular basis of the differential effects of type I and type II pyrethroids on sodium channels remains unknown. Recent studies indicat that a new class of insecticides, oxadiazines, also target sodium channels (Silver and Soderlund, 2005). Pyrethroids have been widely used in controlling many insect pests. Unfortunately, the heavy use of pyrethroids in the last 30 years has led to the development of pyrethroid resistance in many insect pest populations, including mosquitoes and other insect species (Brengues et al., 2003; Dong, 1997; Martinez-Torres et al., 1999a; Martinez-Torres et al., 1999b; Miyazaki et al., 1996; Pridgeon et al., 2003; Liu et al., 2002). One of the most important resistance mechanisms is reduced sensitivity of the sodium channel, which is also known as knock down resistance (*kdr*). Insects exhibiting *kdr* have reduced target-site (sodium channel) sensitivity to pyrethroids and DDT resulting from one or more point mutations in their sodium channel proteins. The important role that sodium channels play in insecticide resistance has prompted many researchers to study insect sodium channel biology.

1.2.1.1.1 Structure and function of sodium channel

Voltage-gated sodium channels are integral trans-membrane proteins responsible for the rapidly-rising phase of action potentials in the membranes of neurons and other excitable cells (Catterall, 2000). Sodium channels open and close within milliseconds when the cell membrane is depolarized by a few millivolts. Influx of sodium ions through the channel depolarizes the membrane further and initiates the rising phase of the action potential (Yu and Catterall, 2003). Sodium channels are special "gates" within the membrane that separates nerve cells (neurons) from the surrounding environment (the extracellular fluid). These channels are opened or closed depending on electrical or chemical stimuli. When they are opened, they let charged sodium ions pass through. Sodium ions flow between the neurons and the extracelluar fluid to set up the state of electrical charge potential when the neuron is at rest (the resting potential) and to release that potential when the neuron is sending a nerve impulse (the action potential) (Dong, 2007) . Sodium channels are opened at the moment the action potential occurs to let sodium ions flow from the extracellular fluid into the neuron. There are two main types of ion channels, voltage-gated channels and chemically-gated channels. Voltage-gated channels open or close in response to the surrounding electro-potential while chemically gated ones open and close in response to chemical stimuli. Sodium channels are almost all voltage-gated (Yu and Catterall, 2003).

The sodium channel protein is a larger, multimeric complex, which is composed of a highly conserved α-subunit, and is associated with one or more smaller β subunits in certain tissues (Catterall, 2000). Sodium-channel proteins in the mammalian brain consist of a highly processed α-subunit that is approximately 260 kDa, and is associated with one

or more accessory subunits (β1, β2 & β3) of 33-36 kDa in certain tissues (Yu and Catterall, 2003). The α-subunit has 4 homologous domains (I-IV) containing six α-helical transmembrane segments (S1-S6). The segments are connected by non-conserved hydrophilic intervening segments. The fourth transmembrane segment (S4) of each domain is highly positively charged, and thought to be a voltage sensor. A reentrant loop between helices S5 and S6 is embedded into the transmembrane region of the channel to form the narrow, ion-selective filter at the extracellular end of the pore. The wider intracellular end of the pore is formed by four S6 segments. Small extracellular loops connect the transmembrane segments, with the largest ones connecting the S5 or S6 segments to the membrane reentrant loop. Larger intracellular loops link the four homologous domains. Interesting structural information on the sodium channel is emerging. Direct structural determination at 19 Å resolution by cryo-electron microscopy and image reconstruction techniques show sodium channels as having a bell-like shape (side view), with a four-fold symmetry of transmembrane masses and large intracellular and extracelluar masses through which several inlets and outlets allow aqueous access (Sato et al., 2001). However, these four-domain sodium channels have not been identified from prokaryotes. Ren et al. (2001) reported a bacterial sodium channel in the salt-loving bacterium *Bacillus halodurans*. This single domain voltage-dependent sodium channel has sequence and pharmacological characteristics resembling those of calcium channel.

Three key features of sodium channels are voltage-dependent activation, rapid inactivation, and selective ion conductance. The outer pore of sodium channel is formed by the reentrant loops between transmembrane segments S5 and S6 of each domain. Two important amino acids in analogous positions in all four domains are thought to form the

negatively charged outer and inner rings that serve as a receptor site for pore-blockers and the selectivity filter. Mutations of these residues have significant effects on binding of neurotoxins, such as tetrodotoxin and saxitoxin (Noda et al., 2000).

1.2.1.1.2 Insect knockdown resistance

In insects, sodium channels are the target site of pyrethroid and DDT insecticides. Pyrethroid insecticides slow the kinetics of sodium channel activation and inactivation, resulting in the prolonged opening of individual channels and leading to paralysis and death of poisoned insects (Narahashi, 2000). The primary structure of insect sodium channels is similar to that of mammalian sodium channel α-subunit. The first insect sodium channel gene, *para*, was identified in *Drosophila melanogaster* (Loughney et al., 1989). Insensitivity of sodium channel results in pyrethroid and DDT insecticide resistance. The term "knockdown resistance (*kdr*)" is used to describe cases of resistance to DDT and pyrethroid insecticides in insects and other arthropods that result from reduced sensitivity of the nervous system. Unlike resistance due to enhanced metabolic detoxication, knockdown resistance is unaffected by synergists that inhibit insect esterases and monooxygenases.

Knockdown resistance was originally characterized and rigorously studied in the house fly (*Musca domestica* L). It has been found that a leucine to phenylalanine replacement at amino acid residue 1014 (designated L1014F) in the S6 segment of domain II (IIS6) of the α subunit is associated with moderate (10-fold to 30-fold) knockdown resistance of *kdr* strains (Williamson et al., 1996). In *super-kdr* strains, a mutation of methionine to threonine replacement at residue 918 (M918T) near the S4-S5

linker in domain II was found along with the L to F mutation, which resulted much higher resistance (500-fold) (Williamson et al., 1996; Miyazaki et al., 1996). To date, the M to T mutation has not been detected in the absence of the L to F mutation in any house fly population. Subsequent studies mapped the *kdr* trait to house fly chromosome 3 (Soderlund et al., 1999). These finding prompted the search for similar mutations in other insect species. Most of these studies focused on the S4-S6 segments of domain II and confirmed the importance of the region of the S4-S6 segments of domain II as a site for knockdown resistance mutations.

The conserved nature of the *kdr* mutation (L to F) in IIS6 has been identified in a wide range of agricultural and medical pest insects. Comparison of insecticide-suseptible and *kdr* resistant German cockroach strains indicated that leucine in IIS6 only occurred in susceptible stains, while phenylalanine was specifically presented with *kdr* resistant strain (Dong, 1997). The same mutation was also found in horn flies (*Haematobia irritans*) (Jamroz et al., 1998), diamondback moths (*Plutella xylostella*) (Schuler et al., 1998), aphids (*Myzus persicae*) (Martinez-Torres et al., 1999a), mosquitoes (*Anopheles gambiae, Culex pipiens and Culex quinquefasciatus*) (Martinez-Torres et al., 1998; Martinez-Torres et al., 1999b; Xu et al., 2005), and Colorado potato beetles (*Leptinotarsa decemlineata*) (Lee et al., 1999). Unlike many examples of L to F mutation in other species, the house fly *super-kdr* trait (M918T in IIS4-5) has only been found in highly resistant population of horn flies (*Haematobia irritan*) (Jamroz et al., 1998). A second mutation at position 1014, the replacement of leucine with histidine (L1014H), was found in pyrethroid-resistant *Heliothis virescens* (Park and Taylor, 1997) and house fly (Liu and Pridgeon, 2002). The third mutation at position 1014, leucine replaced by serine

(L1014S), was documented in mosquitoes *Culex pippiens* and *Anopheles gambiae* (Martinez-Torres et al., 1999b; Ranson et al., 2000).

The effects of the L to F mutation in IIS6, the most common sodium channel gene mutation associated with knockdown resistance, on pyrethroid sensitivity were examined in several sodium channel genes. The house fly sodium channel gene *Vssc1* carrying the L to F mutation has been coexpressed with the *Drsophila melanogaster* tipE protein in *Xenopus* oocytes and assayed by voltage-clamp. Vssc1/tipE channels containing the L to F mutation were approximately 10-fold less sensitive to modification by cismethrin, a type I pyrethroid, than wild type channels (Smith et al., 1997). Similar effect was also observed on *D. melanogaster para* sodium channels coexpressed with the tipE protein using deltamethrin as a test pyrethroid (Vais et al., 2000).

The current literature suggests that point mutations of insect voltage-dependent sodium channel genes are the primary cause of knockdown resistance to pyrethroids.

1.2.1.1.3 Alternative splicing of sodium channel involved in kdr *resistance*

Sodium channel genes have been identified in a variety of animals, including invertebrates and vertebrates. The biophysical characteristics, pharmacology, gene organization, and even intron splice sites of invertebrate sodium channels are largely similar to mammalian sodium channels, adding further support to the idea that the primordial sodium channel was established before the evolutionary separation of invertebrates from vertebrates (Plummer et al., 1999).

The functional diversity of sodium channels in mammals is achieved mainly by expression of distinct sodium channel genes and at least ten genes have been found in

mammals (Yu and Catterall, 2003), including 9 functional genes and one non-voltage-gated channel. The mammalian sodium channel genes exhibit unique tissue distributions, channel properties, and distinct pharmacologies (Goldin, 2001). Southern blot analysis indicated that there are at least 8 sodium channel genes in Zebrafish (Novak et al., 2006). In the model insect *Drosophila melanogaster*, however, only one gene, *para*, has been identified to encode a functional sodium channel. *DSC1* was reported as a putative sodium channel gene in *D. melanogaster* (Salkoff et al., 1987), isolated from a genomic DNA library using a cDNA probe encoding the eel sodium channel. The DSC1 protein shares high sequence similarity with *para* and mammalian sodium channel α- subunits. But the functional identity of the DSC1 channel has never been determined. *BSC1*, an ortholog of *DSC1*, has been cloned from *Blattella germanica* (Liu et al., 2001), which encodes a voltage-gated calcium-selective channel (Zhou et al., 2004). While mammals have evolved a distinct mechanism involving selective expression of multiple sodium channel genes, insects generate sodium channel functional diversity by alternative splicing of a single sodium channel gene. Two functional splicing variants encoding parts of segments 3 and 4 of domain III have been found in the German cockroach sodium channel gene *para* ^{CSMA}, which exhibited different sensitivity to deltamethrin, a pyrethroid insecticide (Tan et al., 2002).

1.2.1.1.4 RNA editing of sodium channel may be involved in kdr *resistance*

The phenomenon of RNA editing was first discovered in trypanosomes based on the insertion of four ribonucleotides into a frameshifted mitochondrial transcript (Benne et al., 1986). Since then, RNA editing has been widely detected in eukaryotes, from

single-celled protozoa to mammals and plants; in all three major type RNA classes, mRNA, tRNA and rRNA; in nucleus-, mitochondrion-, chloroplast- and virus-encoded RNAs (Bass 2001). Like RNA splicing and polyadenylation, RNA editing changes the genomically encoded nucleic acid sequence of RNAs. However, unlike other types of RNA processing, RNA editing usually changes only one or two nucleotides at a time. Furthermore, RNA editing refers to many different processes that have entirely different mechanisms, while other types of RNA processing are grouped by a common mechanism.

Palladino et al. (2000) mutated a candidate RNA editing enzyme in *Drosophila*, dADAR (adenosine deaminase, which acts on RNA), and abolished all A- to -I RNA editing sites in *Drosophila.* The mutant adults were morphologically wild-type but exhibited extreme behavioral deficits including temperature-sensitive paralysis, locomotor uncoordination, and tremors that increased in severity with age. They concluded that A- to -I RNA editing in *Drosophila* was primarily involved in adult nervous system function and integrity. Two A-to-I editing events in the German cockroach sodium channel gene $BgNa_{v1\text{-}2}$ resulted in a Lys to Arg change in domain I segments 2 (IS2) and an Ile to Met change in IVS3 were reported (Song et al. 2004). The Lys to Arg change shifted the voltage dependence of activation in the depolarizing direction. Two U-to -C editing sites were also found in $BgNa_v1$-1 and resulted in a Leu to Pro change in IIIS1 and a Val to Ala change in IVS4. The Leu to Pro change shifted both the voltage dependence of activation and steady-state inactivation in the depolarizing direction. Furthermore, these RNA editing events were presented in a tissue-specific and development-specific situation. Interestingly, a persistent tetrodotoxin (TTX) - sensitive

sodium current resulting from U-to-C RNA editing of German cockroach sodium channel gene $BgNa_v$ (formerly $para^{CSMA}$) was detected by Liu et al (2004b).

1.2.1.2 Insensitivity of acetylcholinesterase

Acetylcholinesterase (AChE) terminates nerve impulses by hydrolyzing the neurotransmitter acetylcholine. AChE in the insect nerve system is the primary target of organophosphate (OP) and carbamate insecticides, which inhibit AchE activity by phosphorylating or carbamylating the serine residue within the active site of the enzyme (Weill et al., 2003). However, insects have developed resistance to OPs and carbamates through modification of their AChE sensitivity to insecticides (Oppenoorth, 1985; Weill et al., 2003; Hemingway et al., 2004; Russell et al., 2004).

The first insect AChE gene *ace* was cloned from *Drosophila* by Fournier et al. (1989). In several resistant *Drosophila* strains, a variety of point mutations that cause various single amino acid replacements led to increased levels of enzyme insensitivity (Fournier et al 1989). Five mutations (F 115 to S, I 199 to V, I 199 to T, G 303 to A, and F 368 to Y) have been found in several insecticide resistant strains of *Drosophila melanogaster* (Mutero et al., 1994). In some cases, several of these mutations occurred in the same strain, indicating that a combination of resistant alleles preexist in natural populations and this is a mechanism by which insects rapidly adapt to insecticide selective pressures (Mutero et al., 1994). Walsh et al. (2001) found five mutations (V 180 to L, G 262 to A, G 262 to V, F 237 to Y, and G 365 to A) in the *ace* gene of insecticide resistant house fly strains. These mutations, either singly or in combination, confer different patterns of insecticide resistance (Walsh et al., 2001). The effects of mutation

combinations appear to be additive, i.e. the individual mutations cause a low level of insensitivity while combinations of mutations generate enhanced resistance (Walsh et al., 2001).

To date, while only a single *ace* gene has been identified in *Drosophila* and *Musca domestica*, different genes, *ace-1* and *ace-2*, have been found in mosquitoes (Bourguet et al., 1996). The presence of 2 different *ace* genes was confirmed from the whole genomic sequence of *Anopheles gambiae* (Holt et al., 2002). The complete *ace-1* coding regions of susceptible and resistant *Culex pipiens* strains were cloned and compared between insecticide resistant and susceptible mosquitoes. A single mutation of glycine to serine (G119S), resulting from a single nucleotide polymorphism (SNP), G to A, has been characterized (Weill et al., 2003). The same mutation was also identified in other insensitive AchE strains of *Anopheles gambiae* and *Culex quinquefasciatus* (Weill et al., 2003; 2004; Liu et al., 2005). This glycine residue lies at the base of the active site, close to the catalytic site and abutting the oxyanion hole (Weill et al., 2003). As the G119S mutation creates an *Alu* I restriction site in the *ace-1* of resistant individuals, a PCR/RFLP test was successfully used to detect its presence in single mosquitoes of both *Culex pipiens* and *Anopheles gambia* (Weill et al., 2004). Another mutation, F455W (corresponding to F331 in *Torpedo*), associated with high OP resistance has been identified from *Culex tritaeniorhynchus* (Nabeshima et al., 2004). The mutation locates near the catalytic histidine within the acyl pocket of the enzyme. This mutation has also been commonly found in carbamate-resistant *Myzus persicae* and OP-resistant two-spotted spider mite *Tetranychus urticae* (Nabeshima et al., 2003; Anazawa et al., 2003).

In contrast, *ace-2* genes of mosquitoes *Culex tritaeniorynchus* and *Culex pipiens* are not involved in resistance (Malcolm et al., 1998; Mori et al., 2001).

1.2.1.3 Insensitivity of gamma aminobutyric acid receptor

Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in nervous systems of both vertebrates and invertebrates (ffrench-Constant et al., 1993). Binding of GABA to its receptor activates chloride ion selective channel (Hemingway et al., 2004). The GABA receptor is the target site of cyclodiene and phenylpyrazoles insecticides (ffrench-Constant et al., 2000). GABA receptors are composed of 5 subunits, which arrange around the central ion channel (Hemingway et al., 2004). *Rdl (Resistance to dieldrin)*, a GABA receptor subunit gene, was cloned from a field-isolated *Drosophila* mutant, which was resistant to dieldrin, a cyclodiene insecticide (ffrench-Constant et al., 1993). Genetic mapping of dieldrin resistance in *Drosophila melanogaster* indicated that resistance was conferred by *Rdl* on the left arm of chromosome III at map position 66F (ffrench-Constant and Roush 1991). A mutation at a single codon in the *Rdl* gene, a replacement of alanine at position 302 with either a serine or a glycine, conferred dieldrin resistance in *Drosophila* (ffrench-Constant et al., 1993). Alanine at position 302, which lies within the second transmembrane region of the Rdl subunit, is crucial for insecticide binding and mutation of this amino acid can cause resistance due to a unique dual effect on insecticide binding (Zhang et al., 1994). The mutation of alanine to serine or glycine has been documented in resistant strains of a wide range of insect pests, such as the mosquito *Aedes aegypti* (Thompson et al., 1993a), the house fly *Musca domestica* (Thompson et al., 1993b), the coffee berry borer *Hypothenemus hampei*

(ffrench-Constant et al., 1994), the sweet potato whitefly *Bemisia tabaci* (Anthony et al., 1995), the green peach aphid *Myzus persicae* (Anthony et al., 1998), the red flour beetle *Tribolium castaneum* (Thompson et al., 1993b; Andreev et al., 1999) and the cat flea *Ctenocephalides felis* (Daborn et al., 2004).

1.2.2 Resistance due to metabolic detoxification

Metabolic detoxification is one of the most common mechanisms of insecticide resistance (Scott, 1991). There are 3 major groups of enzymes, cytochrome P450-dedpendent monooxygenases, hydrolases (esterases), and glutathione S-transferases, involved in resistance (Hemingway et al., 2004).

1.2.2.1 Cytochrome P450-mediated detoxification

The cytochrome P450-dependent monooxygenases are a diverse class of enzymes involved in the catabolism and anabolism of endogenous and exogenous compounds (Scott, 1999). P450s are hemoproteins, which are named because of their characteristic absorbance peak at 450nm when their reduced forms are treated with carbon monoxide. The majority of cytochrome P450s in eukaryotes is embedded in the endoplasmic reticulum. P450s require P450 reductase, and sometimes cytochrome b5, to transfer reducing equivalent from NADPH to P450s, depending on the substrate and/or the P450 that is involved (Scott and Wen, 2001). Cytochrome P450s have been found ubiquitously in aerobic organisms, including bacteria, fungi, plants, insects, birds and mammals (Stegeman and livingstone, 1998). Many P450 genes have been identified in insects. In the fruit fly *Drosophila melanogaster* genome, 90 P450 genes throughout 25 families

have been identified (Tijet et al., 2001). Among them, 15 P450 genes are located in arm of the X chromosome, 14 in 2L, 34 in 2R, nine in 3L, and 17 in 3R (one P450 gene cannot be mapped) (Ranson et al., 2002). In the mosquito *Anopheles gambiae* genome, 111 P450 genes (5 of them are pseudogenes) have been founded. Ten *A. gambiae* P450 genes are located in arm of the X chromosome, 11 in 2L, 47 in 2R, 11 in 3L, and 32 in 3R (Ranson et al., 2002). Insect P450 genes have been assigned to 6 *CYP* families: *CYP* 4, 6, 9, 12, 18, and 28 (Feyereisen, 1999). Both in *D. melanogaster* and *A. gambiae,* the largest groups of P450 genes fall into the *CYP4* and *CYP6* families.

Insect cytochrome P450 monooxgenases are involved in detoxification of insecticides and plant toxins (Scott et al., 1998; Feyereisen, 1999). They also play critical roles in the biosynthesis and metabolism of insect hormones, such as juvenile hormones and ecdysteroids, which are very important for insect growth, development, and reproduction (Feyereisen, 1999; Scott and Wen, 2001). Although P-450 mediated metabolism results in detoxification in most cases, it results in insecticides activation, especially in organophosphates (Feyereisen, 1999; Scott, 1999).

Many P450 proteins and genes have been identified from insecticide resistant strains and their association with resistance have been well documented. The first insect P450 gene, *CYP6A1,* was cloned from the diazinon resistant house fly strain Rutgers (Feyereisen et al., 1989). *CYP6A1* was expressed at about 10-fold higher levels in Rutgers than susceptible strains, and the elevated *CYP6A1* expression was not due to gene amplification (Carino et al., 1994). *CYP6A1* was mapped to autosome 5, and factors responsible for the increased expression level was linked to autosome 2 (Feyereisen et al., 1995). This indicated that the expression of *CYP6A1* was regulated by *trans*-factors.

CYP6A1 was expressed in all life stages (Carino et al., 1994). Phenobarbital treatment increased *CYP6A1* expression about 20-fold in adults of the Rutgers strain (Carino et al., 1992).

A single P450 protein, $P450_{1pr}$, was isolated from house flies resistant strain Learn Pyrethroid Resistant (LPR) (Wheelock and Scott, 1989). $P450_{1pr}$ was overexpressed in the LPR strain compared to the susceptible strain (Scott and Lee, 1993). The gene encoding $P450_{1pr}$ was cloned and name as *CYP6D1* (Tomita and Scott, 1995). *CYP6D1* was confirmed as the gene responsible for monooxygenase-mediated pyrethroid resistance in house flies LPR strain (Wheelock and Scott, 1992; Zhang and Scott, 1994). Southern blots showed similar hybridization intensities between LPR and susceptible flies, while northern blots revealed that the CYP6D1 mRNA was expressed at about a 10-fold higher level in LPR than the susceptible strain (Tomita et al., 1995). This indicates that the elevated level of CYP6D1 mRNA in LPR strain is not due to gene amplification but increased transcription (Liu and Scott, 1998). This elevated mRNA level matches the results of an 8-fold higher level of CYP6D1 protein in microsomes from LPR compared to a susceptible strain (Liu and Scott, 1996). The overexpression of *CYP6D1* is not the result of increased stability of the mRNA in LPR strain (Liu and Scott, 1998). *In vitro* run-on transcription assay was applied to measure the relative transcription rate of *CYP6D1* in LPR and susceptible flies. Only low levels of CYP6D1 mRNA was detected when nuclei from the susceptible strain were used in the run-on assay, while abundant CYP6D1 mRNA was detected when the LPR flies were used (Liu and Scott, 1998). The intensity of the CYP6D1 signals was approximately 10-fold higher in LPR than in susceptible flies (Liu and Scott, 1998), which agrees with previous Northern blot results

(Tomita et al., 1995). *In vitro* run-on transcription assay results provided the first direct evidence for increased transcription as a primary cause of insecticide resistance (Liu and Scott, 1998). *CYP6D1* is located on autosome 1 of house flies (Liu et al., 1995). The increased transcription of *CYP6D1* in the LPR strain is due to factors on autosomes 1 and 2 (Liu and Scott, 1996). Phenobarbital, a well-studied inducer of P450s, resulted in a 4-fold increase of *CYP6D1* in a susceptible stain, but had no effect in LPR flies (Liu and Scott, 1997). High levels of CYP6D1 mRNA were found only in adults both in resistant and susceptible strains, but not eggs, larvae, or pupae (Scott et al., 1996). Thus, *CYP6D1* is regulated by as least 3 factors, i.e. strain, inducers and life stage. Studying these factors may help to understand P450 regulation and insecticide resistance (Scott et al., 1999).

In an insecticide resistant strain of *Drosophila melanogaster*, 91-R, *CYP6A2* was expressed about 20-30 fold higher levels compared to susceptible strain (Waters et al., 1992). *CYP6A2* expressed *in vitro* in a baculovirus system was capable of metabolizing aldrin, heptachlor and diazinon (Dunkov et al., 1997). Point mutations in CYP6A2 protein conferred increasing activity against DDT (Berge et al., 1998). Using microarray analyses of all P450s in *Drosophila melanogaster*, Daborn et al. (2002) demonstrated that DDT-R, a gene conferring resistance to DDT, was associated with overtranscripted *CYP6G1*. This gene was also overexpressed in 28 stains of DDT-resistant *Drosophila melanogaster* collected throughout the world. Transgenic analysis indicated that overtranscription of *CYP6G1* alone was both necessary and sufficient for DDT resistance (Daborn et al., 2002). The resistant allele of *CYP6G1* was identified by the presence of a transposable element at the 5' end of the gene (Daborn et al., 2002).

The elevated mRNA levels of P450s associated with insecticide resistance have also been well documented in other insect pests, such as *CYP6Z1* from *Anopheles gambiae* (NiKou et al., 2003), *CYP6F1* from *Culex quinquefasciatus* (Kasai et al., 2000), *CYP4G8* from *Helicoverpa armigera* (Pittendrigh et al., 1997), and *CYP4G19* from *Blattella germanica* (Pridgeon et al., 2003).

Although in most cases P450-mediated insecticide resistance is due to overexpression of P450 genes in resistant strains, the change in the P450 amino acid sequence may also cause insecticide resistance (Scott, 1999).

1.2.2.2 Hydrolase-mediated detoxication

Hydrolases or esterases are a larger group of enzymes that hydrolyse carboxylate and phosphate esters (Scott, 1991; Hemingway and Karunaratne, 1998). Although organophosphate (OP), carbamate, and pyrethroid insecticides all are substrates for hydrolases, hydrolose- mediated insecticide resistance has mainly been found in OP and carbamate (Hemingway et al., 2004). Mechanisms of hydrolose-mediated insecticide resistance can usually be divided to two groups: quantitatively and qualitatively changed esterase-base mechanisms. Quantitatively changed esterase-base mechanism is based on the over-production of enzyme, while qualitatively changed esterase-base mechanism depends on the change of enzyme function (Hemingway et al., 2004; Hemingway, 2000; ffrench-Constant et al., 2004).

Over-production of carboxylesterases has led to the evolution of insecticide resistance, either via changes in gene regulation and /or gene amplification (ffrench-Constant et al., 2004). In the quantitatively changed esterase-base mechanism, generally,

the esterases have a very high affinity but a low capacity for hydrolyzing OPs and carbamates. The large amounts of these enzymes cause resistance because the activated insecticides are rapidly sequestered before they reach the target site, acetylcholinesterase (AchE) (Hemingway and Karunaratne, 1998). In the peach potato aphid *Myzus persicae*, OP resistance is primarily associated with the amplification of *esterase E4* (Field et al., 1998). Although the metabolic rate of insecticides is slow, the E4 enzyme in this species can comprise up to 3% of the body weight, and acts like a sponge to sequester insecticides (Devonshire et al., 1998). The same mechanism has also been found in the mosquito *Culex pipiens* and *quinquefasciatus* (Raymond et al., 1998; Ferrari and Georghiou, 1990; Small et al., 1998), the greenbug *shizaphis graminum* (Gao and Zhu, 2000), the tobacco budworm *heliothis virescens* (Goh et al., 1995), and the brown planthopper *Nilaparvata lugens* (Vontas et al., 2000).

Qualitatively change of enzyme function was first found in OP resistant strains of house flies (Van Asperen and Oppenoorth, 1959). The observation led to the proposal of the "ali-esterase" hypothesis (Oppenoorth and Van Asperen, 1960), i.e. the resistant mutants gained the ability to hydrolyse OP substrates while the ability to hydrolyse carboxylesterase substrates was reduced. This hypothesis proved valid for the sheep blowfly *Lucilia cuprina* (Newcomb et al., 1997a). A single mutation (Gly 137 to Asp) in esterase E3 of this species is responsible for the loss of the carboxylesterase activity and gain of OP hydrolase activity. The functional assay, expressing mutant enzymes, confirmed that this amino acid mutation leads to an increase of OP hydrolase activity (Newcomb et al., 1997b). The same amino acid replacement has also been documented in the OP-resistant house fly *Musca domestica* (Claudiano et al., 1999). Another amino acid

substitution (Trp 251 to Leu) has been found in the esterase E3 of the sheep blowfly associated with malathion resistance (Campbell et al., 1998). The same mutation (Trp to Leu) has also been reported in malathion-resistant house flies (Claudianos et al., 2002). In the malathion-resistant parasitoid wasp *Anisopteromalus calandrae,* the tryptophan was replaced with a glycine (Zhu et al., 1999).

1.2.2.3 Glutatione S-transfersases-mediated detoxication

The glutathione S-transferases (GSTs) are a group of enzymes with multi-functions. GSTs are involved in the detoxification of a wide range of xenobiotics including insecticides (Salinas and Wong, 1999). GSTs primarily catalyse the conjugation of xenobiotics with endogenous substrates, such as glutathione (GSH), and generally make the resultant products more water soluble so that it can be easily excreted (Habig et al., 1974). GSTs are well studied in mammals because they are very important in cancer epidemiology and drug resistance pathways (Tew, 1994; Hayes and Pulford, 1995). In insects, studies of GSTs have focused on their role in detoxifying foreign compounds, particularly insecticides (Clark et al., 1986; Wang et al., 1991; Fournier et al., 1992; Ranson et al., 2001). Up to now, 6 different classes of GSTs (i.e. Delta, Epsilon, Omega, Theta, Sigma and Zeta) have been identified in insects (Enayati et al., 2005).

Elevated GST activity has been implicated in resistance to at least 4 classes of insecticides (Hemingway et al., 2004). Elevated DDT-dehydrochorinase activity has been found in many DDT-resistant insect species including the mosquitoes *Aedes aegypti* (Grant et al., 1991), *Anopheles gambiae* (Prapanthadara et al., 1993) and *Anopheles dirus* (Prapanthadara et al., 1996). In *Aedes aegypti*, two isoforms of GST are overexpressed in

a DDT-resistant strain. At least one of them may be controlled by a mutation in a *trans*-acting repressor in the DDT-resistant strain (Grant and Hammock, 1992). Eight GSTs have been identified in the Epsilon class from *Anopheles gambiae* (Enayati et al., 2005). Some of these are overexpressed in a DDT-resistant *Anopheles gambiae* strain and one of these, *GSTe2*, encodes an enzyme that has the highest levels of DDT dehydrochlorinase activity (Ortelli et al., 2003). Genetic mapping of the DDT resistant loci in *A. gambiae* indicates that both *cis*- and *trans*-acting factors are involved in the overexpression of the Epsilon class GSTs (Ranson et al., 2000).

Correlation of high levels of GST with high levels of resistance to pyrethroid also occurs in *Aedes aegypti* (Grant and Matsumura, 1989), *Tribolium castaneum* (Reidy et al., 1990), and *Spodoptera littoralis* (Lagadic et al., 1993). However, GSTs have not yet been implicated in the direct metabolism of pyrethroid insecticides. GSTs may play an important role by detoxifying lipid peroxidation products induced by pyrethroids (Vontas et al., 2001). GSTs also are responsible for organophosphate resistance (Hayes and Wolf, 1988).The role of GSTs in the detoxification of parathion and methyl parathion in the diamondback moth *Plutella xylostella* was confirmed by purificating and characterizing 4 GST isozymes, GST-1, GST-2, GST-3, GST-4 (Chiang and Sun, 1993; Ku et al., 1994). Recombinant GST enzymes from the diamondback moth and house fly confirmed that GSTs play an important role in the metabolism of organophosphate insecticides (Huang et al., 1998; Wei et al., 2001).

1.2.3 Other mechanisms of insecticide resistance

1.2.3.1 Behavioral resistance

Behavioral resistance is defined as the developments of behaviors that reduce an insect's exposure to a toxin or that allow an insect to survive in an environment that is harmful and/or fatal to the majority of other insects (Sparks et al., 1989). This mechanism can be divided into 2 groups, stimulus-dependent (requires sensory stimulation) and stimulus-independent (absence of sensory stimulation). Behavioral resistance to fenvalerate and permethrin has been identified in the horn fly (Lockwood et al., 1985). Behavioral resistance to pyrethroids has also been documented in German cockroach (Ross, 1992). Behavioral resistances in mosquitoes have been found by reducing the rate of mosquito entry into houses, increasing the rate of early exit from houses and inducing the changing in biting times (Mbogo et al., 1996; Mathenge et al., 2001).

1.2.3.2 Decreased penetration

In order to reach its target site, insecticides must pass through the insect cuticle. Some insecticide resistant mosquitoes have evolved thicker or altered cuticle, reducing penetration of insecticides (Stone and Brown, 1969; Apperson and Georghiou, 1975). Decreased penetration has also been identified in other resistant insect pests, such as the house fly (Wen and Scott, 1999) and German cockroach (Valles et al., 2000), but the exact mechanisms are largely unknown.

1.2.3.3 Accelerated excretion

Accelerated excretion is also a mechanism that can confer insecticide resistance. It has been found that the excretion rate is much faster in resistant strains than that in susceptible strains of flower thrips *Franliniella occidentalis* (Zhao et al., 1994), Colorado potato beetles *Leptinotarsa decemlineta* (Argentine et al., 1995), tobacco budworms *Heliothis virescens* (Ottea et al., 1995) and western corn rootworms *Diabrotica virgifera virgifera* (Scharf et al., 1999). However, little is know about the molecular basis behind increased rates of excretion.

1.3 Mosquitoes as an insect pest

1.3.1 Brief introduction of mosquitoes

Mosquitoes are two-winged flies (Diptera) belonging to the family Culicidae. Over 3,000 known species of mosquitoes have been documented worldwide and more than 160 species exist in North America (Munstermann and Conn, 1997). The development of mosquitoes is temperature-dependent since they are invertebrates. Mosquitoes are holometabolism insects and have four distinct developmental stages: egg, larva, pupa, and adult. Mosquitoes lay their eggs on water or a moist surface. Eggs are white when first deposited, darkening to a dark brown or black within 12-24 hours. Single eggs are oval and about 0.6mm long (Clements, 1992). Some mosquitoes lay their eggs singly, such as *Anopheles* and *Aedes* mosquitoes. Others lay eggs in attached groups called rafts, like *Culex* mosquitoes (Borror et al., 1992). When environmental conditions are ideal, mosquito eggs may hatch to larvae within 1-3 days. The larvae stages of mosquitoes that live in water have four developmental periods called instars. The length of the larval

stage can range from 4 to 14 days, depending on species, food availability, and water temperature (Clements, 1992). Mosquito pupae are very active and also live in water. The pupae stage is non-feeding and can be completed in as few as 2-3 days (Foster and Walker, 2002). Usually, male mosquitoes emerge first and stay near the breeding site, waiting for females. Mating occurs very soon after emergence due to high adult mortality rates. Female mosquitoes mate only once. Both male and female mosquitoes feed on sugar sources, such as plant nectars, but only females feed on blood to nourish and develop their eggs. However, there are a few exceptions. Some species are autogenous, meaning a female is able to develop a first set of eggs without a blood meal (Mattingly, 1969). Female mosquitoes locate hosts by the carbon dioxide and other trace chemicals exhaled by the host. Mosquitoes are very sensitive to several chemicals, such as carbon dioxide, amino acids, and octenol (Clements, 1999). Most mosquito species search for a blood meal in the evening. However, *Aedes albopictus* is very aggressive, and prefers to feed during the daylight hours. Mosquitoes can feed on bird, mammal, or cold-blooded vertebrates such as reptiles and frogs (Clements, 1992). Following blood feeding, female mosquitoes will find a suitable place to lay eggs. If female mosquitoes survive their egg laying activities, they start a search for another blood meal and lay another batch of eggs. Most mosquitoes that have been found in North America belong to four genera: *Anopheles, Aedes, Culex and Psorophora* (Borror et al., 1992).

1.3.2 Medical importance of mosquitoes

Repeated blooding feedings of female mosquitoes have made them the ideal transmitter of a wide diversity of disease, such as malaria, dengue, yellow fever, West

Nile, lymphatic, filariasis, Japanese encephalitis and many others. The malaria vector *Anopheles gambiae* transferring deadliest parasite, *Plasmodium falcipaurm*, contributes to the death of more than 1 million people every year (Morel et al., 2002). Annually, there are 50-100 million human cases of dengue fever (Gubler and Meltzer, 1999), which is mainly transmitted by *Aedes aegypti*. Recent estimates indicate that there are 200,000 cases per year of yellow fever, which is also transmitted by *Aedes aegypti*, leading to 30,000 deaths annually (------, 2002). West Nile virus (WNV), which causes potentially fatal brain inflammation, is mainly transmitted by *Culex* mosquitoes (Granwehr et al., 2004). The introduction of WNV in the USA was in New York City in 1999. The disease moved rapidly west and is now exploding across the country, reaching Colorado, Nebraske, South Dakota, and Texas in 2003. There were 4156 cases of infection with 284 deaths in 2002 and 9858 new cases and 262 deaths in 2003 in the USA (Granwehr et al., 2004).

1.4 Insecticide resistance in mosquitoes

Vector control is a very important strategy for the control of mosquito-borne disease (WHO, 2000) and insecticide application plays a critically important role in vector control. So far, pyrethroids, OPs and carbamates are the major insecticide groups used to control mosquitoes. Pyrethroids are currently the most widely used as indoor residual house sprays to bednets and curtains (Zaim et al., 2000). Pyrethroids are commonly used due to their low toxicity in humans and high effect against insect pests (Hougard et al., 2002). Insecticide application limits mosquito-borne diseases. However, mosquito-borne diseases are making a resurgence. One of the important reasons is the difficulty in the

control of the mosquito vectors that have developed resistance to insecticides (Hemingway and Ranson, 2000). More than 100 mosquito species have developed resistance to insecticides (Hemingway and Ranson, 2000). Mechanisms of mosquito insecticide resistance can be divided into two major groups, insensitivity of target sites and metabolic detoxification.

1.4.1 Resistance due to insensitivity of target sites

Insensitivity of insecticide target proteins is mainly responsible for resistance through mutations. Sodium channel is the primary target site of DDT and pyrethroid insecticides. Resistance to DDT and pyrethroid due to the insensitivity of the target site, also known as knockdown resistance (*kdr*), is linked to point mutations in the *para*-type sodium channel genes. A single mutation of leucine to phenylalanine (L to F), termed the *kdr* mutation, has been associated with pyrethroid resistant mosquitoes *Anopheles gambiae* (Martinez-Torres et al., 1998), *Anopheles stephensi* (Enayati et al., 2003), *Culex pipiens (*Martinez-Torres et al., 1999b), *Culex quinquefasciatus* (Xu et al., 2005), and *Aedes aegypti* (Brengues et al., 2003). Another mutation at the same position, Leucine replaced by serine (L to S), has been documented in mosquitoes *Culex pipiens* (Martinez-Torres et al., 1999b) and *Anopheles gambiae* (Ranson et al., 2000). AChE in the insect nerve system is the primary target of organophosphate (OP) and carbamate insecticides. The modification of AChE results in OP and carbamate resistance. A single mutation of glycine to serine (G 119 S) in *ace*-1 associated with resistance has been found in mosquitoes A*nopheles gambiae* (Weill et al., 2003), *Culex pipiens* (Weill et al., 2003), and *Culex quinquefasciatus* (Liu et al., 2005). Another mutation, phenylalanine to

tyrptophan (F455W), associated with high OP resistance has been identified from *Culex tritaeniorhynchus* (Nabeshima et al., 2004). GABA receptor is the target sit of cyclodiene and fipronil insecticides. Insensitivity of its subunit gene *Rdl*, resulting from an alanine to serine (A 302 S) substitution, has been found in the mosquito *Aedes aegypti* associated with cyclodiene resistance (Thompson et al., 1993a).

1.4.2 Resistance due to metabolic detoxification

The metabolic mechanisms contribute to a decrease in the effective dose available at the target site. The products of three gene families, cytochrome P450s, hydrolases, and GSTs, are mainly involved in the detoxification of insecticides. Overexpression of P450s has been observed in permethrin–resistant mosquitoes *Anopheles gambiae* (Vulule et al., 1999) and *Anopheles albimanus* (Brogdon et al., 1999). *CYP6Z1,* an adult-specific P450 gene, has been identified from *Anopheles gambiae* associated with pyrethroid reistance (NiKou et al., 2003). This gene is located within the boundaries of a quantitative trait locus on chromosome arm 3R associate with permthrin resistance in this species (Ranson et al., 2004). About 2.5-fold elevated level of *CYP6F1* has been documented in *Culex quinquefasciatus* associated with insecticide resistance (Kasai et al., 2000). Overexpression of *CYP4* family members have been found from *Culex pallens* associated with deltamethrin resistance (Shen et al., 2003). Quantitative changes of esterases (elevated esterases), resulting in a production of abundant proteins in resistant insects, have been documented in *Culex pipens, Culex quinquefaciatus, Culex tritaeniorhynchus* (Hemingway and Karunaratne, 1998), *Anopheles gambiae* (Vulule et al., 1999), *Anopheles albimanus* (Brogdon and Barber, 1990) and *Aedes aegypti* (Mourya et al.,

1993) associated with insecticide resistance. Qualitative changes of esterases, resulting in the increasing activities of esterases, have also been identified in *Anopheles culicifacies, Anopheles stephensi* and *Anopheles arabiensis* (Herath et al., 1987; Hemingway, 1982a, b; 1983). The overexpression of GST genes has been found in resistant *Anopheles gambiae* (Ortelli et al., 2003), *Anopheles sbitctus* associated with OP resistance (Hemingway et al., 1991), *Aedes aegypti* associated with pyrethroid resistance (Grant and Matsumura, 1989), and *Aedes aegypti* associated with DDT resistance (Grant and Hammock, 1992).

1.5 Molecular techniques used in today's mosquito research

1.5.1 Mosquito RNAi techniques

RNA interference (RNAi) was first identified in the nematode *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), which induces the silencing of cognate gene expression (Fire et al., 1998). RNAi results in the degradation of dsRNA and any mRNA with high sequence homology with the dsRNA trigger (Bernstein et al., 2001). dsRNA can be introduced to organisms by transfection or can be produced intracellularly by the introduction of viruses or transposons that generate dsRNA during their replication cycle (Hammond et al., 2001).Recently, RNAi has become a powerful tool for probing gene function. Direct injection of dsRNA of a single gene into organisms can specifically silence that gene's expression (Shin et al., 2003). RNAi techniques are widely used to study reverse genetics in model organisms, such as *Caenorhabditis elegans* (Montgomery et al., 1998), *Drosophila* (Kennerdell and Carthrew, 1998; 2000), zebra fish (Li et al., 2000) and mouse (Wianny and Zernicka-Goetz, 2000). RNAi

techniques have also been utilized to study reverse genetics in mosquitoes. To show the conserved role of a complement-like protein in phagocytosis, dsRNA knockdown was used in hemocyte-like *Anopheles gambiae* cultured cells (Levashina et al., 2001). dsRNA of the *defensin* gene was directly injected into the mosquito *Anopheles gambiae* to knockdown this gene and revealed its function (Blandin et al., 2002). Bettencort et al. (2002) utilized direct injection of the dsRNA of *Hemolin* gene to silence the gene in *Cecropia* and demonstrated its vital role for normal development of embryos in the following generation. Although it is a very important tool for reverse genetics, one drawback that direct applications of dsRNA have is that their effects are transients. Consequently, it cannot be used to generate heritable gene silencing.

1.5.2 Mosquito transgenic technology

Transgenic technology is an effective tool to find, isolate and analyze insect genes and to genetically modify insects for the purposes of pest control. Using transgenic techniques, genetically stable mosquito lines can be established and be crossed with other transgenic and wild-type mosquito to generate new genetic variations (Shin et al., 2003). Genetic transformation was firstly done in *Drosophila melanogaster* using transposable element vectors (Rubin and Spradling, 1982). Transposable elements are mobile genetic units identified across life. Most early transgenic experiments were carried out using P-element based vectors due to its success with *Drosophila*. However, the P-element system only works well in drosophilid insects because of several host factors specific to these species (O'Brachta and Atkinson, 2000). Then scientists tried to find other transposable elements with a wider host range. Currently, four major kinds of

transposable elements, *mariner, Hermes, Minos* and *piggyBac*, are widely used in non-drosophilid insects for genetic transformation (Robinson et al., 2004).

The *mariner* element was discovered in a mutant of *Drosophila mauritiana*. The distribution of *mariner* elements is widespread in insects. In 400 tested insect species, more than 15% of them presented *mariner* elements (Robertson, 1993). Horizontal transmission is an explanation for this widespread distribution (Lohe et al., 1995). The elements also have a very broad host range and been used to produce transgenic microbes, insects and vertebrates (Lampe et al., 2000). This indicates that *mariner* transposition is autonomous requiring few or no host factors (Lampe et al., 1996). Germ-line transformation of the mosquito *Aedes aegypti* has been successfully established using *Mos1*, a characterized *mariner* element (Coates et al., 1998). *Hermes* is also a well studied transposable element used in insect germ-line transformation. The *Hermes* element has been shown to transpose into the germ line of house fly (O' Brochta et al., 1996) and medfly (Michel et al., 2001) by a cut-and-paste mechanism of transposition; i.e. only those sequences on the vector-containing plasmid that are flanked by the terminal inverted repeats of the *Hermes* elements could be integrated into the insect genome (O'Brochta et al., 2003). However, this was not the case in the *Hermes*-mediated transgenic mosquitoes. To date, canonical cut-and paste type transposition events have not been found the mosquito germ lines transformed by *Hermes* vectors. In *Aedes aegypti* (Allen et al., 2001) and *Culex quinquefaciatus* (Jasinskiene et al., 1998), germ-line integrations have involved both sequences delimited by the terminal inverted repeats of *Hermes* and sequences flanking the element on the vector-containing plasmid. The *Minos* element is less extensively used than other transposable vectors. *Minos* transposition

activity has been reported in mouse (Drabek et al., 2003) and the mosquito *Anopheles stephensi* (Catteruccia et al., 2000). *Minos* integrates using a cut-and paste mechanism with no exception (O'Brochta, et al., 2003).

The *piggyBac* element was described as 2472 bp in length with 13bp inverted terminal repeats and codes 594 amino acids (Fraser et al., 1996). *piggyBac* elements insert into the tetranucleotide TTAA sites that are duplicated during integration (Fraser et al., 1996). *piggyBac* elements have been used to transform the germ-line of a wide range of insects, including representatives from the orders Diptera, Lepidoptera, Coleoptera and Hymenoptera (O'Brochta, et al., 2003). In mosquitoes, *piggyBac* transposition have been documented in *Aedes aegypti* (Lobo et al., 2002), *Anopheles gambiae* (Grossman et al., 2001), *Anopheles stephensi* (Ito et al., 2002) and *Anopheles albimanus* (Perera et al., 2002), with transformation efficiencies ranging from 1% in *Anopheles gambiae* to 40% in *Anopheles albimanus*. Recently, evidence for its ability to transpose in mammalian cells and mice was reported (Ding et al., 2005). These data indicate that *piggyBac* elements carrying multiple genes can efficiently transpose in human and mouse cell lines (Ding et al., 2005). In mouse germ-line transposition, *piggyBac* elements can be excised accurately from original insertion site and transpose into the genome at diverse locations (Ding et al., 2005). These results suggest that *piggyBac* elements have a wide host range and can be used to establish a highly efficient transposon system.

Transgenic technology might be applied to produce sterile mosquitoes or to generate mosquitoes that cannot transmit disease (Hemingway, 2004). The immune response is one source of genes that can be engineered to prevent mosquitoes from transmitting pathogens. In the *Anopheles gambiae* genome, 242 genes from 18 gene families that may

be involved in the immune response have been identified (Christophides et al., 2002). The use of functional genomic technologies will help us to find which of these genes are transcriptionally regulated in response to challenge by parasite infection, and then these genes can be tested for their ability to confer refractoriness of parasite on transgenic mosquitoes.

CHAPTER 2: THE RESEARCH GOAL AND OBJECTIVES

2.1 Introduction

Mosquito-borne diseases are one of the main causes of human mortality worldwide. The mosquito *Cx. quinquefasciatus* Say is a nuisance species and important medical pest throughout the wet tropics. In the southeastern United States, this species is the vector of West Nile virus (WNV) (Sardelis et al., 2001) and Saint Louis encephalitis virus (SLEV) in many urban settings (Jones et al., 2002). Conventional approaches to mosquito control have relied on the application of insecticides (McCarroll and Hemingway, 2002). Pyrethroid insecticides are currently the most widely used insecticides for the indoor control of mosquitoes and are the only chemical group recommended for the treatment of mosquito nets, the main tool for preventing malaria in Africa (Hemingway, 2004). However, mosquito-borne diseases are making a resurgence. One of the important reasons is the difficulty in the control of the mosquito vectors that have developed resistance to insecticides. The effectiveness and low mammalian toxicities of pyrethroid insecticides have resulted in these compounds being widely used to control mosquitoes. Unfortunately, the heavy use of pyrethroids has led to the development of resistance in mosquitoes, including *Cx. quinquefasciatus* (Liu et al., 2004a).

2.2 The research goal and specific objectives

To address the need for understanding the development of pyrethorid resistance, the goal of my research is to characterize molecular mechanisms involved in pyrethroid resistance in the mosquito, *Cx. quinquefasciatus*. Five specific objectives will be addressed: 1) To investigate molecular mechanisms involved in the overall pyrethroid resistance in *Cx. quinquefasciatus*; 2) To identify *kdr* allelic variation in pyrethroid resistant mosquitoes, *Cx. quinquefasciatus*; 3) To characterize sodium channel gene expression associated with pyrethroid resistant house flies and German cockroaches; 4) To investigate sodium channel gene copy numbers in *Cx. quinquefasciatus* and the house fly; and 5) To clone and sequence the full length sodium channel cDNA in *Cx. quinquefasciatus.*

2.3 Hypotheses and significance of proposed research

The first objective is to study the molecular mechanisms involved in the overall pyrethroid resistance in *Cx. quinquefasciatus.* I hypothesize that high levels of pyrethroid resistance in mosquitoes are conferred by multiple mechanisms. The results of first objective will contribute to the understanding of the molecular basis underpinning the evolution of insecticide resistance of *Cx. quinquefasciatus*. My second objective focuses on the studying of the transcription of the L-to-F *kdr* mutation in the mosquito, *Cx. quinquefasciatus*. This study will investigate whether the functional polymorphism of the

L-to-F *kdr* mutation that determines the resistance phenotype results from DNA variation or transcriptional regulatory variation. I hypothesize that the L-to-F *kdr* mutation of the sodium channel of the mosquito, *Cx. quinquefasciatus* is due to transcriptional regulation. The third objective is to study the expression of the L-to-F *kdr* mutation in both house flies and German cockroaches, which are two diverse insects from holometabolous and hemimetabolous taxa, respectively. My hypothesis is that the transcriptional regulation of the L-to-F *kdr* mutation is also responsible for insecticide resistance in house flies and German cockroaches. The results of the second and third objectives will shed new light on the role of transcriptional regulation in the *kdr*-mediated resistance in insects and its connection with the genotype-resistance phenotype relationship. In addition, these results will provide new opportunities to study the expression of sodium channel genes in insects. The fourth objective focuses on identifying the sodium channel gene copy numbers in both mosquitoes and house flies. My hypothesis is that there are multiple sodium channel genes in mosquitoes and house flies. Several molecular techniques, such as southern blot and sequencing, will be used to investigate the gene copy numbers and gene expression in these two insects. The results of fourth objective will help us to understand the complexity of the sodium channel gene expression involved in the pyrethroid resistance of insects. Information from the fifth objective will provide data on the first full-length mosquito sodium channel cDNA and will identify potential differences in the sodium channel gene between resistant and susceptible mosquitoes. I hypothesize that there are

other mutations in the sodium channel involved in insecticide resistance besides the well known L-to-F *kdr* mutation. Isolation of full-length sodium channel cDNA is a critical and often difficult step toward understanding insecticide resistance. Full-length sodium channel cDNAs are only available for three insect species: *Vssc1* from the house fly, *BgNa* $_v$ from the German cockroach, and *VmNa* $_v$ from the varroa mite. The availability of a mosquito full-length sodium channel gene will enable us to identify possible polymorphisms involved in insecticide resistance by comparing the sodium channel sequences between resistant and susceptible mosquitoes.

The accomplishment of the proposed research will provide a more comprehensive understanding of the molecular basis of *kdr*-mediated insecticide resistance in mosquitoes and contribute conceptually to the development of successful programs in mosquito management for minimizing or preventing resistance development. For example, we may develop new synergists to target enzymes involved in transcriptional regulation of the L to F *kdr* mutation. Information from these studies will allow us to conduct functional studies needed to further characterize the polymorphisms of resistance, which, in turn, will provide novel information in *kdr*-medited resistance in mosquitoes.

CHAPTER 3: RESISTANCE IN THE MOSQUITO, *CULEX QUINQUEFASCIATUS*, AND POSSIBLE MECHANISMS FOR RESISTANCE

3.1 Introduction

Culex quinquefasciatus (Say) is an important disease vector throughout the wet tropics. In the Southeastern USA, this species is moderately competent as a vector of West Nile virus (WNV) (Sardelis et al., 2001), and is a primary vector of Saint Louis encephalitis virus (SLEV) in many urban settings (Jones et al., 2002). The primary approach used to control transmission of these pathogens and other mosquito-associated diseases has mainly relied on the application of insecticides (McCarroll and Hemingway, 2002). However, mosquito-borne diseases are now resurgent (Gubler, 1998), largely due to the difficulty in controlling vectors that have developed resistance to insecticides. The evolution of insecticide resistance by mosquitoes threatens human welfare as a result of their huge impact as vectors of human diseases.

Mechanisms of resistance in mosquitoes have attracted attention, since they elucidate pathways of resistance development and help those designing novel strategies to prevent or minimize the spread and evolution of resistance. Diverse resistance mechanisms have been identified in several strains of field-collected mosquitoes (Pasteur and Raymond, 1996; Hemingway et al., 1990; Kasai et al., 1998; Brengues et al., 2003) and multi-mechanisms appear to be common phenomena in pyrethroid resistance of these

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insect pests (Brengues et al., 2003), which can interact to increase the levels of resistance. The voltage-gated sodium channel is the primary target of pyrethroid insecticides (Pasteur and Raymond, 1996) and its insensitivity has been associated with pyrethroid resistance in several knockdown resistant (*kdr*) insect species, including mosquitoes (Brengues et al., 2003; Dong, 1997; Martinez-Torres et al., 1999b). A substitution of leucine to phenylalanine, termed the *kdr* mutation, in the S6 hydrophobic segment of domain II of the *para*-type sodium channel is consistently associated with knockdown resistance in several insect species, including mosquitoes (Dong, 1997; Martinez-Torres et al., 1999b; Miyazaki et al., 1996; Liu and Pridgeon, 2002). Nevertheless, a correlation between the presence of the *kdr* mutation and knockdown resistance has been only infrequently observed (Brengues et al., 2003; Dong et al., 1998). Cytochrome P450s, GST, and hydrolases have been important metabolic detoxication mechanisms conferring pyrethroid resistance in mosquitoes. Enhanced esterase activities corresponding to pyrethroid resistance have been reported in different mosquito species (Ranson et al., 2002; Hemingway et al., 2004). Elevated expression of cytochrome P450 genes and/or P450 activities are frequently associated with permethrin resistance in mosquitoes (Kasai et al., 1998; Hemingway et al., 2004; Nikou et al., David et al., 2005). Recently, a strong correlation of up-regulated expression of a GST gene, *GSTE2*, with permethrin resistance has been reported in a mosquito strain of *Anopheles gambiae* Giles (David et al., 2005).

Culex quinquefasciatus is a predominant mosquito species in urban areas of the state of Alabama, USA, especially around Mobile and Huntsville, and it has been a major target for several insecticides, including *Bacillus thuringiensis* var *israelensis* (Bti), malathion, resmethrin and permethrin, and control difficulties have been reported.

Increasing the dosage and frequency of insecticide applications and/or changing to different insecticides have frequently been necessary. Recently, two mosquito strains of *Cx. quinquefasciatus*, MAmCq^{G0} and HAmCq^{G0}, from Mobile and Huntsville, Alabama, respectively, have been established in the laboratory. Both MAmCq^{G0} and HAmCq^{G0} mosquito strains exhibit high levels of resistance to pyrethroids, such as permethrin, deltamethrin and resmethrin, and cross-resistance to chlorpyrifos, an organophosphate insecticide (Liu et al., 2004a). The current study was undertaken to gain information on mechanisms of permethrin resistance in mosquito populations of MAmCq and HAmCq. We selected MAmCq^{G0} and HAmCq^{G0} mosquitoes with permethrin for one and three generations, respectively. The levels of resistance to permethrin in the MAmCq^{G1} (after one-generation selection) and HAmCq^{G3} (after three-generation selection) strains were examined with and without piperonyl butoxide (PBO), *S*,*S*,*S*-tributylphosphorotrithioate (DEF) and diethyl maleate (DEM), inhibitors of cytochrome P450 monooxygenases, hydrolases and glutathione *S*-transferases (GST), respectively. A sodium channel gene fragment was amplified and sequenced from the mosquito strains. The frequency and heterozygosity of the A to T SNP for the *kdr* allele between permethrin-selected and unselected mosquitoes were determined in order to obtain a better understanding of the role of *kdr* mutation in the evolution of permethrin resistance.

3.2 Materials and methods

3.2.1 Mosquito strains

HAmCq^{G0} and MAmCq^{G0} strains of *Cx. quinquefasciatus* were collected from Huntsville and Mobile Counties, Alabama, respectively, in 2002. *Culex quinquefasciatus*

is an important urban pest in Alabama and has been a major target for several insecticides, including Bti, malathion, resmethrin, and permethrin. S-Lab, an insecticide-susceptible strain, was obtained from Dr. Laura Harrington (Cornell University). All mosquitoes were reared at 25 (±2 °C) under a 12:12 h light: dark photoperiod (Nayar and Knight, 1999).

3.2.2 Chemicals and insecticides

Permethrin (95.3%) was supplied by FMC Corp. (Princeton, NJ), DEF was purchased from Chem Service (West Chester, PA), and PBO and DEM were obtained from Aldrich (Milwaukee, WI).

3.2.3 Permethrin selections

$MAmCq^{G0}$ and $HAmCq^{G0}$ mosquitoes were selected with permethrin for one and three generations, respectively, in the laboratory after collection, generating $MAmCq^{G1}$ and $HAmCq^{G3}$ strains. The concentration (Table 3.1) used for each selection was sufficient to kill ≥60% of treated individuals in each generation after 24 h. Toxicity of permethrin was analyzed for each strain before and after selection by standard probit analysis with a computerized version (Raymond, 1985) of the method of Finney (1971).

3.2.4 Bioassays

The stock and serial dilutions of all chemicals were prepared in acetone. Bioassay methods for insecticides were as described previously (Mulla et al., 1982; Ali et al., 1999). Each bioassay consisted of 20 4^{th} instar mosquito larvae in 6 Oz. Sweetheart ice

cream cups (Sweetheart Cup Co., Owings Mills, MD) with regular tap water and 1% permethrin solution in acetone at the appropriate concentration and three or four concentrations that give >0 and <100% mortality. PBO, DEF, or DEM was applied simultaneously with permethrin at the maximum sublethal concentration of 5 ppm, 2 ppm, or 3 ppm, respectively. Control groups received either 1% acetone only or 1% acetone with an appropriate concentration of PBO, DEF, or DEM. Mortality was assessed after 24 h. All tests were run at 25^{o}C and replicated at least 3 times on different days. Bioassay data were pooled and probit analysis was conducted using Abbott's correction for control mortality (Abbott, 1925). Toxicity of permethrin is considered significantly different between susceptible and resistant strains when the probit dose-response is significant at $P \leq 0.05$.

3.2.5 Amplification and sequencing of the sodium channel gene fragment from HAmCq and MAmCq

The genomic DNAs of 30 individual adult mosquitoes from each population ($HAmCq^{G0}$, $HAmCq^{G3}$, $MAmCq^{G0}$, and $MAmCq^{G1}$) were extracted as described previously (Liu et al., 1995). The sodium channel gene fragment from mosquitoes was amplified by PCR with a sense primer S1 (5'-TTTACTCATTTCCATCATGG-3') and an antisense primer AS1 (5'-GACAAAAGCAAGGCTAAGAAAAGG-3'), based on sequences of the wild type *Anopheles gambiae para*-type sodium channel gene (accession number: XM318122). PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen) and sequenced (Research Instrumentation Facilities, Auburn University). Sequence analyses of the sodium channel gene fragment

from each strain were repeated at least three times with different preparation of genomic DNAs.

3.2.6 SNP determination for the kdr *allele*

The frequency and heterozygosity of the A to T for the *kdr* allele between permethrin selected and unselected mosquitoes were investigated by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer with a Genemapper software according to the manufacture's instruction (A&B Applied Biosystems). The chemistry of SNP determination method is based on the fluorescently labeled dideoxy single-base extension of unlabeled oligonucleotide primer. The PCR products used for SNP determination were generated from the genomic DNA of 30 individual mosquitoes of each mosquito strain using sense primer S1 and antisense primer AS1. The primer, 5' GGCCACCGTAGTGATAGGAAATTT 3', for SNP determination was designed according to the sequence immediately upstream of the *kd*r allele.

3.3 Results and discussions

3.3.1 Permethrin selection in HAmCq and MAmCq populations

The levels of resistance to permethrin in field collected parental $HAmCq^{G0}$ and $MAmCq^{G0}$ strains were 100- and 940-fold compared with the susceptible S-Lab strain (Table 3.1). After three generations of selection with permethrin, the level of resistance in $HAmCq^{G3}$ increased to 1300-fold compared with S-Lab (Table 3.1) and the level of resistance in $MAmCq^{G1}$ increased to 1700-fold after a single generation selection. The

changes in permethrin resistance between parental and selected mosquitoes suggest that resistance to permethrin in *Cx. quinquefasciatus* populations, or at least in these two strains, is developed rapidly. Such an ability of resistance development has also been found in house flies (Liu and Yue, 2000). The slope of the dose-response curve was increased for both strains after selection (Table 3.1), indicating that $HAmCq^{G3}$ and $MAmCq^{G1}$ were more homogeneous for the resistance after selection compared with the parental strains. The lower level of resistance to permethrin and the gradual slope of the dose-response curve in the field populations of $HAmCq^{G0}$ and $MAmCq^{G0}$ may indicate that a large portion of the field population avoid exposure to insecticides, providing a pool of susceptible individuals for the repopulation of permethrin resistant *Culex* mosquitoes (Georghiou and Taylor, 1977).

3.3.2 Role of metabolic detoxication in permethrin resistance of HAmCq and MAmCq

When mosquitoes were treated with PBO, an inhibitor of cytochrome P450 monooxygenases, 60-, 50- and 4.5-fold increases in toxicity of permethrin to $HAmCq^{G3}$, $MAmCq^{G1}$, and S-Lab, respectively, were observed, resulting in a decrease of permethrin resistance in $HAmCq^{G3}$ and $MAmCq^{G1}$ by 100- and 150-fold, respectively (Table 3.2, Fig. 3.1). This result suggests that P450 monooxygenase-mediated detoxification contributes to permethrin resistance in both $HAmCq^{G3}$ and $MAmCq^{G1}$. DEF, an inhibitor of hydrolases, enhanced toxicity of permethrin to $HAmCq^{G3}$, $MAmCq^{G1}$, and S-Lab by 2.5-, 3.5-, and 1.3-fold, respectively, and decreased resistance to 690- and 610-fold in $HAmCq^{G3}$ and $MAmCq^{G1}$, respectively (Table 3.2, Fig. 3.1). Although 1.9- and 2.8-fold decreases in resistance to permethrin in $HAmCq^{G3}$ and $MAmCq^{G1}$ by DEF can be easily

attributed to the effect of DEF on hydrolytic metabolism of permethrin in these mosquitoes, implying that hydrolytic metabolism makes a minor contribution to resistance, these data should be interpreted with caution. It has been proposed that DEF is not a completely specific inhibitor of hydrolases and that it can inhibit microsomal oxidases at high concentration (Miyazaki et al., 1996; Liu and Pridgeon, 2002; Scott, 1998). Thus, the larger effect of DEF on P450 monooxygenase-mediated metabolism of permethrin in both HAmCq^{G3} and MAmCq^{G1} than in S-Lab, resulting in decreased permethrin resistance in HAmCq^{G3} and MAmCq^{G1} may merit consideration. DEM, an inhibitor of GST, enhanced toxicity of permethrin to HAmCq^{G3}, MAmCq^{G1}, and S-Lab by 2.5-, 2.4-, and 0.9-fold, respectively, and decreased permethrin resistance to 490- and 620-fold in HAmCq^{G3} and MAmCq^{G1}, respectively, (Table 3.2, Fig. 3.1). DEM caused a 2.7-fold decrease in permethrin resistance in both HAmCq^{G3} and MAmCq^{G1}, indicating that GST-mediated metabolism also makes a minor contribution to resistance. Neither PBO, DEF, nor DEM could completely abolish resistance to permethrin in HAmCq^{G3} and MAmCq^{G1}, suggesting that one or more additional mechanisms are involved in overall resistance that are largely unaffected by these synergists.

3.3.3 Role of the kdr *mutation in permethrin resistance of HAmCq and MAmCq*

The *kdr* mutation associated with pyrethroid resistance has been reported in mosquitoes (Brengues et al., 2003; Martinez-Torres et al., 1999b; Enayati et al., 2003; Martinez-Torres et al., 1998). Martinez-Torres et al. (1999b) reported that L to F substitution at position 1014 detected in resistant *Cx. pipiens* mosquitoes compared to the S-Lab strain of *Cx. quinquefasciatus,* in which the A to T polymorphism was not been

detected. In addition, a substitution of same leucine to serine (L1014S) has been detected in one strain of *Cx. pipiens* that was slightly resistant to pyrethroids and highly resistant to DDT. The same L to S mutation has been identified in *Anopheles gambiae* (Ranson et al., 2000a). It has been suggested that the L to S substitution is involved in a high level of resistance to DDT combined with a low level of resistance to pyrethroid, while L to F is involved in high resistance to both pyrethroid and DDT(Martinez-Torres et al., 1999b; Ranson et al., 2000a). To investigate whether the L to F or L to S substitution was present in HAmCq and MAmCq mosquitoes and understand the evolution and importance of the *kdr* mutation in permethrin resistance in HAmCq and MAmCq strains through insecticide selection, a 341 bp fragment, where *kdr* mutation resides, was generated by PCR from genomic DNAs of *Cx. quinquefasciatus* strains. Sequence analysis indicated that the L to F substitution, resulting from A to T nucleotide polymorphism, was detected in HAmCq and MAmCq mosquito strains, which was consistent with the result reported in resistant *Cx. pipiens* (Martinez-Torres et al., 1999b). We investigated the SNP of *kdr* allele frequency in permethrin selected and unselected MAmCq and HAmCq mosquitoes using a fluorescent dideoxy terminator-based method to distinguish the SNP for the *kdr* allele from individual mosquitoes. Our data showed that heterozygous (A/T) and homozygous (T/T) individuals for the *kdr* allele existed in all tested mosquito populations (Fig. 3.2a). The frequency of heterozygote and homozygote for the *kdr* allele was about 0.87-0.90 and 0.10-0.13, respectively, in all the mosquito populations and there was not significant change in the frequency and heterozygosity of the A to T SNP for the *kdr* allele between permethrin selected and unselected mosquitoes of both MAmCq and HAmCq (Table 3.3). No individuals in MAmCq and HAmCq populations homozygous for the A allele

(A/A) were detected in this study and none of them had L to S substitution as reported previously (Martinez-Torres et al., 1999b). Increased fitness due to heterosis and/or frequent hybridization of susceptible individuals in the field populations that avoid exposure to insecticides with the resistant portion may be the possible explanations for these phenomena. In addition to SNP determination, we selectively sequenced 15 PCR products from each strain of mosquitoes and sequence results were identical to that of SNP determination (Fig. 3.2b). Yet, while most of the overlapping signals of A and T were indicated by an "N" by automated DNA sequencer, sometimes the DNA sequencer might choose the stronger one of two overlapping signals as shown in the sequence of $MAmCq^{G1}$ heterozygous individuals (Fig. 3.2b). This phenomenon has also been pointed out by Gao et al (2003).

The *kdr* mutation in the sodium channel gene associated with pyrethroid resistant insects has also been reported in other insect species, such as house flies (*Musca domestica*) (Miyazaki et al., 1996; Williamson et al., 1996; Lee et al., 1999), horn flies (*Haematobia irritans*) (Jamroz et al., 1998), diamondback moths (*Plutella xylostella*) (Schuler et al., 1998), aphids (*Myzus persicae*) (Martinez-Torres et al., 1999a), mosquitoes (*Anopheles gambiae*) (Martinez-Torres et al., 1998; Ranson et al., 2000a), German cockroaches (*Blattella germanica*) (Dong, 1997; Miyazaki et al., 1996; Dong et al., 1998) and Colorado potato beetles (*Leptinotarsa decemlineata*) (Lee et al., 1999). In addition, a substitution of leucine to serine (L1014S) has been detected in pyrethroid resistant *mosquitoes* (Martinez-Torres et al., 1999b; Ranson et al., 2000a) and a leucine to histidine substitution (L1014H) has been observed in pyrethroid resistant house flies (*Musca domestica*) (Hemingway et al., 2004) and tobacco budworms (*Heliothis*

virescens) (Park and Taylor, 1997). It has been reported that the *kdr* resistance trait is completely recessive (Martinez-Torres et al., 1999b; Ranson et al., 2000a; Huang et al., 2004). However, our results may suggest that the *kdr* resistance trait is incompletely dominant because over 87% individuals in resistant mosquito populations are heterozygous (A/T) for the *kdr* allele. There was not significant change in the frequency and heterozygosity of the A to T SNP for the *kdr* allele between permethrin selected and unselected MAmCq and HAmCq mosquitoes with resistance levels ranged from 100- to 1700-fold, respectively, suggesting other mechanisms involved in the evolution of resistance in mosquitoes selected by permethrin in the laboratory.

Our current study indicates that high levels of permethrin resistance in the mosquitoes from Huntsville and Mobile, Alabama, are conferred by multiple mechanisms. The P450 monooxygenase-, hydrolase-, and/or GST-mediated mechanisms are involved in permethrin resistance in both strains. Incomplete suppression of permethrin resistance by PBO, DEF, and/or DEM suggests that one or more additional mechanisms are involved in overall resistance of MAmCq and HAmCq. The *kdr* mutation is one of the factor playing a role in resistance that are unaffected by the synergists. The high levels of permethrin resistance as well as the potential of permethrin resistance development in mosquitoes from Huntsville and Mobile, Alabama, merit judicial approach to the use of pyrethroids in mosquito control in these areas. Mixing of pyrethroids with PBO for application and alternative use of insecticides, especially Bti and some relatively new insecticides (Liu et al., 2004a), may be valuable for the management of *Cx. quinquefasciatus*.

Acknowledgements

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Table 3.1 Toxicity of permethrin to HAmCq and MAmCq strains compared to the susceptible S-Lab strain before and after permethrin selection in the laboratory

Strains	Selected generation	Selecting concentration (ppm)	n[a]	Survival (%)	LC_{50}(CI) ppm	Slope (SE)	RR[b]
HAmCq	G0[c]				0.8 (0.3-1.4)	1.0 (0.2)	100
	G1	0.5	~4000	~20%	2.7 (2.0-3.6)	1.8 (0.2)	340
	G2	2.0	~3500	~30%	6.4 (4.6-8.9)	2.0 (0.3)	800
	G3	5.0	~4000	~40%	12 (9.3-15)	2.4 (0.3)	1300
MAmCq	G0[c]				7.5 (5.5-9.8)	2.1 (0.4)	940
	G1	5.0	~5000	~30%	15 (11-20)	2.6 (0.4)	1700
S-Lab					0.008 (0.006-0.01)	4.0 (0.9)	

[a] n: Number of selected mosquitoes.

[b] RR: Resistance Ratio= LC_{50} of the resistant strain/ LC_{50} of the S-Lab strain

[c] Parental: Field collected strain before permethrin selection

Table 3.2 Toxicity of permethrin with and without PBO, DEF, and DEM to *Cx. quinquefasciatus* strains

Insecticide	Strains	df	n	X^{2a}	LC_{50}[b](CI)[c]	SR[d]	Slope(SE)
Permethrin	$HAmCq^{G3}$	4	178	1.8	12 (9.3-15)		2.4 (0.3)
	$MAmCq^{G1}$	3	130	2.3	15 (11-20)		2.6 (0.4)
	S-Lab	3	150	0.5	0.009 (0.007-0.01)		3.7 (0.6)
Permethrin+PBO	$HAmCq^{G3}$	4	186	3.1	0.2 (0.1-0.2)	60	1.6 (0.2)
	$MAmCq^{G1}$	3	150	0.2	0.3 (0.2-0.4)	50	2.9 (0.4)
	S-Lab	3	150	2.5	0.002 (0.002-0.003)	4.5	2.6 (0.4)
Permethrin+DEF	$HAmCq^{G3}$	3	162	1.0	4.8 (3.5-6.8)	2.5	1.9 (0.3)
	$MAmCq^{G1}$	4	150	3.1	4.3 (3.0-6.6)	3.5	1.6 (0.2)
	S-Lab	4	210	2.4	0.007 (0.005-0.009)	1.3	1.8 (0.2)
Permethrin+DEM	$HAmCq^{G3}$	3	139	0.7	4.9 (3.8-6.3)	2.5	3.4 (0.5)
	$MAmCq^{G1}$	4	160	4.1	6.2 (3.8-10)	2.4	1.9 (0.2)
	S-Lab	2	120	1.0	0.01 (0.009-0.02)	0.9	3.6 (0.6)

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

[b] LC_{50} values in ppm.

[c] 95% confidence interval.

[d] SR: LC_{50} of insecticides /LC_{50} of synergists + insecticides.

FIGURE LEGENDS

Fig. 3.1 Permethrin resistance ratios (RRs) with and without synergists in HAmCq and MAmCq compared with S-Lab of *Culex quinquefasciatus*.

Fig. 3.2 The allelic (*kdr* allele) genotype of A to T single nucleotide polymorphism in HAmCq and MAmCq mosquitoes with and without permethrin selection. A: SNP determination. B: Sequence analysis.

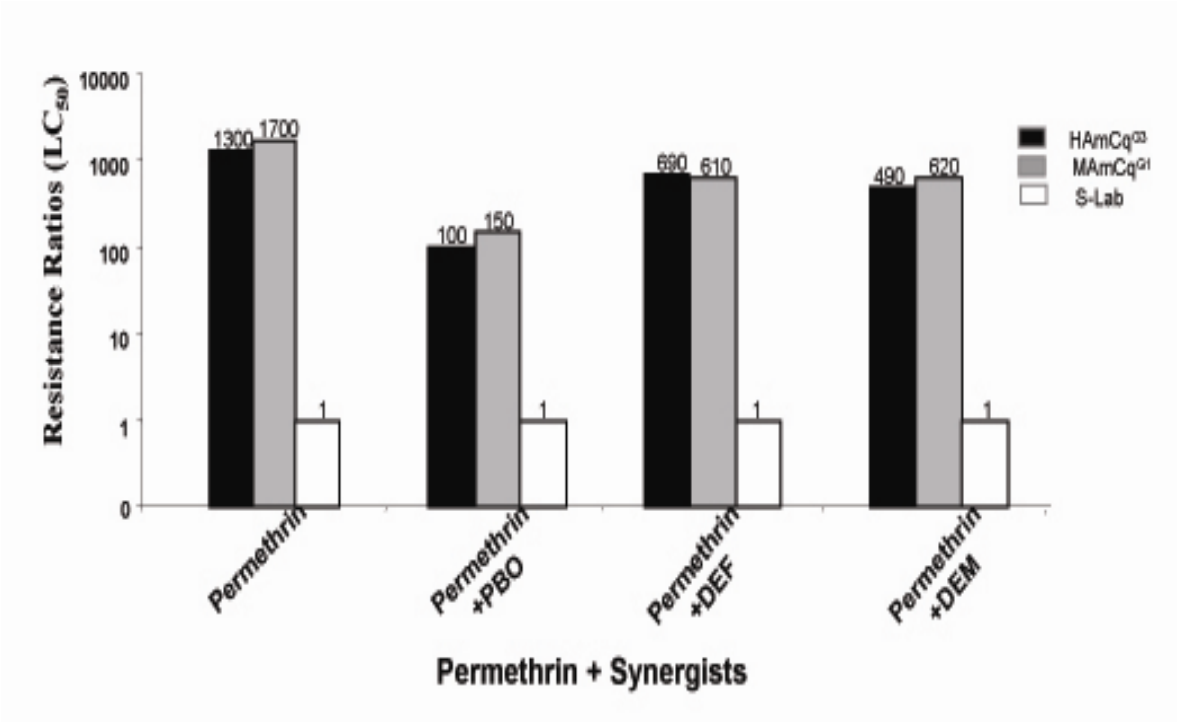
Resistance Ratios (LC_{50})
10000
1000
100
10
1
0
1300
1700
1
100
150
1
690
610
1
490
620
1
HAmCq^03
MAmCq^01
S-Lab
Permethrin
Permethrin +PBO
Permethrin +DEF
Permethrin +DEM
Permethrin + Synergists

Fig. 3.1

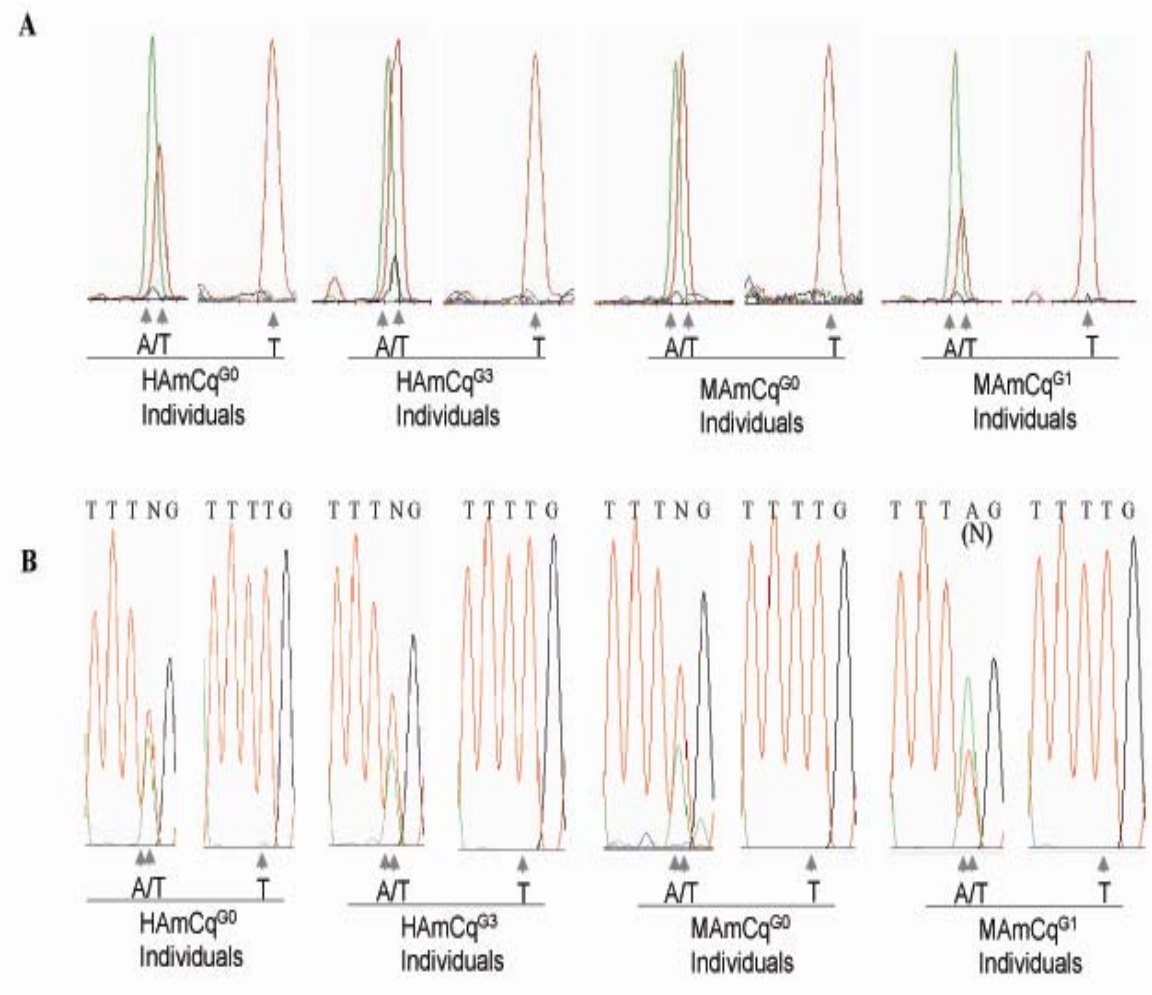
A
A/T
T
HAmCq^G0 Individuals
HAmCq^G3 Individuals
MAmCq^G0 Individuals
MAmCq^G1 Individuals
B
T T T N G
T T T T G
T T T A G
(N)

Fig. 3.2

CHAPTER 4: *KDR* ALLELIC VARIATION IN PYRETHROID RESISTANT MOSQUITOES, *CULEX QUINQUEFASCIATUS* (S.)

4.1 Introduction

The voltage-gated sodium channel is the primary target of pyrethroid insecticides (Narahashi, 1996; Sattelle and Yamamoto, 1988) and its insensitivity is known to be associated with pyrethroid resistance in several knockdown resistant (*kdr*) insect species, including mosquitoes (Brengues et al., 2003; Dong, 1997; Martinez-Torres et al., 1999b). A substitution of leucine to phenylalanine (Leu to Phe) resulting from a single nucleotide polymorphism (SNP), termed the *kdr* mutation, has been identified in the domain II segment 6 of the sodium channel (Knipple et al., 1994; Williamson et al., 1993). This L-to-F *kdr* mutation has been clearly demonstrated to be associated with resistance to pyrethroid and DDT in many insect species (Busvine, 1951; Dong, 1997; Guerrero et al., 1997; Ingles et al., 1996; Martinez-Torres et al., 1998; 1999b; Miyazaki et al., 1996; Schuler et al., 1998; Soderlund and Knipple, 2003; Williamson et al., 1996). Substitutions of leucine to histidine (Leu to His) and leucine to serine (Leu to Ser) at the position corresponding to the L-to-F *kdr* mutation have also been reported in pyrethroid resistant house flies (Liu and Pridgeon, 2002), tobacco budworms (Park and Taylor, 1997), and *Culex pipiens* mosquitoes (Martinez-Torres et al., 1999). In addition, a substitution of

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phenylalanine to serine located just 6 amino acids downstream of the *kdr* mutation has been observed in pyrethroid resistant German cockroaches (Pridgeon et al., 2002). Several other mutations positioned out of the domain II segment 6 of the sodium channel have also been identified as being involved in reducing channel sensitivity to insecticides or neurotoxins (Soderlund and Kinpple, 2003). However, the *kdr* mutation(s) in mosquitoes has attracted a great deal of attention due to the importance of pyrethroid insecticides in the control of mosquitoes worldwide, especially their major role in preventing malaria (Zaim et al., 2000) and serious problems of outbreaks of human diseases that have begun to arise as a result of increased pyrethroid resistance in insects (Sina and Aultman, 2001).

Culex quinquefasciatus is an important vector throughout the wet tropics. In the Southeastern USA, this species is moderately competent as a vector of West Nile virus (WNV) (Sardelis et al., 2001) and is a primary vector of Saint Louis encephalitis virus (SLEV) in many urban settings (Jones et al., 2002). The approach used to control transmission of these pathogens and other mosquito-associated diseases has primarily relied on the application of insecticides (McCarroll and Hemingway, 2002). However, mosquito-borne diseases are now resurgent (Gubler, 1998), largely due to the difficulty in controlling vectors that have developed resistance to insecticides.

Recently, a mosquito strain of *Cx. quinquefasciatus*, $HAmCq^{G0}$, from Huntsville, Alabama, has been established in the laboratory. $HAmCq^{G0}$ mosquito strains exhibit high levels of resistance to pyrethroids (Liu et al., 2004a). In an earlier study, we identified over 87% heterozygous (TTA/T, the genotype of the *kdr* locus for the Leu/Phe site) individuals for a *kdr* allele at the genomic level that were present in the $HAmCq^{G0}$

mosquito population (Xu et al., 2005). There was no significant change in the frequency and heterozygosity of the A to T SNP for the *kdr* allele between parental HAmCq^{G0} mosquitoes and their offspring that had been further selected with permethrin (Xu et al., 2005). This raised the question that if the *kdr* allele is very important in resistance, then its frequency, heterozygosity, and/or homozygosity ought to be correlated with different levels of resistance. In the work reported here, we compared genomic DNA and RNA expression levels within the same individuals from three different mosquito strains of *Cx. quinquefasciatu*s, bearing different resistant phenotypes ranging from susceptible to highly resistance. The goal of the study was to investigate whether the functional polymorphism of the L-to-F *kdr* mutation that determines resistance phenotype undergoes DNA variation or goes through transcriptional regulatory variation, consequently altering protein function (Cowles et al., 2002).

4.2 Materials and methods

4.2.1 Cx. quinquefasciatus *strains*

Three strains of mosquito *Cx. quinquefasciatus* were used in this study. HAmCq^{G0}, collected from Huntsville County, Alabama, in 2002 (Liu at al., 2004a); HAmCq^{G8}, HAmCq mosquitoes that had been selected with permethrin for 8 generations after collection; and S-Lab, a universal insecticide-susceptible strain that was obtained from Dr. Laura Harrington (Cornell University). All mosquitoes were reared at 25 ± 2°C under a photoperiod of 12:12 (L:D) h (Nayar and Knight, 1999).

4.2.2 Permethrin selections

HAmCq^{G0} mosquitoes were selected with permethrin for eight generations in the laboratory after collection, generating the HAmCq^{G8} strain. The concentration (Table 1) for each selection was sufficient to kill ≥ 60% of treated individuals in each generation after 24 h. Toxicity of permethrin was analyzed for each strain before and after selection by standard probit analysis, as described by Liu et al. (2004a) with a computerized version (Raymond, 1985) of the method of Finney (1971).

4.2.3 Amplification, sequencing and SNP determination for the kdr *allele in* Cx. quinquefasciatus

Five males and five females of each mosquito population had their genomic DNA and RNA extracted for each experiment. Three replications were performed, each on a different day, for a total of 30 individual mosquitoes for each population. Three primers were designed for amplifying the sodium channel cDNA fragments from all mosquito populations: KDR S1 (5' CTTACTCATTTCCATCATGG 3'), PG KDR AS5 (5' GCTGGTTGTGCTCCTTGACGC 3'), and KDR AL S1 (5' GCGTTAGGTAATCTG ACGTTTGTGC 3'), which generated amplicons that spanned the intron/exon boundaries but did not amplify genomic DNA. In this way, any influence due to genomic DNA contamination was eliminated. Two rounds of PCR were conducted. The first PCR solution with cDNA template and a primer pair (KDR S1 and PG KDR AS5) was heated to 95°C for 1 min, followed by 40 cycles of PCR reaction (94°C for 30 s, 55°C for 30 s and 72°C for 2 min) and a final extension of 72°C for 10 min. The second PCR, containing 1 μl of the first round PCR reaction solution and a primer pair (KDR AL S1

and PG KDR AS5), was conducted under the same reaction conditions described above except that 38 cycles and a 60°C annealing temperature were used. The sodium channel genomic DNA fragments were amplified using 3 primers: KDR S1, KDR AS1 (5' GACAAAAGCAAGGCTAAGAAAAGG 3'), and KDR AL S1. The primers were designed based on exonic sequences (Martinez-Torres et al., 1999b) that did not generate products in RNA samples without a reverse transcription step. Again, two rounds of PCR were conducted. The first PCR reaction with 1 µg genomic DNA template and a primer pair (KDR S1 and KDR AS1) was heated to 95°C for 5 min followed by 40 cycles of PCR reaction at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with a final extension step at 72°C for 10 min. The second PCR, containing 0.5-1 µl of the first round PCR reaction solution and a primer pair (KDR AL S1 and KDR AS1), was conducted following a PCR cycle of 95°C for 1 min, 38 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, then 72°C for 10 min. The frequency and heterozygosity of the *kdr* allele in mosquitoes were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacture's instructions (A&B Applied Biosystems). The primers for SNP determination were designed according to the sequence immediately upstream of the *kd*r allele. Three replications of the SNP determination of both the genomic DNA and cDNA of each individual were carried out with different preparations of the PCR templates. To confirm that the PCR products that used for the SNP determination were in fact the *kdr* gene fragments, we sequenced the PCR products from both the cDNA and genomic DNA of each individual, at least once each. For the genotype heterozygous individuals that expressed both of the alleles

identified in the SNP determination experiments, four different measurements for each SNP repeat were then performed, resulting in a total of 12 measurements in three SNP repeats for each individual. Peak heights representing the allele-specific extended primer were determined (Bray et al., 2003; Yan et al., 2002) using GeneMapper version 3.5 software (A&B Applied Biosystems) and were used to calculate a ratio of T:A alleles for each sample. The allelic expression ratio of the two alleles was further normalized by the ratio observed from genomic DNA of the same individual. A *t*- test and a one-way analysis of variance (ANOVA) were used to calculate a *P* value for the significance of the allelic variation using the SAS v9.1 software (SAS Institute Inc.).

4.3 Results

4.3.1 Permethrin selection of the HAmCq^{G0} population

The level of resistance to permethrin in the field collected parental HAmCq^{G0} strain was 100-fold compared with the susceptible S-Lab strain (Table 4.1). After eight generations of selection with permethrin, the level of resistance in HAmCq^{G8} increased to 3100-fold compared with S-Lab (Table 4.1). The changes in permethrin resistance between the parental and selected mosquitoes suggest that resistance to permethrin in *Cx. quinquefasciatus* mosquitoes is developed rapidly. The slope of the dose-response curve was also noticeably steeper for the HAmCq^{G8} strain after selection (Table 4.1), indicating that HAmCq^{G8} were much more homogeneous for resistance after selection compared with the parental strain HAmCq^{G0}. The lower level of resistance to permethrin and the gradual slope of the dose-response curve in the field populations of HAmCq^{G0} may indicate that a large portion of the field population successfully avoid exposure to

insecticides, providing a pool of susceptible individuals for the repopulation of permethrin resistant *Culex* mosquitoes (Georghiou and Taylor, 1977).

4.3.2 Identification of the L-to-F kdr *allelic variation at the genomic DNA level*

We first questioned whether the functional polymorphism of the *kdr* mutation in the sodium channel that was associated with insecticide resistance of mosquitoes underwent DNA variation. We investigated the SNP for A/T alleles of the *kdr* locus at the genomic DNA level in 30 individuals of each mosquito population to determine the correlation of the *kdr* allelic genotype and resistance phenotype. The PCR products from both the cDNA and genomic DNA of each individual were sequenced. In all cases, the PCR sequences amplified from cDNA (GenBank accession nos. ***DQ408540*** and ***DQ408541***) and genomic DNA (GenBank accession nos. ***DQ408538*** and ***DQ408539***) were the sodium channel gene fragment. Interestingly, we did not detect any introns in this region as previously reported by Martinez-Torres et al. (1998). We found no correlation for the *kdr* allele at the genomic DNA level with levels of susceptibility and resistance to insecticides (Table 4.2). We did, however, find different genotypes for the *kdr* locus among individuals in the same population of both resistant and susceptible mosquitoes, including individuals that were heterozygous for both susceptible (A) and resistance (T) alleles or homozygous for either the A or T allele, although the major genotype was heterozygous (A/T) in all the *Culex* populations tested (Table 4.2). There was no significant change in the genotype between susceptible and resistant strains or between the field collected resistant parental strain $HAmCq^{G0}$ and its offspring $HAmCq^{G8}$ that had undergone eight generations of selection in the laboratory with permethrin (a

pyrethroid insecticide) although their levels of resistance to permethrin are dramatically different (Table 4.2).

4.3.3 Transcriptional regulation in the functional polymorphism of the L-to-F kdr *mutation*

We next questioned whether the functional polymorphism of the *kdr* mutation was in fact due to transcriptional regulation. We assessed the RNA expression variation of the *kdr* allele in order to investigate the role of regulatory variation in the *kdr* mutation associated with insensitivity and resistance. As expected, there was a strong correlation between the *kdr* allelic expression and the levels of pyrethroid resistance in *Culex* mosquitoes. In the susceptible *Cx. quinquefasciatus* strain S-Lab, we found 23/23 (100%) of the genomic heterozygous (A/T) individuals expressed the susceptible allele A through monoallelic expression and 3/3 (100%) homozygous individuals for the *kdr* allele (T) expressed the susceptible allele A through a U-to-A RNA editing (Fig. 4.1, Table 4.3), generating a codon encoding Leu corresponding to the susceptibility in this strain to insecticides. The genetically homozygous individuals for the A allele all expressed the same allele. In contrast, in the highly resistant *Culex* strain of $HAmCq^{G8}$ with 8 generations of selection in the laboratory after collection from the field, 23/25 (93%) of the genomically heterozygous (A/T) individuals showed monoallelic expression of the T allele, resulting in a change of Leu to Phe (Fig. 4.1), while the genetically homozygous individuals for the *kdr* allele all expressed the same allele. The consequence of 93% of the $HAmCq^{G8}$ individuals presenting this expression of the *kdr* allele strongly correlated with the high level of resistance in this population. Thus, our study, for the first time,

showed that transcriptional regulation through RNA allelic variation expression and RNA editing is involved in the changes of genetic information in the L-to-F *kdr* locus.

4.3.4 The L-to-F kdr *allelic expression variation in the field population of* Culex *mosquitoes*

It has been suggested that there is inevitably a large portion of field insect populations that avoid exposure to insecticide selection pressure, thus providing a pool of susceptible individuals that then repopulate the resistant portions selected by insecticides (Georghiou and Taylor, 1977). We hypothesized that these directly collected field insect populations would present intermediate levels of expression for the *kdr* allele that corresponded to their proportions of both susceptible and resistant individuals and intermediate levels of resistance. To test our hypothesis, we examined the RNA expression levels of *Cx. quinquefasciatus* $HamCq^{G0}$, which had relatively low levels of resistance and was not further selected with insecticides in the laboratory (Liu et al., 2004a). We identified, as expected, a pattern of allelic variation in the individuals of this field collected parental population (Fig. 4.1), with frequencies ranging from 0.2 for individuals that expressed only the susceptible allele, 0.43 for those that expressed both alleles, and 0.37 for those that expressed the *kdr* allele. The allelic variation pattern for the *kdr* locus in this field collected mosquito population strongly correlated with its intermediate levels of resistance (Liu et al., 2004a) and the proportions of both susceptible and resistant individuals, as suggested by Georghiou and Taylor (1977). Among the genomic heterozygous individuals of $HAmCq^{G0}$, 6/25 (24%) individuals expressed the A allele and 10/25 (40%) expressed the T allele through monoallelic

expression regulation, while 9/25 (36%) individuals expressed both A and T alleles. In contrast, 4/5 (80%) individuals in the genomic homozygotes for the *kdr* allele (T) expressed both the A and T alleles through a U-to-A editing event (Table 4.3). The field collected mosquitoes clearly represent a transitional status between susceptible and highly resistant through their intermediate expression condition for the *kdr* locus, which closely corresponded to their relatively low levels of resistance and the gradual slope of their dose-response curve (Liu et al., 2004a). This result clearly indicates the existence of inter-individual variability of the *kdr* allelic expression in natural populations, which thus provide a rich substrate for evolutionary insecticide selection (Stamatoyannopoulos, 2004; Wray et al., 2003) and for adaptation to changing environments (Hamilton, 2002).

4.3.5 Biallelic expression or allelic imbalances of the L-to-F kdr *mutation in resistance*

Our study has shown that monoallelic expression from genomic heterozygotes is very common in the insect populations tested. However, several heterozygous individuals in the *Culex* HAmCq^{G8} and HAmCq^{G0} strains did show biallelic expression (Fig. 4.1). These small differences or subtle changes in the level of variation between two alleles (~20% difference in the gene expression) may be of physiological importance (Bray et al., 2003; Yan et al., 2002). To investigate the role of biallelic expression variation in resistance, we next examined the allele-specific transcript levels of a total of 11 genomic heterozygotes. Significant differences (P<0.05) in allelic variation were observed in the only 2 heterozygotes in HAmCq^{G8} tested that showed significant preferential expression for the T allele (Fig. 4.2). However, in the parental strain of HAmCq^{G0}, 3 of the 9 heterozygotes showed a significant preferential expression for the A allele, 4 showed

nearly equal expression between the two alleles, and 2 showed a significant preferential expression for the T allele. These results showed a correspondence between the preferential expression of alleles in the *kdr* locus and levels of resistance. As suggested by the role of variation expression in the sensitivity to human diseases (Bray et al., 2003; Yan et al., 2002), these differences in the levels of *kdr* allelic variation expression may have dramatic phenotypic consequences for selection (Stamatoyannopoulos, 2004).

4.4 Discussion

This study examined genomic DNA and RNA sequences within and among resistant and susceptible mosquito populations of *Cx. quinquefasciatus*. We found no clear correlation between the *kdr* allelic genotyping and either levels of susceptibility and/or resistance to insecticide in any of insect populations tested. This agrees with previous studies, where no correlation between the genotype of the *kdr* mutation and knockdown resistance phenotype has also been observed in several other insect species, including mosquitoes *Aedes aegypti and Culex pipiens pipiens*, house flies, and German cockroaches (Bass, et al., 2004; Brengues et al., 2003; Dong et al., 1998; McAbee et al., 2003; Miyazaki et al., 1996). These researchers also found different genotypes presented in the same populations of insects. Furthermore, Miyazaki et al. (1996) observed both *kdr* and susceptible allelic genotypes in susceptible house fly strains. These earlier studies strongly support our findings that the DNA variants at the *kdr* locus are not correlated with insecticides susceptibility and resistance.

However, comparison of the allelic expression in the *kdr* locus among the susceptible populations that expressed solely the susceptible allele, parental populations

directly collected from the field that present intermediate levels of expression for the *kdr* allele, and highly resistant populations that expressed mostly or solely the *kdr* allele, clearly reveals a strong correlation between *kdr* allelic expression and levels of insecticide resistance. This correlation is probably regulated through RNA variation and RNA editing. These findings highlight the extraordinary ability of insects to adapt to evolutionary selection by regulating a target site with different transcripts and provide a comprehensive picture of posttranscriptional regulation of the insect sodium channel gene expression. This study also indicates that the posttranscriptional control of gene expression and function is far more elaborate and extensive than previously thought.

RNA editing, including site-specific base modification or the insertion or deletion of nucleotides, has recently been revealed as a posttranscriptional regulatory mechanism that modulates the sodium channel function in insects (Liu et al., 2004b; Palladino et al., 2000; Reenan, 2005; Song et al., 2004). A-to-I editing events identified in the *para* transcripts of *Drosophila* are known to affect adult nervous system function, integrity, and behavior (Palladino et al., 2000), while RNA editing of A-to-I and U-to-C sites in the German cockroach's sodium channel has been proposed as a possible mechanism that generates functional variants of sodium channels (Liu et al., 2004b; Song et al., 2004). Nevertheless, the impact of RNA editing on the regulation of sodium channel mutations associated with insecticide resistance is unclear. Our results suggest that different isoforms of the insect sodium channel that are generated by A-to-U editing events in insects might be a response to insecticide sensitivity in susceptible mosquitoes.

More significantly, our study shows that RNA allelic variation is one of the principle molecular mechanisms governing changes in the genetic information contained

in the *kdr* locus and regulating the function of the sodium channel in the insect nervous system. Unlike RNA editing, which modifies a single site-specific base to another, allelic variation consists of either differential expression between the two alleles of a heterozygous individual or preferential expression of one of the two alleles in samples. RNA allelic variation is an important posttranscriptional modification that has received increasing attention by those working on the human genome (Pastinen et al., 2004). Recent studies have indicated that such allelic variation in gene expression is common and an individual's allelic expression status may result in a change in the expression level of the gene (Tilghman, 1999; Yan et al., 2002). The allelic variation in human gene expression leads to phenotypic variability between individuals and serves as the primary cause of complex diseases (Bray et al., 2003; Lin et al., 2005; Yan et al., 2002). Yet, allelic variation in gene expression that affects gene function has not been well defined in the insects, especially in insecticide resistance. Our finding of monoallelic or biallelic variation of the L-to-F *kdr* locus could shed new light on the role of allelic variation of gene expression in *kdr*-mediated resistance in mosquitoes.

While we have known that the L-to-F *kdr* mutation is very important in the evolution of resistance development, it is unlikely to be the sole mechanism responsible for such a widespread phenomenon as *kdr*-mediated resistance. In particular, more than 20 sodium channel mutations have already been identified as being involved in reducing channel sensitivity to insecticides or neurotoxins (Soderlund and Knipple, 2003). Whether these mutations in the channel are regulated via the same mechanisms as those implicated in the L-to-F *kdr* mutation remains a subject for further investigation. However, our findings have important implications for the molecular mechanisms that

regulate these mutations in resistance and for understanding the mechanisms of insecticide selection and adaptation.

In conclusion, this study found a strong correlation between allelic expression regulation for the *kdr* locus and levels of insecticide resistance and/or susceptibility through RNA allelic variation and RNA editing. The role of transcriptional regulation through RNA allelic variation in the evolution of insecticide resistance in mosquitoes *Cx quinquefasciatus* is clearly revealed by the results reported here, showing the importance of this mechanism in connecting the genotype-resistance phenotype relationship. This study not only highlights the extraordinary ability of mosquitoes to adapt to insecticide selection by regulating a target site with different transcripts, but also gives a comprehensive view of gene expressional regulation in insecticide resistance. It should now be feasible to apply the approaches discussed above to investigate the *kdr* allelic expression in other insect species in order to discover whether the posttranscriptional regulation of the *kdr* mutation identified in mosquitoes is a common molecular mechanism in insecticide resistance generally.

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Table 4.1 Toxicity of permethrin to HAmCq and MAmCq strains compared to the susceptible S-Lab strain before and after permethrin selection in the laboratory

Strains	Selected generation	Selecting concentration (ppm)	n[a]	Survival (%)	LC_{50}(CI) (ppm)	Slope (SE)	RR[b]
HAmCq	G0[c]				0.8 (0.3-1.4)	1.0 (0.2)	100
	G1	0.5	~4000	~20%	2.7 (2.0-3.6)	1.8 (0.2)	340
	G2	2.0	~3500	~30%	6.4 (4.6-8.9)	2.0 (0.3)	800
	G3	5.0	~4000	~40%	12 (9.3-15)	2.4 (0.3)	1300
	G4	8.0	~4000	~40%	15 (8.0-24)	2.7 (0.4)	1700
	G5	13	~4500	~40%	18 (13-22)	2.9 (0.4)	2000
	G6	16	~5000	~30%	23 (19-27)	3.2 (0.5)	2600
	G7	18	~4000	~30%	25 (22-28)	3.3 (0.5)	2900
	G8	19	~5000	~20%	26 (24-29)	3.5 (0.5)	3100
Slab					0.008 (0.006-0.01)	4.0 (0.9)	1

[a]n: Number of selected mosquitoes.

[b]RR: Resistance Ratio= LC_{50} of the resistant strain/ LC_{50} of the S-Lab strain

[c]Parental strain before permethrin selection

Table 4.2 The genotype of the *kdr* locus (the L/F site) in mosquitoes, *Cx. quinquefasciatus*

Insect Species	Strain	n[a]	Phenotype	Genotype[b] (total individuals)		
				TTA	TTA/T	TTT
Cx. quinquefasciatus	S-Lab	30	Susceptible	4	23	3
	HAmCq^{G0}	30	100-fold resistance	0	25	5
	HAmCq^{G8}	30	3,100-fold resistance	0	27	3

[G0]The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

[a] The total number of tested mosquitoes (three replicates for each of 5 males and 5 females)

[b] The nucleotides in the *kdr* locus changed in the genotypes are underlined

Table 4.3 RNA editing events identified in the *kdr* locus of the sodium channel gene of different *Cx. quinquefasciatus* strains

Insects	Nucleotide changes*	RNA editing event	Resulting amino acid
Culex S-Lab	TTT to TTA	U-to-A	Leucine
Culex HamCq^{G0}	TTT to TTA/T	U-to-A	Leucine

* The nucleotides edited in the *kdr* locus are underlined

FIGURE LEGENDS

Fig. 4.1 Allele-specific transcription for two alleles in the *kdr* locus in mosquitoes *Cx. quinquefasciatus* compared with the corresponding genotypes for the same individuals. Thirty mosquitoes in each population were used for a total of 30 SNP determinations, with each of the 10 SNP determinations being repeated three times. [a]All the individuals with the specific genotype (DNA) allele(s) in the *kdr* locus (data presented in Table 4.2). [b]Individuals with specific expression (cDNA) of allele(s) for the corresponding genotype of the same individuals. [c]The overall expression frequency of specific alleles in the whole population (30 individuals).

Fig. 4.2 Variation of allelic expression of A and T in the *kdr* locus for heterozygous *Cx. quinquefasciatus* individuals of $HAmCq^{G8}$ and $HamCq^{G0}$ that expressed both alleles. Average allele ratios were calculated from a total of 12 different measurements, four repeats of each of the 3 SNP replications, for the samples from each individual. The *y* axis represents the expression ratio of the T-allele compared with the A-allele, corrected by the corresponding average genomic DNA ratio. *Statistically significant difference of allele expression between two alleles at $P < 0.05$. **Statistically significant difference of allele expression between two alleles at $P < 0.01$.

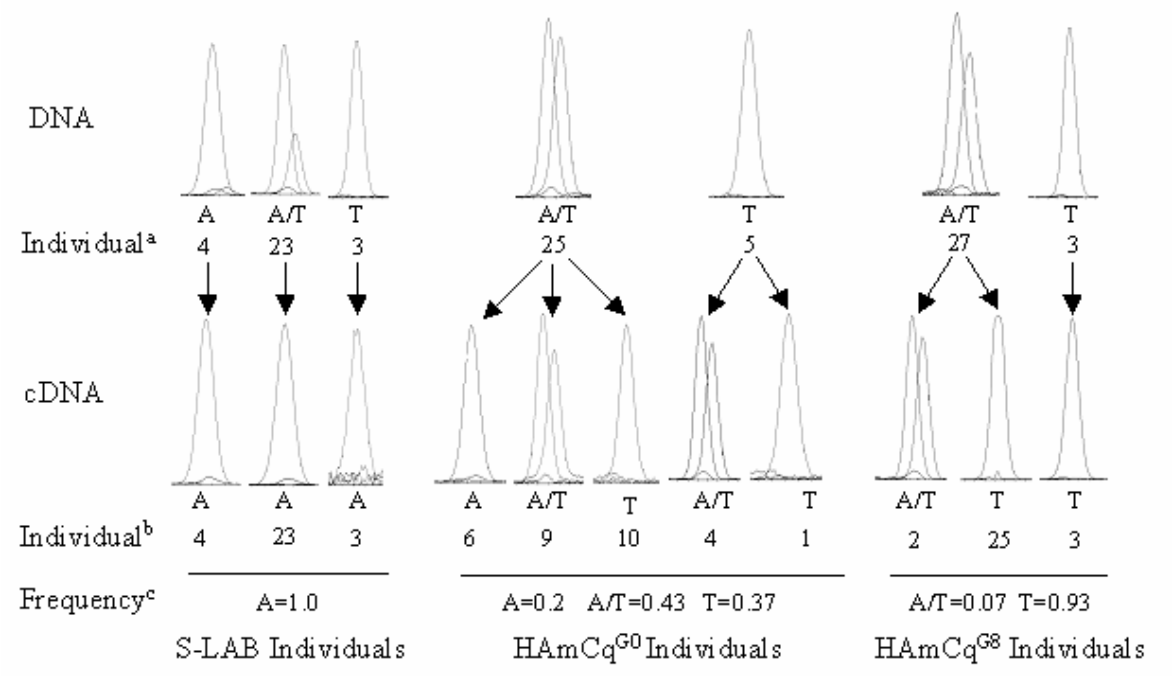
DNA
A
A/T
T
A/T
T
A/T
T
Individual[a]
4
23
3
25
5
27
3
cDNA
A
A
A
A
A/T
T
A/T
T
A/T
T
T
Individual[b]
4
23
3
6
9
10
4
1
2
25
3
Frequency[c]
A=1.0
A=0.2
A/T=0.43
T=0.37
A/T=0.07
T=0.93
S-LAB Individuals
HAmCq^G0 Individuals
HAmCq^G8 Individuals

Fig. 4.1

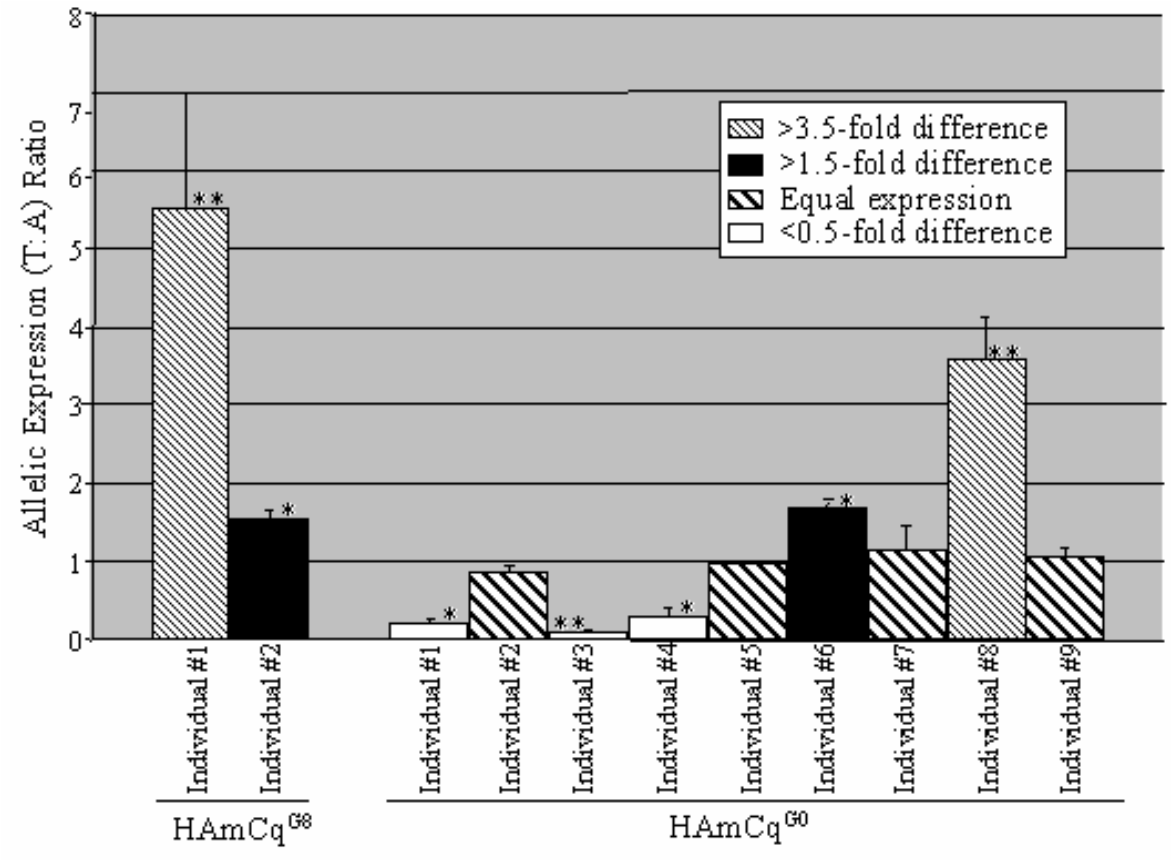
Allelic Expression (T:A) Ratio
8
7
6
5
4
3
2
1
0
>3.5-fold difference
>1.5-fold difference
Equal expression
<0.5-fold difference
**
*
*
**
*
*
**
Individual #1
Individual #2
Individual #1
Individual #2
Individual #3
Individual #4
Individual #5
Individual #6
Individual #7
Individual #8
Individual #9
HAmCq G8
HAmCq G0

Fig. 4.2

CHAPTER 5: SODIUM CHANNEL GENE EXPRESSION ASSOCIATED WITH PYRETHROID RESISTANT HOUSE FLIES AND GERMAN COCKROACHES

5.1 Introduction

Most of insecticides are neurotoxins and are targeted on the nervous system of insects. Nevertheless, target site insensitivity resulting from point mutations within the voltage-gated sodium channel, acetylcholinesterase, and the ligand-gated chloride channel of the insect nervous system has been documented to be of primary importance in insecticide resistance (ffrench-Constant et al., 1993; Ingles et al., 1996; Williamson et al., 1996; Dong, 1997; Weill et al., 2003) which poses a severe threat to our efforts to control agriculturally and economically important insect pests, as well as the insect vectors of human and animal diseases (Scott, 1990; Hemingway et al., 2002). Yet, the molecular basis underlying the genotype and target site insensitivity-mediated resistance phenotype relationship is paid little attention.

Voltage-gated sodium channels are responsible for the depolarization phase of action potentials in the membranes of neurons and most electrically excitable cells (Catterall, 2000). The overall organization of sodium channel proteins is conserved among invertebrates and vertebrates and consists of four homologous domains (I-IV), each containing six α-helical transmembrane segments (S1-S6) (Tan et al., 2002).

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* Published in Gene 2006, 379: 62-67.

Sodium channels typically activate and then completely inactivate within a few milliseconds, the later of which results in the rapid repolarization of action potentials (Liu et al., 2004b). Pyrethroid insecticides and DDT are known to exert their insecticidal effects by altering the function of voltage-gated sodium channels in the nerve membranes of insects and preventing the repolarization phase of action potentials (Sattelle and Yamamoto, 1988; Narahashi, 1996). A substitution of leucine to phenylalanine (Leu to Phe) resulting from a single nucleotide polymorphism (SNP), termed the *kdr* mutation, in the domain II segment 6 of the sodium channel (Williamson et al., 1993; Knipple et al., 1994) has been clearly demonstrated to be associated with resistance to pyrethroid and DDT in many insect species (Busvine, 1951; Ingles et al., 1996; Williamson et al., 1996; Dong, 1997).

The *kdr* mutation has attracted a great deal of attention and has been extensively studied in different insect species due to the importance of pyrethroids in the control of insect pests worldwide, especially their major role in preventing malaria (Zaim et al., 2000; Sina and Aultman, 2001). We chose the *kdr* mutation as a model system with which to address the general issue of whether the functional polymorphisms in genes that are associated with insensitivity of the insect nervous system undergo DNA variation or go through transcriptional regulatory variation, consequently altering protein function. Here we show that posttranscriptional regulation through RNA allelic variation and RNA editing is involved in the *kdr* allelic expression in insects in general.

5.2 Materials and Methods

5.2.1 Insect samples

House fly strains: $ALHF^{G6}$, a house fly strain (wild-type) collected from a poultry farm, near Grant, Marshall County, Alabama, in 1998, selected with permethrin for 6 generations after collection, and then maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001); and CS, a wild type insecticide-susceptible strain. German cockroach strains: ACY (American Cyanamid Co., Clifton, NY) is a susceptible strain; and Apyr-R^{G0}, collected directly from Opelika, Alabama, in 1999 (Wei at al., 2001).

5.2.2 Genomic DNA and RNA extraction and cDNA preparation

Five males and five females of each insect population had both genomic DNA and RNA extracted for each experiment. Three replications were performed, each on a different day, for a total of 30 individual insects for each population. Genomic DNA was extracted using a modified version of the protocol described by Takada and Konami (1992). Individual insects were collected on dry ice and homogenized in ice-cold homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl and 10 mM EDTA (pH 8.0)). SDS and proteinase K (Sigma Chemical Co.) were added to final concentrations of 1% and 200 μg/ml, respectively. The samples were incubated at 50°C overnight. The samples were then extracted three times with equal volumes of phenol: chloroform and once with an equal volume of chloroform. RNase (50 μg/ml) was added to the aqueous phase and the samples were incubated at 37°C for 1 h followed by another 2 h of incubation at 50°C with addition of proteinase K (100 μg/ml). The mixture was again

extracted with phenol: chloroform (1:1). The aqueous phase was collected and NaCl was added to a final concentration of 0.1 M. DNA was precipitated with 2 volumes of 100% ethanol. Total RNA was extracted using the acidic guanidine thiocyanate and phenol-chloroform method (Sambrook et al., 1989; Liu and Scott, 1997). cDNA synthesis was carried out by reverse transcription-mediated polymerase chain reaction (RT-PCR). First strand cDNA was synthesized with SuperScript II reverse transcriptase (Stratagene) and an antisense 5'-anchored oligo(dT) primer (5' TAATACGACTCACTATAGGGAGAT TTTTTTTTTTTTTTT 3') (Tomita and Scott, 1995) using total RNA as the template.

5.2.3. Amplification, sequencing, and SNP determination for the kdr allele in insects

The sodium channel cDNA fragments from house flies and german cockroaches were amplified by primer pairs of Kdr(HF) F1/Kdr(HF) R3 and Kdr(GC) F1/Kdr(GC) R3, respectively (Table 5.1). The amplicons that generated by these primer pairs span intron/exon boundaries but do not amplify genomic DNA. In this way, any influence due to genomic DNA contamination was eliminated. The sodium channel DNA fragments of house flies and German cockroaches were amplified using primer pairs Kdr(HF) F1/Kdr(HF) R4 and Kdr(GC) F5/Kdr(GC) R1 based on exonic sequences (Table 5.1) that did not generate products in RNA samples without a reverse transcription step. The frequency and heterozygosity of the *kdr* allele in insects were investigated by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacture's instructions (A&B Applied Biosystems). The primers for SNP determination were designed according to the sequence immediately upstream of the *kdr*

allele. Three replications of the SNP determination were carried out using the same genomic DNA and cDNA of each same individual, with different preparations of the PCR templates. To confirm our PCR products that used for the SNP determination were the *kdr* gene fragments, we sequenced the PCR products both from cDNA and genomic DNA of each same individual, with at least once.

5.3. Results

5.3.1 *The* kdr *allelic variation at the genomic DNA level*

To determine whether the functional polymorphism for the L-to-F *kdr* mutation undergoes DNA variation, we conducted a systematic study to examine SNPs in association with the *kdr* mutation. To accomplish this, we compared genomic and cDNA sequences within and between insecticide resistant and susceptible house fly and German cockroach populations. The SNPs of 30 individuals from each insect population were used to determine the correlation between the *kdr* allelic genotype and its mutation-mediated resistance phenotype. The PCR products from both genomic DNA and cDNA of each individual were sequenced. In all cases, the PCR sequence was the sodium channel gene fragment. Interestingly, using the primers designed in our study, we did not detect any introns in this region in both house flies and German cockroaches as previously reported (Dong et al., 1998; Rinkevich et al., 2006). Except in the German cockroach ACY strain, different genotypes for the *kdr* locus were observed among individuals in the same population of each insect species. All the individuals in the ACY strain had the genotype homozygous for the susceptible allele (TTG) at the *kdr* locus, which correspond to a codon encoding Leu. Nevertheless, individuals in susceptible (CS)

and resistant (ALHF^{G6}) house fly populations and in the German cockroach resistant (Apyr-R^{G0}) population were heterozygous for both susceptible and resistance alleles or homozygous for either the susceptible or resistance allele (Table 5.2). The major genotype was homozygous for the susceptible allele in both resistant and susceptible strains of house flies and German cockroaches (Table 5.2). There was no significant change in the genotype between susceptible and resistant strains of both insect species tested although the levels of resistance to pyrethroids between them are dramatically different (Liu and Yue, 2001; Wei at al., 2001). These results strongly indicate no correlation for the *kdr* allele at the genomic DNA level with levels of susceptibility and/or resistance to insecticide in both insect species.

5.3.2. The kdr *allelic expression variation in house flies*

To investigate whether the functional polymorphism of the *kdr* mutation is in fact due to posttranscriptional modification, we first assessed the RNA expression variation of the *kdr* allele in both susceptible and resistant house flies. We identified a strong correlation between the *kdr* allelic expression and the levels of pyrethroid resistance (Fig. 5.1A). In the susceptible CS house fly population, 23/23 (100%) of the genomically heterozygous (C/T) individuals expressed C allele (Fig. 5.1A) through the regulation of the monoallelic preferential expression. Homozygous individuals for the *kdr* allele T in the CS strain all expressed C allele via a U-to-C RNA editing event (Fig 5.1A, Table 5.3), while the genetically homozygous individuals for the C allele all showed the expression for the same allele. The expression of C allele in the *kdr* locus of CS house flies generates a codon encoding Leu that corresponds to the susceptibility in this strain to insecticides.

In contrast, in the highly resistant house fly population of ALHF^{G6} with 6 generations of selection in the laboratory after collection from the field, 4/4 (100%) of the genomically heterozygous (C/T) individuals expressed T allele through monoallelic preferential expression of T. Genomically homozygous individuals with the susceptible allele C also expressed T through a C-to-U RNA editing event (Fig 5.1A, Table 5.3). While the genomically homozygous individuals for the *kdr* allele T all showed the expression for the same allele (T). The expression of T allele in the *kdr* locus of ALHF^{G6} house flies results in a change of Leu to Phe (Fig 5.1A, Table 5.3), corresponding to the resistance phenotype of this strain. These results strongly indicate that the correlation between the expression of alleles for the *kdr* locus and the levels of susceptibility or resistance to pyrethroids is regulated by RNA allelic variation and RNA editing.

5.3.3 The kdr *allelic expression variation in the field population of German cockroaches*

We next examined the RNA expression levels of the German cockroach Apyr-R^{G0} strain, which had relatively low levels of resistance in the absence of further selection with insecticides in the laboratory (Wei et al., 2001). It has been proposed that there is inevitably a large portion of the field insect populations that avoid exposure to insecticide selection pressure, thus providing a pool of susceptible individuals for the repopulation of the resistant portions selected by insecticides (Georghiou and Taylor, 1977). We hypothesized that this directly collected field German cockroach population would present intermediate levels of expression for the *kdr* allele that corresponded to their intermediate levels of resistance. We identified that 17% of individuals in this field collected population expressed only the susceptible allele G, 43% of them expressed the

kdr allele C, and 40% expressed both G and C alleles (Fig. 5.1B). Clearly, the allelic expression pattern for the *kdr* locus in this field collected German cockroach population strongly correlated with its intermediate levels of resistance (Wei et al., 2001) and its mixture portions of both susceptible and resistant individuals as suggested by Georghiou and Taylor (1977). Among 24 genomic homozygous individuals of Apyr-R^{G0} for the susceptible allele G, 12/24 (50%) of them expressed both G and C alleles and 7/24 (29%) expressed the C allele at the corresponding position through a G-to-C editing event (Fig. 1B, Table 3), resulting in a Leu to Phe change (Fig 5.1B, Table 5.3). While 5/24 (21%) of Apyr-R^{G0} individuals showed the expression for the same allele, generating a codon encoding Leu. One genomically heterozygous individual of Apyr-R^{G0} expressed C allele through monoallelic expression regulation (Fig. 5.1B, Table 5.3). This result further confirmed the involvement of transcription regulation through RNA allelic expression variation and RNA editing in the expression of alleles in the *kdr* locus of the insect sodium channel gene. The study also suggest that inter-individual variability in the regulation of *kdr* allelic expression of the sodium channel gene is a regular feature in the natural populations of insects, providing a rich substrate for evolutionary insecticide selection (Wray et al., 2003; Stamatoyannopoulos, 2004) and for adaptation to changing environments (Hamilton, 2002).

5.4. Discussion

The steady accumulation of information from studies on toxicology, genetic linkage, gene cloning and sequencing, and functional analysis for over the last 15 years has clearly demonstrated that the L-to-F *kdr* mutation of the sodium channel gene is very

important in pyrethroid and DDT resistance in a wide range of different insect species. Left unanswered, however, has been the question of whether the polymorphism of the L-to-F *kdr* mutation in the sodium channel gene that determines resistance phenotypic variability undergoes DNA variation or experiences transcriptional regulatory variation.

This study compared genomic DNA and RNA sequences within and among resistant and susceptible house fly and German cockroach populations. We found no correlation between the *kdr* allelic genotypes with levels of susceptibility and/or resistance to insecticides in any of insect populations tested. This agrees with previous studies, where no correlation between the genotype of the L-to-F *kdr* mutation and knockdown resistance phenotype were observed in several insect species, including house flies, German cockroaches, mosquitoes *Aedes aegypti* and *Culex pipiens pipiens*, and cat fleas *Ctenocephalides felis* (Miyazaki et al., 1996; Dong et al., 1998; Brengues et al., 2003; Bass et al., 2004; McAbee et al., 2003). These researchers also reported different genotypes were present in the same populations of insects. Furthermore, Miyazaki et al. (1996) found both *kdr* and susceptible allelic genotypes in susceptible house fly strains. These studies support our findings that the DNA variants at the *kdr* locus are not clearly correlated with insecticide susceptibility and resistance. However, the results reported here reveal a strong correlation between the *kdr* allelic expression and the levels of insecticide resistance. This correlation is probably regulated through RNA variation and RNA editing. Together, these findings not only highlight the extraordinary ability of insects to adapt to evolutionary selection by regulating a target site with different transcripts, but also give a comprehensive view of posttranscriptional regulation of insect sodium channel gene expression. This study also indicates that the

posttranscriptional control of gene expression and function is far more elaborate and extensive than previously thought (Moore, 2005).

The role of RNA editing as an important mechanism for modulating the sodium channel function in insects has recently been revealed (Palladino *et al.*, 2000; Liu et al., 2004; Reenan, 2005). Nevertheless, the impact of RNA editing of the sodium channel gene on insecticide resistance is unknown. Here we discovered different isoforms of insect sodium channel generated by U-to-C, C-to-U and G-to-C editing events in insects in response to insecticide sensitivity and resistance. Furthermore, our discovery of the G-to-C editing resulting in the functional amino acid substitution has never been reported in the animal kingdom before our study, indicating that our current knowledge of RNA editing is far from comprehensive. More significantly, our study for the first time showed that RNA allelic variation expression is another principle molecular basis governing changes in the genetic information contained in the *kdr* locus and regulating the function of the sodium channel in the insect nervous system.

While we demonstrated that the *kdr* mutation is very important in the evolution of resistance development, it is unlikely to be the sole mechanism connected with such a widespread phenomenon as *kdr*-mediated resistance. Especially, more than 20 sodium channel mutations have been identified as being involved in reducing channel sensitivity to insecticides or neurotoxins (Soderlund and Kinpple 2003). Whether these mutations in the channel are regulated via the same mechanisms as those implicated in the *kdr* mutation remains a subject for further investigation. However, our findings have important implications for the evaluation of the molecular mechanisms that control these mutations in resistance and for understanding the mechanisms of selection and adaptation

in evolution. In addition, Point mutations associated with target site insensitivity have also been well documented for *Ace*-encoded acetylcholinesterase (Weill et al., 2003), the target of organophosphates (OP) and carbamates, and the *Rdl*-encoded ligand gated chloride channel (ffrench-Constant et al., 1993). Our findings may offer a new approach to explore how these target site genotypes and their insensitivity-mediated resistance phenotypes are coupled.

In conclusion, our results suggest that posttranscriptional modification of *kdr* allelic expression may be involved in altering the genetic information of the *kdr* locus in the insect sodium channel and, subsequently, may also be involved in the development of insecticide resistance. It is possible that this finding may point to a common molecular mechanism connecting an insect's genotype with the resistance phenotype conferred by the target site insensitivity of the insect nervous system. A comprehensive understanding of sodium channel gene expression also requires an appreciation of how the transcripts of the sodium channel gene are influenced by these diverse regulatory mechanisms. However, it is important to note that the resistance phenotype in the selected strains may not be caused solely by sodium channel insensitivity. Other mechanisms, such as metabolic detoxification, also play an important role in the development of resistance in these insect strains (Pridgeon et al., 2002; Xu et al., 2005). The high levels of resistance in house flies and German cockroaches (i.e. 6600- and 97-fold, respectively) support that multiple mechanisms or genes are most likely responsible for the overall resistance. Whether these resistance genes share a similar transcriptional regulation in response to insecticide selection pressure remains to be determined.

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Table 5.1 Oligonucleotide primers used for amplifying the sodium channel cDNA and genomic DNA fragments from the house fly *Musca domestica* and the German cockroach *Blattella germanica*

Insects	Template	Primer Sequence
M.	gDNA	Kdr (HF) F1: 5' CAATGGGTGCATTGGGTAATCTGAC 3'
domestica		Kdr (HF) R4: 5'GGGGCTGATAAACTAGATGAACCG 3'
	cDNA	Kdr (HF) F1: 5' CAATGGGTGCATTGGGTAATCTGAC 3'
		Kdr (HF) R3: 5' AATGATGATGTCACCATCCAGCTG 3'
B.	gDNA	Kdr (GC) F5: 5' TGGAACTTTACGGACTTCATGCAC 3'
germanica		Kdr (GC) R1: 5' GCTGACAGATTGGATGAACC 3'
	cDNA	Kdr (GC) F1: 5' AGGTCTGTCCGTGCTGCGATCG 3'
		Kdr (GC) R3: 5' CTCAAATGCCTCAGCAATCTTG 3'

Table 5.2 The genotype of the *kdr* locus (the L/F site) in house flies, *M. domestica*, and German cockroaches, *B. germanica*

Insect Species	Strain	n[a]	Phenotype	Genotype[b] (total individuals)		
M. domestica	CS	30	Susceptible	CTT (23)	C/TTT (6)	TTT (1)
	ALHF^{G6}	30	6,600-fold resistance	CTT (22)	C/TTT (4)	TTT (4)
B. germanica	ACY	30	Susceptible	TTG (30)	0	0
	Apyr-R^{G0}	30	97-fold resistance	TTG (24)	TTG/C (1)	TTC (5)

^{G0}The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

[a] The total number of tested insects (three replicates for each of 5 males and 5 females)

[b] The nucleotides in the *kdr* locus changed in the genotypes are underlined

Table 5.3 RNA expression regulation events identified in the *kdr* locus of the sodium channel gene of house flies, *M. domestica*, and German cockroaches, *B. germanica*

Insects	Nucleotide changes*	RNA regulation event	Resulting amino acid
Musca CS	CTT to CTT	-	Leucine
	C/TTT to CTT	Monoallelic Expression	Leucine
	TTT to CTT	U-to-C	Leucine
Musca ALHF^{G6}	CTT to TTT	C-to-U	Phenylalanine
	C/TTT to TTT	Monoallelic Expression	Phenylalanine
	TTT to TTT	-	Phenylalanine
Blattella ACY	TTG to TTG	-	Leucine
Blattella Apyr-R^{G0}	TTG to TTG	-	Leucine
	TTG to TTC/G	G-to-C	Phenylalanine/Leucine
	TTG to TTC	G-to-C	Phenylalanine
	TTG/C to TTC	Monoallelic Expression	Leucine
	TTC to TTC	-	Phenylalanine

* The nucleotides regulated in the *kdr* locus are underlined

FIGURE LEGEND

Fig. 5.1 Detection of allele-specific transcription for two alleles in the *kdr* locus house flies *M. domestica* (A) and German cockroaches *B. germanica* (B) compared with the corresponding genotypes for the same individuals. Thirty insects in each population were used for a total of 30 SNP determinations, with each of the 10 SNP determinations being repeated three times. [a]The total individuals with the specific genotype (DNA) allele(s) in the *kdr* locus (the data as presented in Table 2). [b]The individuals with specific expression (cDNA) of allele(s) for the corresponding genotype of the same individuals. [c]The total expression frequency of specific alleles in the whole population (30 individuals).

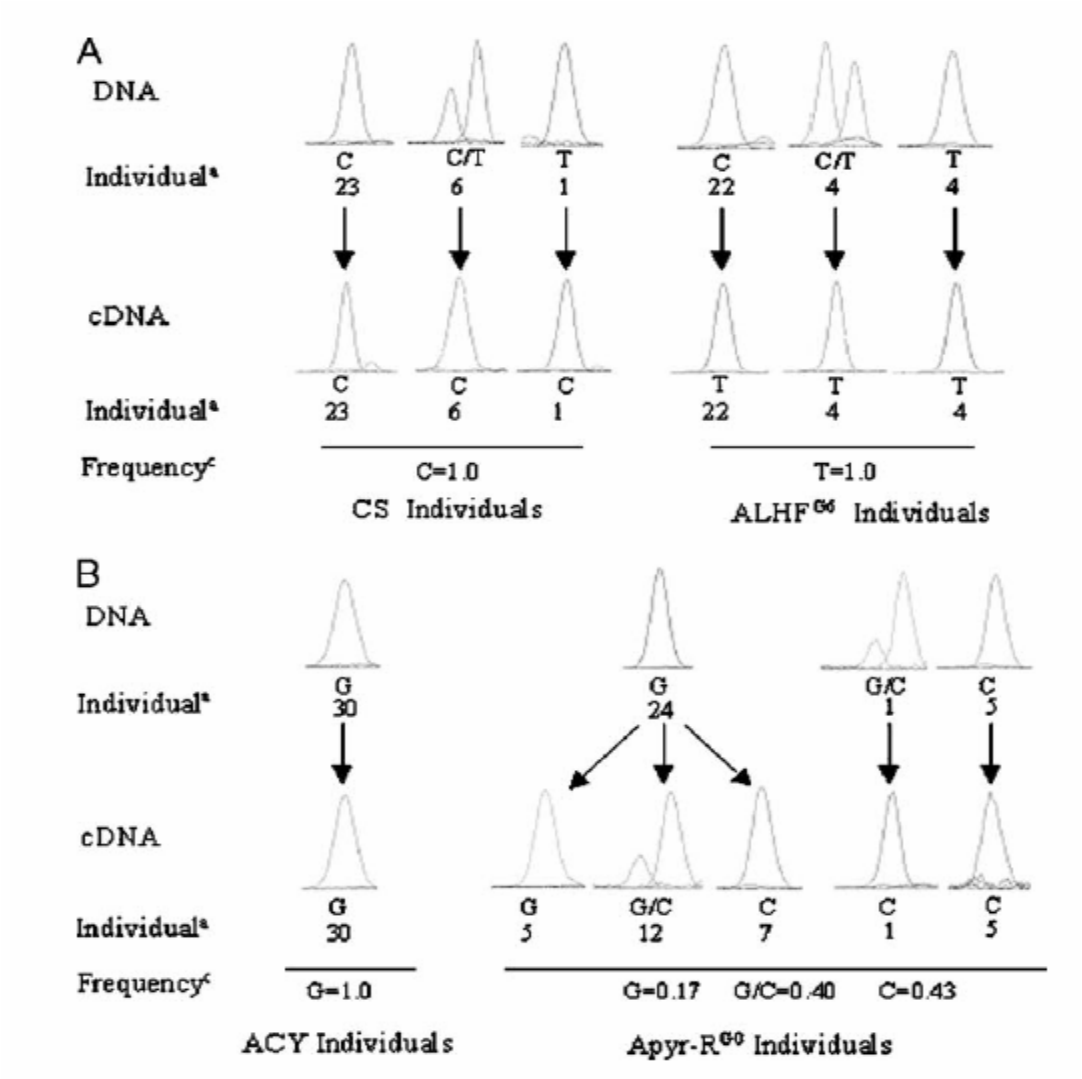
A
DNA
Individual[a]
C 23
C/T 6
T 1
C 22
C/T 4
T 4
cDNA
Individual[a]
C 23
C 6
C 1
T 22
T 4
T 4
Frequency[c]
C=1.0
T=1.0
CS Individuals
ALHF[66] Individuals
B
DNA
Individual[a]
G 30
G 24
G/C 1
C 5
cDNA
Individual[a]
G 30
G 5
G/C 12
C 7
C 1
C 5
Frequency[c]
G=1.0
G=0.17
G/C=0.40
C=0.43
ACY Individuals
Apyr-R[60] Individuals

Fig. 5.1

CHAPTER 6: IDENTIFICATION OF MULTIPLE SODIUM CHANNEL GENES IN MOSQUITOES AND HOUSE FLIES

6.1 Introduction

Voltage-gated sodium channels are responsible for the depolarization phase of action potentials in membranes of neurons and most electrically excitable cells (Catterall, 2000). The overall organization of the sodium channel α-subunit is conserved among invertebrates and vertebrates and consists of four homologous domains termed I-IV. Within each domain, there are six transmembrane segments (i.e., S1–S6), and a hairpin-like P loop between S5 and S6 that comprises part of the channel pore (Goldin, 2002). Molecular characterization has revealed that 10 genes, including one non-functional gene, present in the mammalian sodium channel gene family (Catterall, 2000; Goldin, 2002; Yu and Catterall 2003). Eight sodium channel genes have been documented in zebra fish (Novak et al., 2006) and six sodium channel genes have been cloned from the electric fish (Lopreato et al., 2001). In contrast to the fairly well defined vertebrate sodium channel genes, the diversity of sodium channel genes in invertebrate is not fully characterized. In the model insect *Drosophila melanogaster*, only one confirmed sodium channel gene, *para*, and one putative sodium channel gene, *DSC1*, have been identified. Nevertheless, the *DSC1* ortholog, *BSC1*, from the German cockroach, *Blattella germanica*, was recently documented as a novel family of Ca^{2+}-selective cation channel

genes, but not a sodium channel gene (Zhou et al., 2004).

The *para*-type sodium channel gene in insects was first identified based on temperature-sensitive paralysis phenotypes displayed by mutant alleles (Loughney et al., 1989) and has been functionally expressed in *Xenopus* oocytes. The results confirmed that *para* is a true voltage-gated sodium channel with biophysical and pharmacological properties similar to mammalian voltage-gated sodium channels (Warmke et al., 1997). It appears that *para* is the only gene that encodes a sodium channel in insects according to recent research. The *para*-type sodium channel is the primary target of pyrethroid insecticides (Narahashi, 1996) and its insensitivity has been associated with pyrethroid resistance in several knockdown resistant (*kdr*) insect species (Brengues et al., 2003, Dong, 1997, Martinez-Torres et al., 1999). A substitution of leucine to phenylalanine (Leu to Phe) resulting from a single nucleotide polymorphism (SNP), termed the L-to-F *kdr* mutation, in domain II segment 6 of the *para*-type sodium channel (Williamson et al., 1993, Knipple et al., 1994) has been proposed to be associated with resistance to pyrethroid and DDT in many insect species (Dong, 1997, Ingles et al., 1996 and Williamson et al., 1996). Recent studies by Xu et al. (2006a; 2006b) indicated that posttranscriptional regulation through RNA allelic variation and RNA editing of the L-to-F *kdr* mutation may be involved in altering the genetic information of the *kdr* locus in the insect sodium channels.

In the current study, I have identified multiple *para*-type sodium channel gene isoforms in the mosquito *Culex quinquefasciatus* and the house fly *Musca domestica* through Southern blot and DNA sequencing analysis. Yet, only one *para*-type transcript, albeit multiple *para*-type sodium channel gene isoforms, has been identified by Northern

blot analysis. Posttranscriptional regulation might be an important factor involved in formation of a single functional transcript of sodium channel genes in both mosquitoes and house flies.

6.2 Materials and Methods

6.2.1 Insect strains

Three strains of mosquito *Cx. quinquefasciatus* were used in this study. $HAmCq^{G0}$, collected from Huntsville County, Alabama, in 2002 (Liu at al., 2004a); $HAmCq^{G8}$, HAmCq mosquitoes that had been selected with permethrin for 8 generations after collection; and S-Lab, a universal insecticide-susceptible strain that was obtained from Dr. Laura Harrington (Cornell University). Two strains of house flies were used in this study: $ALHF^{G6}$, an insecticide resistant house fly strain collected from Alabama in 1998 and selected with permethrin for 6 generations after collection; and CS, an insecticide-susceptible house fly strain obtained from Dr. J. G. Scott (Cornell University). All insects were reared at 25 ± 2 °C under a photoperiod of 12:12 (L:D) h.

6.2.2 Genomic DNA preparation

Genomic DNA was extracted from adult mosquitoes and/or house flies as described by Liu et al (2004). Briefly, insects were collected and homogenized on dry ice with ice-cold homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl and 10 mM EDTA; pH 8.0). SDS and proteinase K were added to final concentrations of 1% and 200μg/ml, respectively. Samples were incubated at 50 °C over night. The samples were then extracted three times with equal volumes of phenol: chloroform (1:1) and once with an

equal volume of chloroform. RNase (50 μg/ml) was added to the aqueous phase, and incubated at 37°C for 1 h, followed by another 2 h of incubation at 50°C with the addition of proteinase K (100 μg/ml). The mixture was again extracted with phenol: chloroform. The aqueous phase was collected and precipitated with 100% ethanol.

6.2.3 Southern blot analysis

Genomic DNAs (40 μg) was digested completely with 260 units *Eco*RI or *Hin*dIII in a 170 μl solution. Samples were precipitated with ethanol, and then resuspended in TE buffer prior to electrophoresis on 0.8% agarose gels in 0.5× TBE. DNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH, USA) as described by Sambrook *et al*. (1989). To optimize the detection of a single band but not multiple bands per sodium channel gene, the probe design met the following 2 criteria: (1) lack of internal *Eco*RI and *Hin*dIII sites and (2) inclusion within a single exon. The mosquito and house fly *para* type sodium channel cDNA fragments were amplified using primers pairs: KDR S1S (5′ GACAACGTGGACCGCTTCCCGGACA 3′)/KDR AS1S (5′ ACGACAA AATTTCCTATCACTACGGTG 3′) and KDR (HF) F1S 5′ ACCACAAGGATCGCTTC AAGGACCA 3′)/KDR (HF) R1S (5′ ACCACAAGATTGCCGATCACGACCG 3′), respectively. These cDNA fragments were labeled with [α-^{32}P] dCTP using the High Prime Labeling Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions and hybridized with DNA blots using QuickHyb solution (Stratagene, La Jolla, CA). Blots were prehybridized and hybridized at 68°C for 30 min and 1hr, respectively. The blots were then washed in 2×SSC and 0.1% SDS for 15 min at 60°C three times, and in 0.2×SSC and 0.1% SDS once. Kodak films with intensifying screens

were used for autoradiography. All Southern blot analyses were repeated three times with different preparations of DNA samples.

6.2.4 RNA preparation and Northern blot analysis

Total RNA from whole bodies of house flies were extracted using the acidic guanidine thiocyanate and phenol–chloroform method (Sambrook et al., 1989). Poly(A)$^+$ RNA was isolated with Oligotex-dT suspension (QIAGEN). Three micrograms of poly(A)$^+$ RNA from each sample were fractionated on a 1% formaldehyde denaturing agarose gel containing ethidium bromide and transferred to Nytran membranes (Schleicher and Schuell) as described by Sambrook et al. (1989). Northern hybridization was performed with QuickHyb solution (Stratagene) and the same probe used for house fly Southern blot analysis. The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA (rRNA) band on the agarose gel under UV light before transfer (Spiess and Ivell, 1998). Northern blot analyses were repeated three times with different preparations of mRNA samples.

6.2.5 Amplification of para*-type sodium channel gene genomic DNA and cDNA fragments in mosquitoes and house flies*

The individual genomic DNAs and cDNAs of mosquitoes and house flies for PCR amplification were prepared as described by Xu et al (2006a, 2006b). Primers KDR S14 (5′ GGAACTTCACCGACTTCATGCAC TC 3′) and KDR AS1 (5′ GACAAAAGCAAG GCTAAGAAAAGG 3′) were designed to amplify the sodium channel genomic DNA fragments from templates of all mosquito populations. To amplify house fly sodium

channel genomic DNA fragments, primers KDR (HF) F4 (5′ TCGCTTCAAGGACCATG AAYTACCGCGCTG 3′) and KDR (HF) R5 (5′ CCGAAGTTGGACAAAAGCAAAGC TAAGAAAAG 3′) were used to carry out PCR. The PCR reactions for amplifying mosquito sodium channel gene fragments were heated at 95 °C for 5 min, followed by 42 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min) and a final extension of 72 °C for 10 min. The PCR reaction with house fly genomic DNA template was conducted under the same reaction conditions described above except a 60 °C annealing temperature was used. The sodium channel cDNA fragments in both mosquitoes and house flies were amplified using the same methods as described in Section 4.2.3, Chapter 4 and Section 5.2.3, Chapter 5 (Xu et al., 2006a; 2006b).

6.2.6 SNP determination for the kdr *allele in mosquitoes and house flies*

The frequency and heterozygosity of the *kdr* allele in mosquitoes and house flies were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to manufacturer's instructions (A&B Applied Biosystems). The primers, Cx_SNP2 (5′ GCCACCGTAGTGATAGGAAATTT 3′) and HF_SNP2 (5′ TGGCCACG GTCGTGATCGGCAAT 3'), used for SNP determination of mosquitoes and house flies, respectively, were designed according to the sequence immediately upstream of the *kdr* allele. Three replications of the SNP determination were carried out with different preparations of the PCR templates. To confirm that the PCR products used for the SNP determination were in fact the *para*-type sodium channel gene fragments, these PCR products were sequenced.

.6.3 Results

6.3.1 Identification of multiple sodium channel genes in mosquitoes and house flies

The copies number of *para*-type sodium channel gene in mosquitoes and house flies were investigated using Southern blot analysis. Probes were generated according to a single exon of mosquito and house fly sodium channel genes. Two and three DNA bands were identified in pyrethroid resistant $HAmCq^{G8}$ mosquitoes and insecticide susceptible S-Lab mosquitoes, respectively, (Fig. 6.1A) when mosquito genomic DNAs were digested with restriction enzymes *Eco*RI or *Hin*dIII (Fig. 6.1A). The different number and size of genomic DNA bands detected in $HAmCq^{G8}$ and S-Lab may result from polymorphisms at restriction enzyme cutting site(s) in these two strains. These results strongly suggest that at least two sodium channel genes are present in the mosquito *Cx. quinquefasciatus*. Two DNA bands were detected in both pyrethroid resistant $ALHF^{G6}$ and susceptible CS house flies when *Eco*RI was used to digest house fly genomic DNAs (Fig. 6.1B). When house fly DNAs were digested with *Hin*dIII enzyme, two strong hybridization bands were found in CS house flies. These results indicate that there are at least two *para*-type sodium channel genes in house flies (Fig. 6.1B). Taken together, the results strongly suggest that there are multiple *para*-type sodium channel genes present in mosquitoes and house flies.

6.3.2 Identification of para*-type sodium channel gene transcripts in house flies*

The *para*-type sodium channel gene transcript in house flies was examined using Northern blot analyses. Only one band displayed a strong hybridization signal in both pyrethroid resistant ($ALHF^{G6}$) and susceptible (CS) house flies (Fig. 6.2A), suggesting

that, most likely, there was only one *para*-type sodium channel gene transcript present in house flies. Northern blot results also demonstrated that the levels of *para*-type sodium channel gene expression were similar in pyrethroid resistant ALHF^{G6} and susceptible CS house flies (Fig. 6.2B). These results suggest that, unlike other resistance genes, such as cytochrome P450 genes (Liu and Scott, 1998), hydrolase genes (Hemingway et al., 2004), and glutathione S-transferase genes (Ranson et al., 2001), whose expression are overexpressed in resistant insects, overexpression of the *para*-type sodium channel gene is not a factor involved in the pyrethroid resistance of ALHF^{G6} house flies.

6.3.3 Sequencing analysis of multiple sodium channel genes in mosquitoes and house flies

Two DNA products (~500 bp and ~200 bp) were generated from each mosquito sample of HAmCq^{G8}, HAmCq^{G0} and S-Lab by PCR using primers of KDR S14 and KDR AS1 and single mosquito genomic DNA as the template (Fig. 6.3). These PCR products from each of individual samples were sequenced. Sequencing analysis indicated that the ~ 500bp PCR product was the mosquito *para*-type sodium channel gene sequence with an intron sequence reported by Martinez-Torres et al. (1998), while the ~200 bp PCR product did not obtain the intron sequence. A similar result was also discovered in the house fly sodium channel gene. Two DNA products (~300 bp and ~200 bp) were also identified from ALHF^{G6} and CS house flies (Fig. 6.3) by PCR using primers KDR (HF) F4 and KDR (HF) R5 and single house fly genomic DNA as the template. The ~300 bp PCR product was house fly *para*-type sodium channel gene sequences with an intron sequence (Rinkevich et al., 2006), and the ~200 bp PCR product was house fly *para*-type

sodium channel gene sequences without the intron sequence. These sequencing results, thus, further suggest that there are at least two sodium channel genes presented in the mosquito, *Cx. quinquefasciatus* and house flies, *M. domestica*.

6.3.4 Identification of the L-to-F kdr *allelic variation at the genomic DNA and RNA levels in three mosquito strains*

I next investigated the SNP for A/T alleles at the *kdr* locus of two *para*-type sodium channel genes in both genomic DNA and RNA levels in 30 individuals of each mosquito population to determine the correlation of the *kdr* allelic variation and resistance phenotype. Fifty percent of the individual PCR products from both cDNA and genomic DNA were sequenced and found to be all mosquito *para*-type sodium channel gene fragments. No correlation was detected for the *kdr* allele of the *para*-type sodium channel gene that lacked the intron at the genomic DNA level with levels of gene expression and resistance to insecticide (Table 6.1), which was consistent with our previous results (Xu et al 2006a). In the susceptible S-Lab strain, I found different genotypes for the *kdr* locus of the sodium channel gene that lacked the intron among individuals in the same population, including individuals that were heterozygous for both susceptible (A) and resistance (T) alleles or homozygous for either the A or T allele, although the major genotype was heterozygous (A/T) in the S-Lab population (Table 6.1). In contrast, I found that all the individuals were genotype homozygous for the A allele in the *para*-type sodium channel gene that obtained the intron, which was corresponded with their sodium channel gene transcriptional level, i.e., all individual expressed susceptible allele A (Table 6.1). The expression of susceptible allele A at the *kdr* locus of the sodium channel

gene with the intron sequence in susceptible mosquitoes resulted in a codon TTA encoding Leu, which corresponds to insecticide susceptibility in this strain. This result indicates a strong correlation among the genotype of sodium channel gene with the intron, the gene expression, and the level of insecticide susceptibility in susceptible S-Lab strain. Similar patterns were also identified in the highly resistant strain $HAmCq^{G8}$ with 8 generations of selection in the laboratory after collection from the field. In $HAmCq^{G8}$, 8/30 (27%) individuals were genotype heterozygous for both susceptible (A) and resistance (T) alleles, and 22/30 (73%) individuals were homozygous for the resistant T allele, which is strongly correlated with the frequency of allelic expression at the *kdr* locus of the sodium channel gene (Table 6.1). In the $HAmCq^{G0}$ population, the frequency of the gene expression was more correlated with the frequency of the genotype of the sodium channel gene that obtained the intron (Table 6.1). These results strongly suggest that the sodium channel gene possessing the intron is the functional copy of the sodium channel gene that is selected for transcription and translation of the sodium channel protein in mosquitoes.

6.3.5 Identification of the L-to-F kdr *allelic variation at the genomic DNA and RNA levels in two house fly strains*

To further characterize functional sodium channels in insects, I examined alleles (C/T) at the *kdr* locus of two *para*-type sodium channel gene copies at the genomic DNA and RNA levels in 30 individuals of each house fly strain. I found that *kdr* genotypes of the *para*-type sodium channel gene without the intron sequence in house flies were the same as our previous report (Xu et al., 2006b). The major genotype was homozygous for

the susceptible allele C in both resistant ALHF^{G6} and susceptible CS house flies (Table 6.2). These results strongly indicate no correlation for the *kdr* allele at the genomic DNA level with levels of gene expression and susceptibility and/or resistance to insecticide in both house fly strains. In contrast, a strong correlation was found among the genotypes for alleles at the *kdr* locus of *para*-type sodium channel gene with intron sequence, the frequency of the gene expression, and the levels of susceptibility and/or resistance to insecticide in both house fly strains (Table. 6.2). All individuals in the insecticide susceptible CS population were genotype homozygous for susceptible allele C, whereas, all individuals in the resistant ALHF^{G6} population were genotype homozygous for resistance allele T (Table 6.2). These results strongly correlated with the allelic expression of the gene and the level of susceptibility and/or resistance in house flies, suggesting that the sodium channel gene with the intron may be the functional sodium channel gene in house flies.

6.4. Discussion

The crucial role of *para*-type sodium channel in insecticide resistance has prompted many researchers to study insect sodium channel biology. Knowledge on the functional diversity and pharmacology of insect sodium channels has accumulated in the past decades (Dong, 2007). *Para*-type sodium channel genes have been isolated from many medically or economically important insect species (Soderlund and Knipple 2003). However, the number of *para*-type sodium channel genes present in the insect genome remains unclear.

This study examined *para*-type sodium channel genes in *Culex* mosquitoes and house flies using different molecular techniques. Both Southern blot and sequencing results reveals at least two *para*-type sodium channel genes exist in the mosquito, *Cx. quinquefasciatus* and house fly, *M. domestica*. This is the first time that multiple *para*-type sodium channel genes have been documented in insects. Although there are multiple *para*-type sodium channel genes in the house fly genome, only one transcript was found in the house fly. Unlike other insecticide resistance genes, such as cytochrome P450 genes, hydrolase genes, and glutathione S-transferase genes, the up-regulation of sodium channel gene expression does not appear to be a possible factor involved in the pyrethroid resistance in $ALHF^{G6}$ house flies because both resistant $ALHF^{G6}$ and susceptible CS house flies show equal expression of sodium channel genes.

Comparison of *kdr* genotypes in two *para*-type sodium channel genes and their corresponding transcriptional expression in three mosquito strains and two house fly strains clearly reveals sodium channel gene that possesses the intron may be the functional sodium channel gene in insects since their *kdr* genotypes are close or identical to their transcripts (Table 6.1; 6.2). Nevertheless, no-correlation among genotype, transcriptional expression or level of resistance has been identified for the sodium channel gene that lacks the intron sequence. One hypothesis is that the gene without the intron may be a non-functional gene or pseudogene presents in the genome of insects. Recent studies have proposed that pseudogenes are present in the genome with the loss of the functions (Zheng et al., 2005; Harrison and Gerstein 2002). Pseudogenes can be generated by either direct DNA duplication or retrotransposition. A pseudogene resulting from the latter is called a processed pseudogene (or retro-pseudogene). Processed

pseudogenes arise from reverse-transcription of mRNA and re-integration into the genome. It has been proposed that processed pseudogenes are generated from reverse transcribing spliced mRNAs into cDNAs using the reverse transcriptase of long interspersed nuclear element (LINE) and re-integrating into the human genome (Esnault et al., 2000; Weiner 2000). About 8000 processed pseudogenes have been reported in the human genome through exhaustive sequence comparisons with known human proteins (Zhang et al., 2003). The characters of processed pseudogenes include the absence of introns, the presence of flanking direct repeats, and a 3'-polyadenylation tract (Zheng et al., 2005). In this study, one of documented *para*-type sodium channel gene both in mosquito and house fly doesn't have intron sequence and their individual genotypes are not correlated with allelic expression in cDNA. Nevertheless, it was found that the L-to-F *kdr* mutation can be translated from this non-intron *para*-type sodium channel gene via post-transcriptional regulation of RNA allelic variation and RNA editing. Thus, whether this non-intron *para*-type sodium channel gene is a pseudogene or a functional gene with a post transcriptional regulation remains a subject for further investigation.

In conclusion, it is found in this study, for the first time, that there are multiple *para*-type sodium channel genes in the mosquito *Cx. quinquefasciatus* and the house fly *M. domestica.* Only the *kdr* genotypes of the *para*-type sodium channel gene with the intron sequence in individual insects were consistent with the expression of their transcripts. This research reveals the complexity of sodium channel gene expression and its role in pyrethroid resistance of insects. It also provides a new framework to study the sodium channel gene expression regulation and its importance in insecticide resistance of insects.

Acknowledgements

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Table 6.1 The genotypes and transcripts of the *kdr* locus (the L/F site) in mosquitoes, *Cx. quinquefasciatus*

Strain	n[a]	Phenotype	Template source	*Kdr* allele (individual numbers)		
				A	A/T	T
S-Lab	30	Susceptible	gDNA w/o intron	4	23	3
			gDNA w/ intron	30	0	0
			cDNA	30	0	0
HAmCq[G0]	30	100-fold resistance	gDNA w/o intron	0	25	5
			gDNA w/ intron	7	14	9
			cDNA	6	13	11
HAmCq[G8]	30	3,100-fold resistance	gDNA w/o intron	0	27	3
			gDNA w/ intron	0	8	22
			cDNA	0	2	28

[G0]The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

[a] The total number of tested mosquitoes (three replicates for each of 5 males and 5 females)

Table 6.2 The genotypes and transcripts of the *kdr* locus (the L/F site) house flies, *M. domestica*

Strain	n[a]	Phenotype	Template source	*Kdr* allele (individual numbers)		
				C	C/T	T
CS	30	Susceptible	gDNA w/o intron	23	6	1
			gDNA w/ intron	30	0	0
			cDNA	30	0	0
$ALHF^{G6}$	30	6600-fold resistance	gDNA w/o intron	22	4	4
			gDNA w/ intron	0	0	30
			cDNA	0	0	30

[a] The total number of tested house flies (three replicates for each of 5 males and 5 females)

FIGURE LEGENDS

Fig. 6.1 Southern blot analysis of the sodium channel gene in the genome of mosquitoes and house flies. (A) Genomic DNAs from HAmCq^{G8} and S-Lab mosquitoes were digested with *Eco*RI and *Hin*dIII. Mosquito membrane was hybridized with ^{32}P-labelled mosquito sodium channel gene fragment generated from a single exon. (B) Gnomic DNAs from ALHF^{G6} and CS were house flies digested with *Eco*RI and *Hin*dIII. House fly membrane was hybridized with ^{32}P-labelled house fly sodium channel gene fragment generated from a single exon. All Southern blot analyses were repeated three times with different preparations of DNA samples.

Fig. 6.2 Northern blot and expression analysis of sodium channel gene in ALHF^{G6} and CS house flies. (A) Northern blot analysis of sodium channel gene in house flies. The blot was hybridized with the same probe used in house fly Southern blot analysis. The ethidium bromide stain of 18S ribosomal RNA in agarose gel is shown at the bottom. (B) Relative sodium channel gene mRNA in ALHF^{G6} and CS house flies. The blots from three independent experiments were scanned. The results are shown as mean ± S.E.

Fig. 6.3 PCR products obtained using genomic DNA of single insects as templates after separation on a 2% agarose gel.

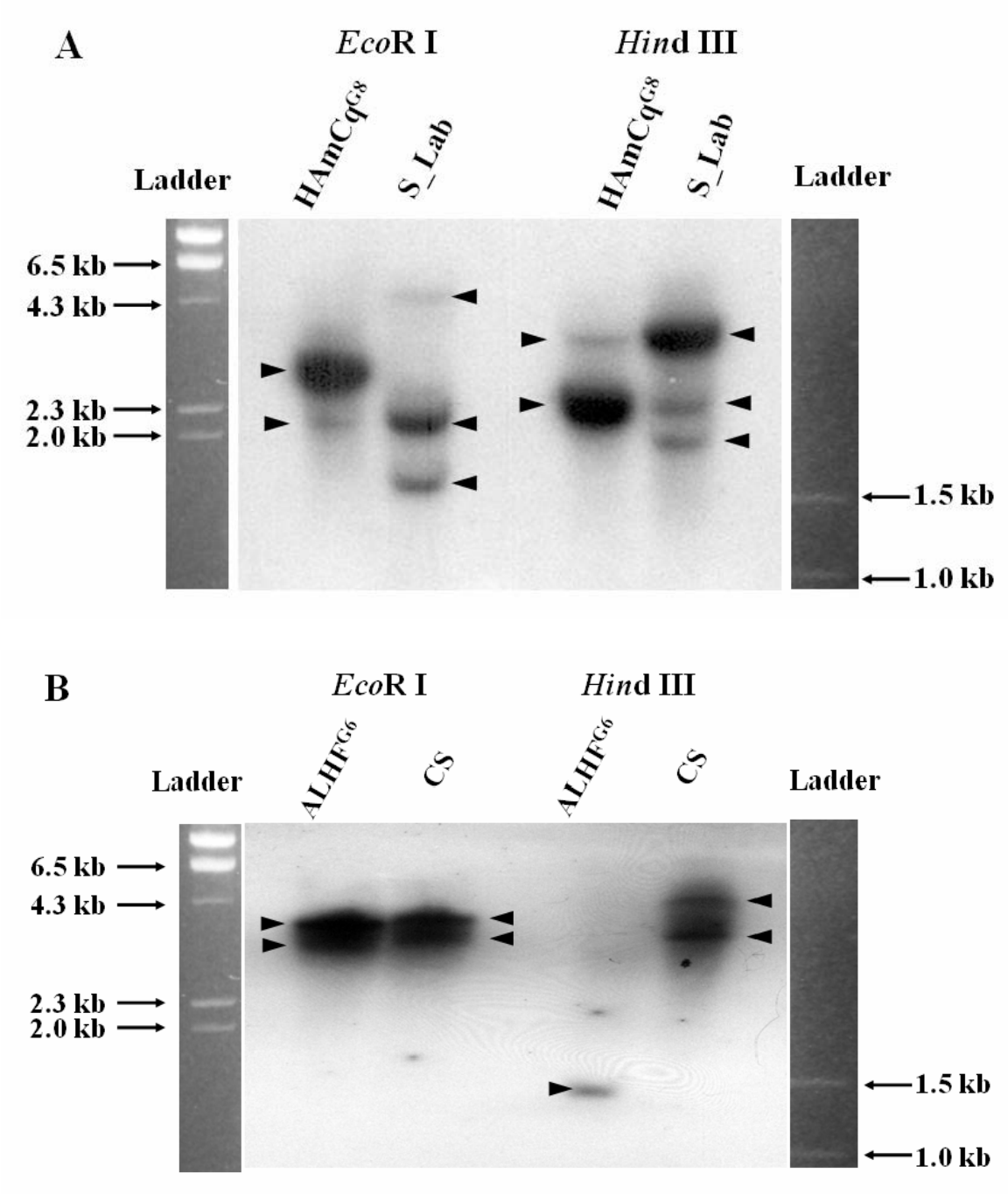
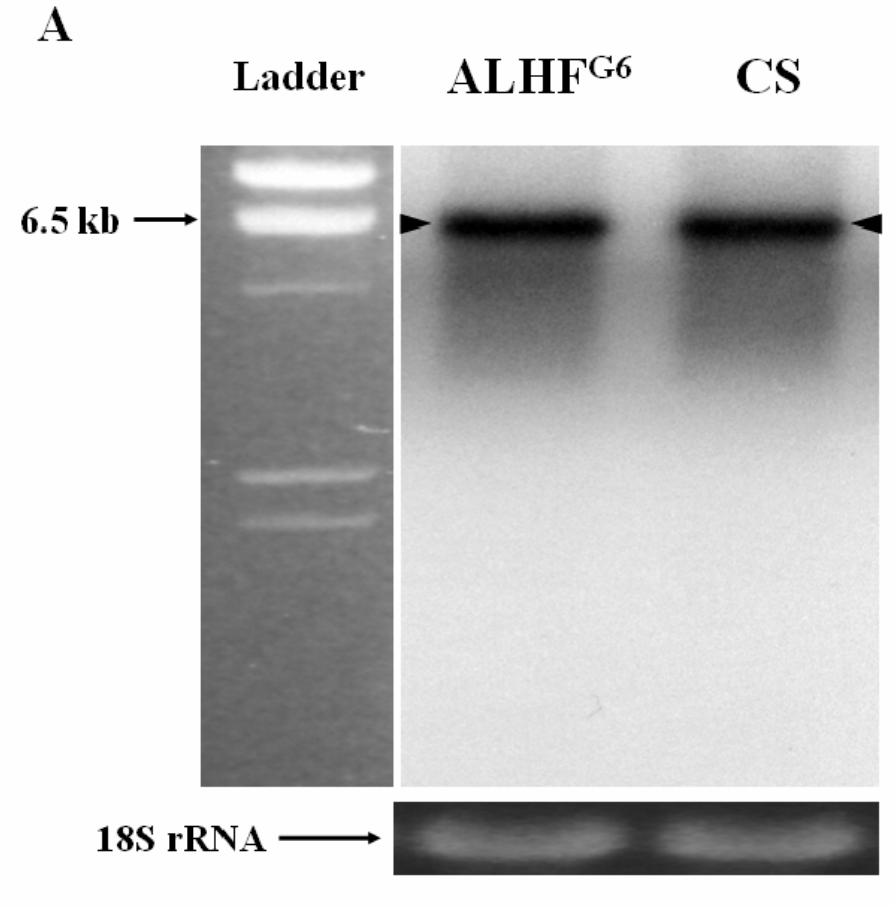

A
EcoR I
Hind III
Ladder
HAmCqG8
S_Lab
HAmCqG8
S_Lab
Ladder
6.5 kb
4.3 kb
2.3 kb
2.0 kb
1.5 kb
1.0 kb
B
EcoR I
Hind III
Ladder
ALHFG6
CS
ALHFG6
CS
Ladder
6.5 kb
4.3 kb
2.3 kb
2.0 kb
1.5 kb
1.0 kb

Fig 6.1


A
Ladder
ALHF^G6
CS
6.5 kb
18S rRNA

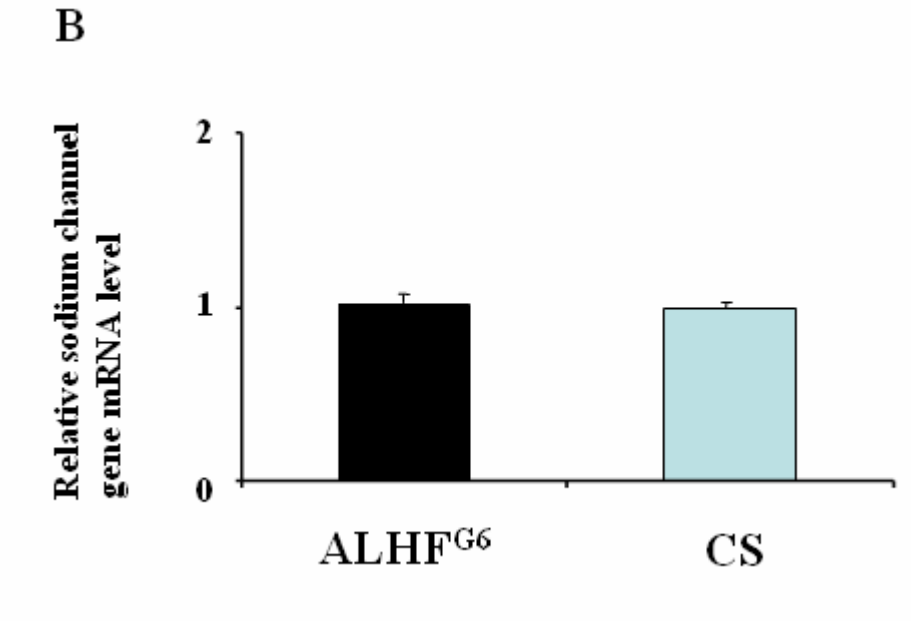

B
Relative sodium channel gene mRNA level
2
1
0
ALHF^G6
CS

Fig. 6.2

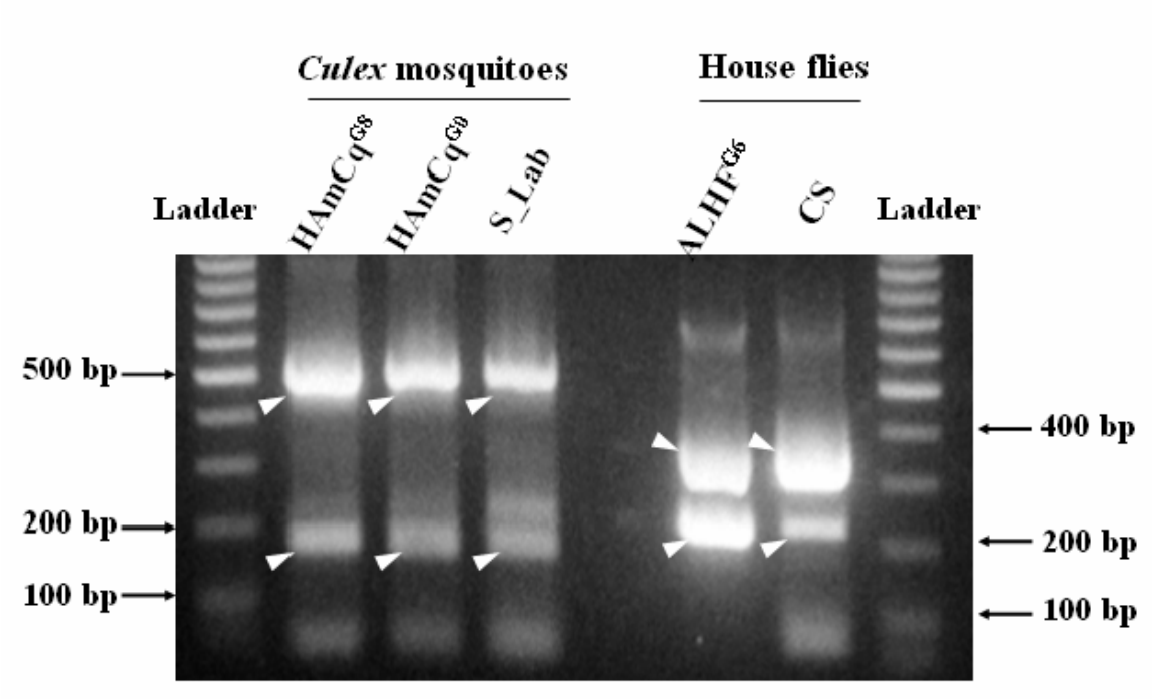

Culex mosquitoes
House flies
Ladder
HAmCq^{G8}
HAmCq^{G0}
S_Lab
ALHF^{G6}
CS
Ladder
500 bp
200 bp
100 bp
400 bp
200 bp
100 bp

Fig. 6.3

CHAPTER 7: MUTATIONS IN THE SODIUM CHANNEL GENE ARE ASSOCIATED WITH PYRETHROID RESISTANCE IN THE MOSQUITO, *CULEX QUINQUEFASCIATUS* (S.)

7.1 Introduction

Mosquito-borne diseases, with the most well known being malaria, are some of the main causes of human mortality worldwide (Marshall, 2000; Jacob, 2001). Conventional approaches to mosquito control have relied on the application of insecticides and pyrethroid insecticides are currently the most widely used for mosquito control because of their high effectiveness, relatively low mammalian toxicity, and environmental safety (McCarroll and Hemingway, 2002; Hemingway et al., 2004). However, mosquito-borne diseases are now resurgent largely because of the difficulty in controlling mosquito vectors that have developed resistance to insecticides (Hemingway et al., 2002). Identification and characterization of resistance mechanisms and genes involved in resistance in mosquitoes will be extremely important toward understanding the molecular basis of resistance and will provide important information for designing novel strategies in controlling resistant mosquito populations.

The voltage-gated sodium channel is a primary target of pyrethroid insecticides (Pasteur et al., 1996) and its insensitivity has been associated with pyrethroid resistance in several knockdown resistant (*kdr*) insect species (Brengues et al., 2003; Dong, 1997;

Martinez-Torres et al., 1999b). Unlike enhanced metabolic detoxification, *kdr*-mediated resistance is unaffected by synergists. The current literature suggests that structural changes in insect voltage-dependent sodium channels are the primary cause of knockdown resistance to pyrethroids (Soderlund and Knipple, 2003). The overall organization of the sodium channel α-subunit is conserved among invertebrates and vertebrates and consists of four homologous domains termed I-IV. Within each domain, there are six transmembrane segments (i.e., S1–S6), and a hairpin-like P loop between the S5 and S6 hydrophobic segments that comprises part of the channel pore (Goldin, 2002). A substitution of leucine (L) to phenylalanine (F), termed the *kdr* mutation, in the S6 of domain II of the sodium channel is consistently associated with knockdown resistance in several insect species, including mosquitoes (Williamson et al., 1996; Dong, 1997; Martinez-Torres et al., 1999b; Miyazaki et al., 1996; Liu and Pridgeon, 2002). The *super-kdr* resistance in the house fly is associated with an additional mutation of methionine (M) to threonine (T) in the linker region between S4 and S5 of domain II (Williamson et al., 1996). Functional studies, i.e., expression of house fly sodium channel carrying both *kdr* and *super-kdr* mutations in *Xenopus* oocytes, reveal that the mutant sodium channel is much less sensitive to pyrethroids than wild-type sodium channel (Smith et al., 1997; Lee et al., 1999). Taken together, these previous studies strongly indicate that mutations in the sodium channel gene play an important role in insecticide resistance. Thus, sodium channel genes have been obtained from many medically or economically important insect pest species (Soderlund and Knipple, 2003). Nevertheless, in most cases, only partial cDNA sequences were isolated (Dong, 2007). Full-length sodium channel cDNA sequences are available only in four insect species: *para* from fruit fly (Loughney et al.,

1989), *Vssc1* from the house fly (Ingles et al. 1996), $BgNa_v$ from the German cockroach (Dong, 1997), and $VmNa_v$ from the varroa mite (Wang et al., 2003). The availability of the full-length sodium channel cDNA from mosquitoes will make it possible to identify the mutations involved in resistance, which is a critical step toward an understanding of insecticide resistance in mosquitoes.

Culex quinquefasciatus is a mosquito species predominant in urban areas of the state of Alabama. It has been a major target for several insecticides, including pyrethroids (Liu et al., 2004a). However, difficulties in controlling *Culex quinquefasciatus* with insecticides have been reported (Liu et al., 2004a). Recently, a strain of *Cx. quinquefasciatus*, $HAmCq^{G0}$, from Huntsville, Alabama, has been established in our laboratory. $HAmCq^{G0}$ exhibited elevated levels of resistance to pyrethroids, such as permethrin, deltamethrin and resmethrin (Liu et al., 2004a). $HAmCq^{G0}$ mosquitoes were further selected by permethrin for 8 generations in the laboratory and the level of resistance in $HAmCq^{G8}$ increased dramatically compared with the parental strain, $HAmCq^{G0}$ (Xu et al., 2006a). The levels of resistance to permethrin in the HAmCq strains were partially suppressed by piperonyl butoxide (PBO), S,S,S-tributylphosphorotrithioate (DEF), and diethyl maleate (DEM), the inhibitors of cytochrome P450 monooxygenases, hydrolases, and glutathion S-transferases (GSTs), respectively, suggesting that, besides the increased metabolic detoxication, one or more mechanisms are involved in the mosquito resistance that cannot be suppressed by inhibitors (Xu et al. 2005). In this study, I focused on the isolation of sodium channel gene(s) from this mosquito species and characterization of mutations that may be of importance in resistance. Three overlapping cDNA clones were isolated and the entire

coding region of the sodium channel gene was revealed from mosquitoes, *Cx. quinquefasciatus*, thus providing the first complete cDNA sequence of a mosquito sodium channel gene. Several novel mutations, including silent mutations, were correlated with the levels of resistance in these *Culex* mosquitoes.

7.2 Materials and Methods

7.2.1 Mosquito samples

Three strains of the mosquito *Cx. quinquefasciatus* were used in this study. $HAmCq^{G0}$, collected from Huntsville County, Alabama, in 2002; $HAmCq^{G8}$, HAmCq mosquitoes that had been selected with permethrin for eight generations after collection; and S-Lab, a universal insecticide-susceptible strain that was obtained from Dr. Laura Harrington (Cornell University). All mosquitoes were reared at 25 ± 2 °C under a photoperiod of 12:12 (L:D) h.

7.2.2 RNA isolation and cDNA synthesis

Total RNAs from whole bodies of 30 mosquitoes of *Culex* mosquito strains: S-Lab, $HAmCq^{G0}$ and $HAmCq^{G8}$ were extracted using the acidic guanidine thiocyanate and phenol–chloroform method (Sambrook et al., 1989). $Poly(A)^+$ RNAs (mRNAs) of three strains were isolated using Oligotex-dT suspension (QIAGEN), according to the manufacturer's instructions. cDNA synthesis was carried out by reverse transcription-mediated polymerase chain reaction (RT-PCR). First strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and an antisense 5'-anchored oligo(dT)

primer (5’ TAATACGACTCACTATAGGGAG ATTTTTTTTTTTTTTTT 3’) (Tomita and Scott, 1995) using poly(A)$^+$ RNAs as templates.

7.2.3 Reverse transcription-polymerase chain reaction (RT-PCR) and RACE (rapid amplification of cDNA ends)

The first strand cDNA was used as template to amplify mosquito sodium channel cDNA. The PCR solution with cDNA template and a primer pair, PG KDR S4 and KDR AS02 (Table 7.1) were heated to 94°C for 2 min, followed by 40 cycles of PCR reaction (94°C for 45 s, 60°C for 45 s and 72°C for 3 min) and a final extension of 72°C for 10 min. 3′- and 5′-RACE was carried out using the Marathon™ cDNA Amplification Kit (Clontech) as described by the manufacturer. The first-stranded cDNAs were synthesized with AMV reverse transcriptase using *Culex* mosquito mRNAs as templates. The double-stranded cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of the double-stranded cDNA using T4 DNA ligase as described by the manufacturer. cDNA was then used as a template in PCR to amplify the 3’ end using adaptor primer AP1 and gene specific primer KDR S03 (Table 7.1). The 5’ end was amplified using adaptor primer AP1 and gene specific primer KDR AS 34 (Table 7.1). PCR conditions were 35 cycles of 94°C for 1 min, 58°C for 1 min, and 68°C for 4min.

7.2.4 DNA sequencing

The RT-PCR and RACE-PCR products were cloned into PCR™ 2.1 TA cloning vector (Invitrogen). All clones were sequenced by primer walking (Genomic and

Sequencing Lab of Auburn University). Sequence analyses of sodium channel gene fragments were repeated at least three times with different preparations of mRNAs. In addition, PCR products from three different preparations of mRNAs were directly sequenced once each.

7.2.5 Amplification and SNP determination for the mutations in individual mosquitoes, Cx. quinquefasciatus

Thirty individual mosquitoes for each population of S-Lab, HAmCq^{G0} and HAmCq^{G8}, had their total RNA extracted. Individual first strand cDNAs were synthesized with SuperScript II reverse transcriptase (Invitrogen) and an antisense 5'-anchored oligo(dT) primer (5' TAATACGACTCACTATAGGGAGATTTTTTTTTTTT TTTT 3') (Tomita and Scott, 1995) using the total RNAs as templates. Three pairs of primers were designed for amplifying three sodium channel cDNA fragments from all individual mosquitoes (Fig. 7.1). Fragment 1 was amplified using primers KDR S16 and KDR AS34 (Table 7.1); fragment 2 was generated using primers PG_KDR S4 and KDR AS02 (Table 7.1); and fragment 3 was amplified using primers KDR S03 and KDR AS09 (Table 7.1). The PCR reactions with cDNA template and a primer pair were heated to 94°C for 2 min, followed by 40 cycles of PCR reaction (94°C for 45 s, 60°C for 45 s and 72°C for 3 min) and a final extension of 72°C for 10 min. Mutations or single nucleotide polymorphisms (SNPs) among the three *Culex* mosquito stains of S-Lab, HAmCq^{G0} and HAmCq^{G8} were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (A&B Applied

Biosystems). Primers for SNP determination were designed according to the sequence immediately upstream of identified mutations. The primer Cx_SNP4 (Table 7.1) was used to detect the SNP in the fragment 1 of three populations; primers Cx_SNP13-16 (Table 7.1) were used to detect SNPs in the fragment 2 of three populations; and primers Cx_SNP6, 18, 19 (Table 7.1) were used to detect SNPs in the fragment 3.

7.3 Results

7.3.1 Characterization of the sodium channel gene in Cx. quinquefasciatus

In this study, an entire coding region sequence of *Cx. quinquefasciatus* sodium channel gene was obtained using a combination of RT-PCR and 3' and 5' RACE. A ~2 kb cDNA fragment 2 (Fig. 7.1) of sodium channel gene was amplified by RT-PCR using primers PG KDR S4 and KDR AS02 designed from the partial sequence of the sodium channel gene of *Cx. quinquefasciatus* (Xu et al., 2006a). Sequence analyses indicated that this fragment encoded the middle section of the sodium channel. Subsequently, a ~2kb fragment and a ~2.5 kb fragment were generated by 3' and 5' RACE, respectively, using primer pairs of KDR S03/AP1 (the adaptor primer) and AP1/ KDR AS34. KDR S03 and KDR AS34 were designed according to the 3' and 5' end sequences of fragment 2 (Fig. 7.1). In total, the full length of the *Cx. quinquefasciatus* sodium channel cDNA contains an open reading frame (ORF) of 6303 nucleotides encoding 2101 amino acids (Fig. 7.2), with a putative protein molecular mass of 235 kDa. The first ATG is proposed to be the start codon because it is the first in-frame methionine codon in the ORF that yields a sodium channel-like amino acid sequence. A putative stop codon (TGA) is located at nucleotide 6304. The deduced amino acid sequence of the *Cx. quinquefasciatus* sodium

channel gene has high similarity with other sodium channel proteins: approximately 81% overall identity with the corresponding sequence of the fruit fly, *Drosophila melanogaster* (Loughney et al., 1989), 81% identity with the house fly, *Musca domestica* (Williamson et al., 1996), 78% identity with the German cockroach, *Blattella germanica* (Dong, 1997) and 57% identity with the varroa mite, *Varroa destructor* (Wang et al., 2003).

7.3.2 Comparison of Cx. quinquefasciatus *sodium channel gene nucleotide and amino acid sequences among HAmCq^{G8}, HAmCq^{G0} and S-Lab strains*

The study of Xu et al. (2006a) demonstrated a strong correlation between *kdr* allelic expression and levels of pyrethroid resistance in *Culex* mosquitoes, indicating that the L to F *kdr* mutation plays a crucial role in resistance. Besides the *kdr* mutation, more than 20 sodium channel mutations have been identified as being involved in reducing channel sensitivity to insecticides or neurotoxins in insects (Soderlund and Kinpple 2003). To identify other possible mutations in mosquito sodium channel gene associated with pyrethroid resistance in *Cx. quinquefasciatus*, the full length sodium channel cDNA was isolated from HAmCq^{G8} (3100-fold resistance to permethrin), HAmCq^{G0} (100-fold) and S-Lab (susceptible strain). cDNAs and deduced amino acid sequences of the sodium channel gene were compared among the three strains (Fig. 7.2). As expected, the L to F *kdr* mutation at nt 3081 (Fig. 7.2) correlated with levels of resistance was identified in both HAmCq^{G8} and HAmCq^{G8} strains, which was consistent with previous results (Xu et al., 2006a). Comparison of the sodium channel genes among these 3 strains also revealed two other mutations, besides the *kdr* mutation, which were corresponded with levels of

resistance. One nucleotide polymorphism of G to T at nt 325 (G in S-Lab, T in HAmCq^{G0}, and T in HAmCq^{G8}) resulted in an amino acid change from alanine^{109} (in S-Lab) to serine^{109} (in HAmCq^{G0}and HAmCq^{G8}) (Fig. 7.2). The other nucleotide polymorphism of T to C at nt 4774 (T in S-Lab, C+T (Y) in HAmCq^{G0}, and C in HAmCq^{G8}) resulted in an amino acid substitution of tryptophan^{1592} (in S-Lab) to arginine^{1592} (in HAmCq^{G8}) and both arginine^{1592} and tryptophan^{1592} (in HAmCq^{G0}) (Fig. 7.2). Interestingly, besides these three mutations, seven silent mutations (nucleotide changes that did not result in amino acid change) showed a correlation with the levels of resistance in these 3 *Culex* mosquito strains. These silent mutations were located at nt 2691 (A in HAmCq^{G8}, A+G (R) in HAmCq^{G0}, and G in S-Lab), nt 2808 (A in HAmCq^{G8}, A+C (M) in HAmCq^{G0}, and C in S-Lab), nt 3780 (G in HAmCq^{G8} and HAmCq^{G0}, and A in S-Lab), nt 3792 (T in HAmCq^{G8} and HAmCq^{G0}, and C in S-Lab), nt 3804 (A in HAmCq^{G8} and HAmCq^{G0}, and G in S-Lab), nt 5256 (G in HAmCq^{G8} and HAmCq^{G0}, and A in S-Lab), and nt 5439 (G in HAmCq^{G8}, G+C (S) in HAmCq^{G0}, and C in S-Lab) (Fig. 7.2). Although the levels of resistance to pyrethroids were very high in HAmCq^{G8} and HAmCq^{G0} mosquitoes, the *super-kdr* mutation documented in house flies and horn flies (Williamson et al., 1996; Guerrero et al., 1997) was not found in these two strains.

7.3.3 Two mutations associated with pyrethroid resistance in Cx. quinquefasciatus

To further characterize correlation of mutations with pyrethroid resistance in *Cx. quinquefasciatus*, I first investigated the polymorphism of G to T at nt 325 that resulted in an amino acid change from alanine^{109} to serine^{109} in 30 individuals of each mosquito population using the SNP determination method. There was a correlation between the

allelic expression at nt 325 and levels of pyrethroid resistance in *Culex* mosquitoes. In the susceptible S-Lab strain, 19/30 (63%) individuals expressed the allele G generating a codon encoding alanine, and 11/30 (37%) individuals expressed the both allele G and T (Table 7.2). In contrast, in the resistant HAmCq^{G0} and HAmCq^{G8} strains, 30/30 (100%) individuals showed expression of the T allele, resulting in an amino acid change to serine (Table 7.2, Fig. 7.2). This alanine^{109} to serine^{109} mutation might be responsible for the low level of pyrethoid resistance because all the individuals in both intermediately resistant strain HAmCq^{G0} and highly resistant strain HAmCq^{G8} expressed the resistant allele T. I then examined the polymorphism of T to C at nt 4774 that resulted in an amino acid substitution of tryptophan^{1592} to arginine^{1592}. All individuals in the susceptible S-Lab strain expressed the allele T, producing a codon encoding tryptophan. In contrast, 15/30 (50%) individuals in the highly resistant HAmCq^{G8} strain expressed allele C, resulting in a change to arginine (Table 7.2, Fig. 7.2); 20% (6/30) individuals expressed susceptible allele T; and 30% (9/30) expressed both alleles in HAmCq^{G8} mosquitoes (Table 7.2). An intermediate level of expression was found in HAmCq^{G0} mosquitoes with 5/30 (17%) individual expressed allele C, (Table 7.2); 57% (17/30) individuals expressed susceptible allele T; and 26% (8/30) expressed both alleles in HAmCq^{G0} mosquitoes (Table 7.2), which was corresponded to their intermediate level of resistance. The high frequency of expression of C allele in the highly resistant strain (3100-fold) of HAmCq^{G8} suggests that this tryptophan^{1592} to arginine^{1592} mutation might be involved in the observed high level of pyrethoid resistance.

7.3.4 Seven silent mutations associated with pyrethroid resistance in Cx. quinquefasciatus

Allelic expression was then examined at the mRNA level in 30 individuals of each mosquito population for the seven silent mutations, which were found from the comparison of three sodium channel genes among HAmCq^{G8}, HAmCq^{G0} and S-Lab. Interestingly, SNP determination results showed strong correlations of these 7 silent mutations and levels of pyrethroid resistance in *Cx. quinquefasciatus* (Table 7.2). Some of these silent mutations were associated with the high level of pyrethroid resistance, such as mutations at nt 2691 and 2808. At nt 2691, all individuals in the S-Lab population expressed susceptible allele G while all HAmCq^{G8} individuals expressed resistant allele A (Table 7.2). However, in the field population HAmCq^{G0}, none of them expressed resistant allele A (Table 7.2). A similar situation was also found for the mutation of nt 2808 (Table 7.2). These results strongly suggest that the silent mutations at nt 2691 and 2808 might contribute to the pyrethroid resistance generated after eight generations of permethrin selection in the laboratory. The mutation at nt 5256 might also be important to low levels of pyrethroid resistance. In the susceptible strain S-Lab, 18/30 (60%) individuals transcribed allele A, and 12/30 (40%) individual expressed both allele A and G at nt 5256 (Table 7.2). In contrast, all the individual mosquitoes in HAmCq^{G8} and HAmCq^{G0} expressed only resistant allele G (Table 7.2). These data suggest that the silent mutation at nt 5256 may be involved in relatively low resistance in the field mosquito population HAmCq^{G0}. Other silent mutations located at nt 3780, 3792, 3804 and 5439 also showed a strong correspondence of the RNA allelic expression and levels of resistance in *Culex* mosquitoes (Table 7.2). Unlike the expression of mutations at nt

3792, 3804 and 5439 in these populations (Table 7.2), no individual expressed both alleles at nt 3780 in all three strains. It was found that all individuals in the S-Lab expressed the A allele and all individuals in HAmCq^{G8} expressed the G allele, whereas 33% (10/30) individuals expressed A allele and 67% (20/30) individuals expressed G allele in HAmCq^{G0} strain.

7.4 Discussion

This study is the first time to isolate a first full-length mosquito sodium channel gene and systematically characterized mutations among three mosquito strains ranging from susceptible to highly resistant. The *Cx. quinquefasciatus* sodium channel gene shows high similarity to other insects' sodium channel genes, such as fruit flies (Loughney et al., 1989), house flies (Williamson et al., 1996; Ingles et al., 1996) and German cockroaches (Dong, 1997). Comparison of resistant and susceptible sodium channel genes in *Cx. quinquefasciatus* revealed three mutations that result in amino acid substitutions. Besides the well known L to F *kdr* mutation at nt 3081, which has been documented to be associated with pyrethroid resistance (Xu et al., 2006a), two other mutations of alanine^{109} to serine^{109} and tryptophan^{1592} to arginine^{1592} were identified to be correlated with the levels of pyrethroid susceptibility or resistance among 3 mosquito populations of S-Lab, HAmCq^{G0}, and HAmCq^{G8}.

Although silent mutations in sodium channel genes have been found in several pyrethroid-resistant insects, such as house flies (Ingles et al., 1996) and German cockroaches (Dong, 1997; Pridgeon et al., 2002), they were assumed to exert no discernible effect on the sodium channel gene function or resistant phenotype because

these silent mutations do not change the amino acid composition of the protein product. However, according to recent research, such mutations or SNPs are not always silent. For example, these mutations can lead to inactivation of the native splicing donor site, which results in a premature stop codon or exon skipping, yielding a shorter mRNA (Thi Tran et al., 2005; Adachi et al., 2003). Silent mutations can also affect levels of gene expression as described by Wang et al. (2005). Recently, Kimchi-Sarfaty et al. (2007) provide strong evidence that silent mutations or SNPs in the mammalian membrane transport protein gene *MDR1* can affect *in vivo* protein folding and, consequently, function. In this study, it is demonstrated, for the first time, that the allelic expression of seven silent mutations in the mosquito sodium channel gene is strongly correlated with levels of insecticide susceptibility or resistance in the mosquito populations of S-Lab (susceptible phenotype), $HAmCq^{G0}$ (intermediately resistant phenotype) and $HAmCq^{G8}$ (highly resistant phenotype). An important question arising from this study is the role these silent mutations in the function of sodium channel genes. Comparison of mosquito resistant and susceptible sodium channel genes did not identify a premature stop codon or exon skipping (Fig. 7.2), indicating that these seven silent mutations might not have these effects. Recent studies suggest that codon usage is not randomly assigned in prokaryote organisms (Supek and Vlahovicek, 2005). The timing of co-translational folding is affected by silent mutations and might result in altered function when frequent codons are changed to rare ones (Anthony and Skach, 2002; Kimchi-Sarfaty et al., 2007). The silent mutations in the mosquito sodium channel gene may play similar roles as described above and differentiate the functions between the resistant and susceptible sodium channel proteins. It would be very interesting to investigate the sensitivity of the *Cx.*

quinquefasciatus sodium channel that carries these mutations (including silent ones) to pyrethroid insecticides in the *Xenopus* oocyte expression system as described by Tan et al. (2002).

In conclusion, this study, for the first time, provides a mosquito full-length sodium channel gene. The availability of the full-length sodium channel gene will make it possible to successfully express the gene in *Xenopus* oocytes and to demonstrate its functional difference. Besides the L to F *kdr* mutation, another two mutations in the sodium channel are documented in the *Cx. quinquefasciatus*. These will help to elucidate the molecular basis of pyrethroid resistance in mosquitoes. More significantly, by demonstrating the correlation between allelic expression of silent mutations and levels of pyrethroid resistance in *Culex* mosquitoes, this study opens up a new avenue of insecticide resistance research and suggests that silent mutations may contribute to the insensitivity of sodium channels to pyrethroid insecticides. In addition, point mutations associated with target site insensitivity also have been well documented for *Ace*-encoded acetylcholinesterase (Weill et al., 2003; Hemingway et al., 2004), the target of organophosphates (OPs) and carbamates, two large groups of insecticides, and the *Rdl*-encoded ligand gated chloride channel (ffrench-Constant et al., 1993). It should now be feasible to screen silent mutations in *Ace* and *Rdl* genes toward understanding their significance in insecticide resistance in general. Characterization of silent mutations in the sodium channel, *Ace* and *Rdl* genes may offer new approaches to the study of resistance and provide fresh information with which to combat the development of insecticide resistance, a serious problem now affecting the control of medically, agriculturally, and economically important insect pest world wide.

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Table 7.1 Oligonucleotide primers[a] used for amplifying the sodium channel cDNA fragments and for detecting SNP (single nucleotide polymorphism) from the mosquito, *Cx. quinquefasciatus*

Primer name	Nucleotide sequence (5' to 3')
Primers used to do RT-PCR and RACE	
KDR S16	TGTTGGCCATATAGACAATGACCGA
KDR AS34	GTAATACTGACAATCCCTGAACGC
PG_KDR S4	GCGGTAACTACTTCTTCACGGC
KDR AS02	CCAKCCYTTRAAKGTGGCYACTTG
KDR S03	TGAACTTYGACCACGTGGGG
KDR AS09	GCTTCTGAATCTGAATCAGAGGGAG
AP1	CCATCCTAATACGACTCACTATAGGGC
Primers used to do SNP determination	
Cx_SNP 4	CTCGAGGATATTGACGCTTTTTAC
Cx_SNP 6	TGAAGGCCATTCCGCGGCCCAAG
Cx_SNP 13	TCCATCATGGGCCGAACGATGGG
Cx_SNP 14	AACTGCTACAAGCGGTTCCCGGC
Cx_SNP 15	GGTTCCCGGCRCTGGCCGGCGA
Cx_SNP 16	TGGCCGGCGAYGACGACGCGCC
Cx_SNP 18	ATGTTCATCTTCGCCATCTTCGG
Cx_SNP 19	CGGATAACGACAAGGGTTACCC

[a]Designation of oligonucleotide mixtures: R = A+G; Y = C+T; K = G + T.

Table 7.2 The single nucleotide polymorphisms (SNPs) in mosquitoes, *Cx. quinquefasciatus* at mRNA level

Locus	Strain	n[a]	Phenotype	SNPs (total individuals)		
Mutations						
nt 325	S-Lab	30	Susceptible	G (19)	G/T (11)	T (0)
	HAmCq^{G0}	30	100-fold resistance	G (0)	G/T (0)	T (30)
	HAmCq^{G8}	30	3,100-fold resistance	G (0)	G/T (0)	T (30)
nt 4774	S-Lab	30	Susceptible	T (30)	T/C (0)	C (0)
	HAmCq^{G0}	30	100-fold resistance	T (17)	T/C (8)	C (5)
	HAmCq^{G8}	30	3,100-fold resistance	T (6)	T/C (9)	C (15)
Silent mutations						
nt 2691	S-Lab	30	Susceptible	G (30)	G/A (0)	A (0)
	HAmCq^{G0}	30	100-fold resistance	G (20)	G/A (10)	A (0)
	HAmCq^{G8}	30	3,100-fold resistance	G (0)	G/A (0)	A (30)
nt 2808	S-Lab	30	Susceptible	C (30)	C/A (0)	A (0)
	HAmCq^{G0}	30	100-fold resistance	C (19)	C/A (11)	A (0)
	HAmCq^{G8}	30	3,100-fold resistance	C (0)	C/A (0)	A (30)
nt 3780	S-Lab	30	Susceptible	A (30)	A/G (0)	G (0)
	HAmCq^{G0}	30	100-fold resistance	A (10)	A/G (0)	G (20)
	HAmCq^{G8}	30	3,100-fold resistance	A (0)	A/G (0)	G (30)
nt 3792	S-Lab	30	Susceptible	C (30)	C/T (0)	T (0)
	HAmCq^{G0}	30	100-fold resistance	C (12)	C/T (9)	T (9)
	HAmCq^{G8}	30	3,100-fold resistance	C (0)	C/T (0)	T (30)
nt 3804	S-Lab	30	Susceptible	G (30)	G/A (0)	A (0)
	HAmCq^{G0}	30	100-fold resistance	G (6)	G/A (12)	A (12)
	HAmCq^{G8}	30	3,100-fold resistance	G (0)	G/A (0)	A (30)
nt 5256	S-Lab	30	Susceptible	A (18)	A/G (12)	G (0)
	HAmCq^{G0}	30	100-fold resistance	A (0)	A/G (0)	G (30)
	HAmCq^{G8}	30	3,100-fold resistance	A (0)	A/G (0)	G (30)
nt 5439	S-Lab	30	Susceptible	C (27)	C/G (3)	G (0)
	HAmCq^{G0}	30	100-fold resistance	C (0)	C/G (9)	G (21)
	HAmCq^{G8}	30	3,100-fold resistance	C (0)	C/G (2)	G (28)

^{G0}The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

[a] The total number of tested insects (three replicates for each of 5 males and 5 females)

FIGURE LEGENDS

Fig. 7.1 Amplification of *Cx. quinquefasciatus* cDNA fragments. Top: a diagram of *Cx. quinquefasciatus* sodium channel protein showing the four homologous domains (I-IV), each divided into six transmembrane segments (S1–S6). Fragment 1, 2, and 3 were isolated by RT–PCR. The size of each fragment is indicated.

Fig. 7.2 Alignment of the cDNA/deduced amino acid sequence of the full length *Cx. quinquefasciatus* sodium channel gene from HAmCq^{G8}, HAmCq^{G0} and S-Lab. Numbering is according to nucleotide sequence. The nucleotide changes and corresponding amino acid substitutions are in shade face. Silent mutations, only nucleotide changes, are in bold face. Identical sequences are indicated as dashes (-). Designation of nucleotides: R = A+G; M = A+C; W = A+T; Y = C+T; S = G + C.

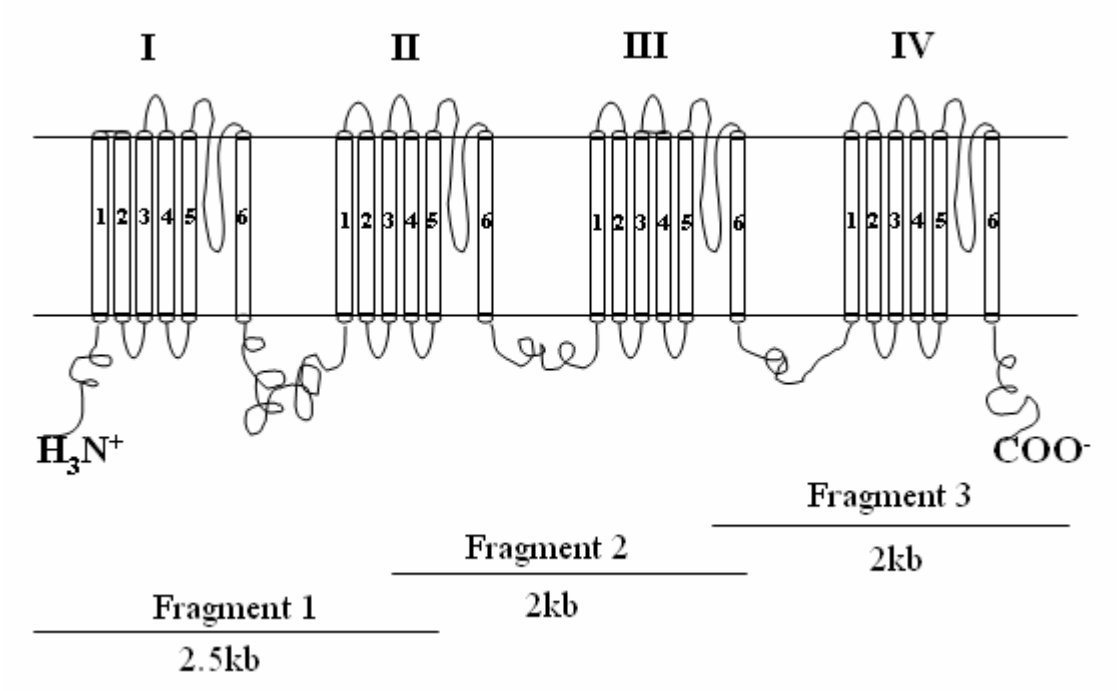
I
II
III
IV
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
H3N+
COO-
Fragment 3
2kb
Fragment 2
2kb
Fragment 1
2.5kb

Fig. 7.1

```
ATGACCGAAGACCTCGATTCGATATCTGAGGAAGAACGTAGTTTGTTCCGTCCTTTCACC      60    HAmCq^G8
M  T  E  D  L  D  S  I  S  E  E  E  R  S  L  F  R  P  F  T

CGTGAATCATTACTTGTTATTGAAGAACGCATCGCAAATGAACAGGCAAAACAGCGCGAA     120
R  E  S  L  L  V  I  E  E  R  I  A  N  E  Q  A  K  Q  R  E

TTGGAAAAGAAGCGAGCCGAGGGGGAGACTGGTTTTGGTCGGAAGAAAAAGAAAAAAGAA     180
L  E  K  K  R  A  E  G  E  T  G  F  G  R  K  K  K  K  K  E

ATACGGTACGACGATGAGGACGAGGATGAAGGTCCCCAGCCGGACTCCACACTCGAGCAG     240
I  R  Y  D  D  E  D  E  D  E  G  P  Q  P  D  S  T  L  E  Q

GGAGTGCCGATCCCTGTTCGAATGCAGGGCAGCTTCCCTCCGGAATTGGCCTCCACGCCT     300
G  V  P  I  P  V  R  M  Q  G  S  F  P  P  E  L  A  S  T  P

CTCGAGGATATTGACGCTTTTTACTCAAACATTAAGACATTCGTAGTAGTTAGTAAAGGA     360    HAmCq^G8
------------------------T-----------------------------------     HAmCq^G0
------------------------G-----------------------------------     S-Lab
L  E  D  I  D  A  F  Y  S  N  I  K  T  F  V  V  V  S  K  G       HAmCq^G8
-  -  -  -  -  -  -  -  S  -  -  -  -  -  -  -  -  -  -  -       HAmCq^G0
-  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -       S-Lab

AAGGATATTTTTCGTTTCTCCGCAACCAATGCATTATATGTACTCGATCCGTTCAATCCT     420
K  D  I  F  R  F  S  A  T  N  A  L  Y  V  L  D  P  F  N  P

ATACGTCGTGTAGCTATTTATATTTTAGTACATCCATTATTTTCATTTTTTATAATAACA     480
I  R  R  V  A  I  Y  I  L  V  H  P  L  F  S  F  F  I  I  T

ACCATTCTTGGTAATTGTATTTTGATGATCATGCCATCCACGCCGACAGTCGAATCTACC     540
T  I  L  G  N  C  I  L  M  I  M  P  S  T  P  T  V  E  S  T

GAGGTGATATTCACCGGCATCTACACGTTCGAATCAGCTGTAAAAGTGATGGCGCGAGGT     600
E  V  I  F  T  G  I  Y  T  F  E  S  A  V  K  V  M  A  R  G

TTCATATTACAACCGTTTACTTATCTTAGAGATGCATGGAATTGGTTGGACTTCGTAGTA     660
F  I  L  Q  P  F  T  Y  L  R  D  A  W  N  W  L  D  F  V  V

ATAGCATTAGCATATGTAACTATGGGTATAGATTTGGGTAATCTCGCTGCATTGAGAACA     720
I  A  L  A  Y  V  T  M  G  I  D  L  G  N  L  A  A  L  R  T

TTCAGGGTACTACGAGCTCTCAAAACAGTGGCCATCGTTCCAGGTCTCAAGACCATCGTC     780
F  R  V  L  R  A  L  K  T  V  A  I  V  P  G  L  K  T  I  V

GGCGCTGTCATCGAGTCCGTAAAGAATCTCAGAGATGTGATAATTTTAACAATGTTTTCG     840
G  A  V  I  E  S  V  K  N  L  R  D  V  I  I  L  T  M  F  S

TTGTCGGTGTTTGCTTTAATGGGGCTGCAGATCTACATGGGCGTGCTGACGCAAAAGTGC     900
L  S  V  F  A  L  M  G  L  Q  I  Y  M  G  V  L  T  Q  K  C

ATCAAGGAGTTCCCGACGGACGGCTCGTGGGGCAACCTGACCCACGAGAACTGGGAGCGG     960
I  K  E  F  P  T  D  G  S  W  G  N  L  T  H  E  N  W  E  R

CACCATTCGAACGATTCCAATTGGTACTTTTCCGAAACCGGGGACACGCCCCTCTGCGGC    1020
H  H  S  N  D  S  N  W  Y  F  S  E  T  G  D  T  P  L  C  G

AATTCGTCGGGTGCTGGCCAATGTGAGGAAGGATATGTATGTTTACAAGGTTTTGGAGAT    1080
N  S  S  G  A  G  Q  C  E  E  G  Y  V  C  L  Q  G  F  G  D

AATCCAAATTACGGGTATACAAGTTTTGATACTTTCGGATGGGCATTCTTATCTGCCTTT    1140
N  P  N  Y  G  Y  T  S  F  D  T  F  G  W  A  F  L  S  A  F

CGTCTCATGACCCAGGACTACTGGGAGAATTTATATCAACTGGTGTTACGATCAGCTGGA    1200
R  L  M  T  Q  D  Y  W  E  N  L  Y  Q  L  V  L  R  S  A  G

CCGTGGCACATGCTCTTCTTCATTGTGATTATCTTCTTGGGTTCGTTCTACCTTGTAAAT    1260
P  W  H  M  L  F  F  I  V  I  I  F  L  G  S  F  Y  L  V  N

TTGATCTTGGCCATTGTCGCCATGTCGTACGACGAACTCCAGAAGAGGGCCGAAGAGGAG    1320
L  I  L  A  I  V  A  M  S  Y  D  E  L  Q  K  R  A  E  E  E
```

```
GAGGCCGCCGAGGAAGAAGCGCTTCGGGAAGCGGAAGAAGCGGCCGCAGCGAAACAGGCC    1380
E  A  A  E  E  E  A  L  R  E  A  E  E  A  A  A  A  K  Q  A

AAACTCGAGGCCCACGCAGCGGCGGCGGCGGCCGCGGCCAACCCGGAGATCGCCAAGAGC    1440
K  L  E  A  H  A  A  A  A  A  A  A  A  A  N  P  E  I  A  K  S

CCGTCGGACTTTTCCTGCCACAGTTACGAGCTGTTCGTGGGCCAGGAGAAGGGCAACGAC    1500
P  S  D  F  S  C  H  S  Y  E  L  F  V  G  Q  E  K  G  N  D

GACAACAACAAGGAGAAGATGTCGATCCGGAGCGAAGGATTGGAGTCGGTGAGCGAAATC    1560
D  N  N  K  E  K  M  S  I  R  S  E  G  L  E  S  V  S  E  I

ACAAGAACAACCGCACCAACAGCTACTGCAGCTGGCACTGCAAAAGCCCGTAAAGTGAGC    1620
T  R  T  T  A  P  T  A  T  A  A  G  T  A  K  A  R  K  V  S

GCGGCTTCACTTTCATTACCTGGTTCACCATTTAATCTTCGTAGAGGATCTAGAGGATCA    1680
A  A  S  L  S  L  P  G  S  P  F  N  L  R  R  G  S  R  G  S

CATCAGTTTACGATACGTAACGGTAGAGGACGTTTCGTGGGCGTACCTGGTAGCGATAGA    1740
H  Q  F  T  I  R  N  G  R  G  R  F  V  G  V  P  G  S  D  R

AAACCATTGGTACTCTCAACATATCTCGATGCACAAGAACACTTGCCATACGCCGATGAC    1800
K  P  L  V  L  S  T  Y  L  D  A  Q  E  H  L  P  Y  A  D  D

TCGAACGCGGTCACACCGATGTCGGAGGAAAATGGTGCAATCATCGTTCCAGTATACTAT    1860
S  N  A  V  T  P  M  S  E  E  N  G  A  I  I  V  P  V  Y  Y

GCTAATTTAGGTTCGCGACACTCATCGTACACATCGCATCAATCGCGCATCTCGTACACA    1920
A  N  L  G  S  R  H  S  S  Y  T  S  H  Q  S  R  I  S  Y  T

TCGCACGGCGACCTGCTCGGCGGCATGACGAAGGAGAGCCGGCTGCGGAGCCGAACCCAG    1980
S  H  G  D  L  L  G  G  M  T  K  E  S  R  L  R  S  R  T  Q

CGCAACACGAACCACTCGATCGTGCCGCCGGCGAACATGGCGGCCTCGGCGGCGTCGGTG    2040
R  N  T  N  H  S  I  V  P  P  A  N  M  A  A  S  A  A  S  V

ACGGGTGCCGGCTCGGGCGCGCCCAACATGTCCTACGTCGACACCAACCACAAGGGCCAG    2100
T  G  A  G  S  G  A  P  N  M  S  Y  V  D  T  N  H  K  G  Q

CAGCGGGACTTTGATCAGTCCCAAGACTACACAGATGATGCTGGTAAAATAAAACACAAC    2160
Q  R  D  F  D  Q  S  Q  D  Y  T  D  D  A  G  K  I  K  H  N

GACAATCCTTTCATCGAGCCCTCTCAAACCCAAACCGTAGTAGATATGAAAGACGTAATG    2220
D  N  P  F  I  E  P  S  Q  T  Q  T  V  V  D  M  K  D  V  M

GTGTTAAACGATATCATTGAGCAAGCTGCTGGTCGGCATAGTAGAGCTAGTGATCATGGA    2280
V  L  N  D  I  I  E  Q  A  A  G  R  H  S  R  A  S  D  H  G

GTCTCTGTTTACTACTTCCCCACAGAGGACGACGACGAGGACGGTCCGACGTTCAAGGAC    2340
V  S  V  Y  Y  F  P  T  E  D  D  D  E  D  G  P  T  F  K  D

AAGGCGGTCGAGTTCGGGATGCGGATGATCGACATCTTCTGCGTGTGGGACTGCTGCTGG    2400
K  A  V  E  F  G  M  R  M  I  D  I  F  C  V  W  D  C  C  W

GTGTGGCTCAAGTTCCAGGAGTGGGTGTCCTTTATCGTGTTCGACCCGTTCGTCGAGCTG    2460
V  W  L  K  F  Q  E  W  V  S  F  I  V  F  D  P  F  V  E  L

TTCATCACGCTCTGCATCGTGGTCAACACGCTGTTCATGGCGCTCGACCACCACGACATG    2520
F  I  T  L  C  I  V  V  N  T  L  F  M  A  L  D  H  H  D  M

AACCCGGACATGGAGCGGGCGCTCAAGAGCGGTAACTACTTCTTCACGGCGACGTTCGCG    2580
N  P  D  M  E  R  A  L  K  S  G  N  Y  F  F  T  A  T  F  A

ATCGAAGCGACGATGAAGCTGATCGCGATGAGCCCCAAGTGGTACTTCCAGGAAGGTTGG    2640
I  E  A  T  M  K  L  I  A  M  S  P  K  W  Y  F  Q  E  G  W

AACATTTTCGATTTCATCATCGTGGCCCTTTCGCTGCTCGAGCTCGGTCTAGAGGGCGTT    2700    HAmCq^G8
----------------------------------------------R---------              HAmCq^G0
----------------------------------------------G---------              S-Lab
N  I  F  D  F  I  I  V  A  L  S  L  L  E  L  G  L  E  G  V
```

```
CAGGGATTGTCAGTATTACGTTCATTCCGTTTGCTTCGAGTGTTCAAGCTAGCAAAGTCG    2760
Q  G  L  S  V  L  R  S  F  R  L  L  R  V  F  K  L  A  K  S

TGGCCAACGCTGAACTTACTCATTTCCATCATGGGCCGAACGATGGGAGCGTTAGGTAAT    2820    HAmCq^G8
----------------------------------------M-----------           HAmCq^G0
----------------------------------------C-----------           S-Lab
W  P  T  L  N  L  L  I  S  I  M  G  R  T  M  G  A  L  G  N

CTGACGTTTGTGCTCTGCATTATCATCTTCATCTTTGCCGTGATGGGGATGCAGCTGTTC    2880
L  T  F  V  L  C  I  I  I  F  I  F  A  V  M  G  M  Q  L  F

GGCAAGAACTACATCGACAACGTGGACCGCTTCCCGGACAAGGACCTGCCACGGTGGAAC    2940
G  K  N  Y  I  D  N  V  D  R  F  P  D  K  D  L  P  R  W  N

TTCACCGACTTCATGCACTCATTCATGATCGTGTTCCGGGTGCTGTGCGGCGAGTGGATC    3000
F  T  D  F  M  H  S  F  M  I  V  F  R  V  L  C  G  E  W  I

GAATCCATGTGGGACTGCATGCTGGTGGGCGACGTGTCCTGCATTCCGTTCTTCTTGGCC    3060
E  S  M  W  D  C  M  L  V  G  D  V  S  C  I  P  F  F  L  A

ACCGTAGTGATAGGAAATTTTGTCGTTCTTAACCTTTTCTTAGCCTTGCTTTTGTCCAAC    3120    HAmCq^G8
--------------------W-----------------------------------------           HAmCq^G0
--------------------A-----------------------------------------           S-Lab
T  V  V  I  G  N  F  V  V  L  N  L  F  L  A  L  L  L  S  N              HAmCq^G8
-  -  -  -  -  -  L/F -  -  -  -  -  -  -  -  -  -  -  -  -              HAmCq^G0
-  -  -  -  -  -  L  -  -  -  -  -  -  -  -  -  -  -  -  -              S-Lab
TTTGGTTCCTCGAGTTTGTCGGCGCCCACAGCCGACAACGAAACGAACAAGATCGCCGAG    3180
F  G  S  S  S  L  S  A  P  T  A  D  N  E  T  N  K  I  A  E

GCGTTCAACCGGATATCGCGCTTCTCCAACTGGATCAAGGCGAACATCGCGGCCGCGCTC    3240
A  F  N  R  I  S  R  F  S  N  W  I  K  A  N  I  A  A  A  L

AAGTTCGTGAAAAACAAGTTAACAAGCCAGATTGCGTCCGTGCAGCCCGCAGGCAAAGGG    3300
K  F  V  K  N  K  L  T  S  Q  I  A  S  V  Q  P  A  G  K  G

GTATGTCCATGTATATCTGCAGAGCATGGTGAAAATGAGCTGGAATTAACTCCAGATGAC    3360
V  C  P  C  I  S  A  E  H  G  E  N  E  L  E  L  T  P  D  D

ATCCTGGCCGACGGGCTGCTGAAAAAGGGCGTCAAGGAGCACAACCAGCTGGAGGTGGCG    3420
I  L  A  D  G  L  L  K  K  G  V  K  E  H  N  Q  L  E  V  A

ATCGGCGACGGGATGGAGTTTACGATACACGGCGACCTCAAGAACAAGGGCAAGAAGAAC    3480
I  G  D  G  M  E  F  T  I  H  G  D  L  K  N  K  G  K  K  N

AAGCAGCTGATGAACAATTCCAAGGACGATGATACTGCCAGTATAAAGTCCTATGGCAGT    3540
K  Q  L  M  N  N  S  K  D  D  D  T  A  S  I  K  S  Y  G  S

CACAAGAATCGCCCCTTCAAGGACGAAAGCCACAAGGGCAGTGCCGAAACGCTGGAGGGC    3600
H  K  N  R  P  F  K  D  E  S  H  K  G  S  A  E  T  L  E  G

GAAGAAAAGCGCGACGCCAGCAAGGAGGACCTAGGAATTGACGAAGAACTCGACGACGAG    3660
E  E  K  R  D  A  S  K  E  D  L  G  I  D  E  E  L  D  D  E

TGCGAGGGTGAGGAGGGTCCTCTGGACGGGGAAATGATCATCCACGCGGAAGAGGACGAG    3720
C  E  G  E  E  G  P  L  D  G  E  M  I  I  H  A  E  E  D  E

GTGATCGAGGACGCGCCGGCCGACTGCTTCCCGGACAACTGCTACAAGCGGTTCCCGGCG    3780    HAmCq^G8
----------------------------------------------------------G           HAmCq^G0
----------------------------------------------------------A           S-Lab
V  I  E  D  A  P  A  D  C  F  P  D  N  C  Y  K  R  F  P  A

CTGGCCGGCGATGACGACGCGCCATTCTGGCAGGGCTGGGGCAACCTGCGGCTCAAGACG    3840    HAmCq^G8
-----------T-----------A----------------------------------           HAmCq^G0
-----------C-----------G----------------------------------           S-Lab
L  A  G  D  D  D  A  P  F  W  Q  G  W  G  N  L  R  L  K  T

TTCCAGCTGATCGAGAACAAGTACTTCGAGACGGCCGTCATCACGATGATCCTGCTGAGT    3900
F  Q  L  I  E  N  K  Y  F  E  T  A  V  I  T  M  I  L  L  S

AGTTTGGCCCTGGCCCTCGAGGATGTGCACCTGCCGCACCGACCAATCTTGCAGGACGTC    3960
S  L  A  L  A  L  E  D  V  H  L  P  H  R  P  I  L  Q  D  V
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CTGTACTACATGGACAGGATATTCACGGTGATCTTTTTTTTAGAGATGTTGATCAAGTGG    4020
L  Y  Y  M  D  R  I  F  T  V  I  F  F  L  E  M  L  I  K  W

TTGGCGCTCGGCTTCCGGGTGTACTTTACGAACGCCTGGTGCTGGCTCGATTTCATCATT    4080
L  A  L  G  F  R  V  Y  F  T  N  A  W  C  W  L  D  F  I  I

GTGATGGTGTCCTTAATCAACTTCGTGGCTTCACTCTGTGGAGCGGGTGGTATTCAAGCA    4140
V  M  V  S  L  I  N  F  V  A  S  L  C  G  A  G  G  I  Q  A

TTCAAAACTATGCGAACTCTTAGGGCACTGCGTCCGCTACGTGCCATGTCCCGTATGCAG    4200
F  K  T  M  R  T  L  R  A  L  R  P  L  R  A  M  S  R  M  Q

GGTATGAGGGTTGTCGTCAATGCATTGGTACAGGCTATACCGTCCATCTTCAACGTGTTA    4260
G  M  R  V  V  V  N  A  L  V  Q  A  I  P  S  I  F  N  V  L

TTGGTGTGTTTGATCTTTTGGTTGATTTTCGCCATCATGGGCGTCCAGCTGTTTGCCGGA    4320
L  V  C  L  I  F  W  L  I  F  A  I  M  G  V  Q  L  F  A  G

AAGTACTTCAAGTGCGTCGACACGAACAAGACGACACTGTCGCACGAGATCATCCCGGAC    4380
K  Y  F  K  C  V  D  T  N  K  T  T  L  S  H  E  I  I  P  D

GTGAACGCGTGCATCGCGGAGAACTACACCTGGGAGAACTCCCCGATGAACTTTGACCAC    4440
V  N  A  C  I  A  E  N  Y  T  W  E  N  S  P  M  N  F  D  H

GTGGGGAAGGCCTACCTGTGTTTGTTCCAGGTGGCCACGTTCAAGGGATGGATCCAGATC    4500
V  G  K  A  Y  L  C  L  F  Q  V  A  T  F  K  G  W  I  Q  I

ATGAACGACGCGATCGACTCGCGGGACATCGGAAAGCAGCCCATCCGCGAAACCAACATC    4560
M  N  D  A  I  D  S  R  D  I  G  K  Q  P  I  R  E  T  N  I

TACATGTACTTGTACTTTGTGTTCTTCATCATCTTCGGATCGTTCTTCACGCTGAACCTC    4620
Y  M  Y  L  Y  F  V  F  F  I  I  F  G  S  F  F  T  L  N  L

TTCATCGGTGTCATTATTGACAACTTTAACGAACAGAAGAAGAAGGCTGGGGGATCGCTC    4680
F  I  G  V  I  I  D  N  F  N  E  Q  K  K  K  A  G  G  S  L

GAGATGTTTATGACGGAGGACCAAAAAAAGTACTACAACGCAATGAAGAAGATGGGCTCG    4740
E  M  F  M  T  E  D  Q  K  K  Y  Y  N  A  M  K  K  M  G  S

AAGAAGCCACTGAAGGCCATTCCGCGGCCCAAGCGGCGACCACAAGCAATAGTGTTCGAA    4800   HAmCq^G8
----------------------------------Y----------------------------   HAmCq^G0
----------------------------------T----------------------------   S-Lab
K  K  P  L  K  A  I  P  R  P  K  R  R  P  Q  A  I  V  F  E       HAmCq^G8
-  -  -  -  -  -  -  -  -  -  -  R/W -  -  -  -  -  -  -  -       HAmCq^G0
-  -  -  -  -  -  -  -  -  -  -  W  -  -  -  -  -  -  -  -       S-Lab
ATCTGCACAAACAAAAAAGTTCGACATGATCATCATGTTGTTCATCGGCTTCAACATGTTG    4860
I  C  T  N  K  K  F  D  M  I  I  M  L  F  I  G  F  N  M  L

ACGATGACGCTGGATCACTACAAGCAGACGGAAACGTTCAGCGCGGTGCTGGACTACCTG    4920
T  M  T  L  D  H  Y  K  Q  T  E  T  F  S  A  V  L  D  Y  L

AACATGATCTTCATCTGTATCTTCAGTAGCGAGTGTCTGATGAAGATCTTCGCGCTGCGC    4980
N  M  I  F  I  C  I  F  S  S  E  C  L  M  K  I  F  A  L  R

TACCACTACTTTATCGAACCGTGGAACCTGTTCGATTTCGTCGTCGTCATCCTGTCCATT    5040
Y  H  Y  F  I  E  P  W  N  L  F  D  F  V  V  V  I  L  S  I

TTGGGCCTGGTGCTGAGCGACCTGATCGAAAAGTACTTCGTCTCGCCGACGCTGCTCCGT    5100
L  G  L  V  L  S  D  L  I  E  K  Y  F  V  S  P  T  L  L  R

GTGGTGCGCGTGGCCAAGGTCGGTCGGGTGCTGCGTCTCGTCAAGGGCGCCAAGGGCATC    5160
V  V  R  V  A  K  V  G  R  V  L  R  L  V  K  G  A  K  G  I

CGGACGTTGCTGTTTGCGCTGGCCATGTCGCTGCCGGCGCTGTTCAACATCTGTCTGCTG    5220
R  T  L  L  F  A  L  A  M  S  L  P  A  L  F  N  I  C  L  L

CTGTTCCTGGTGATGTTCATCTTCGCCATCTTCGGGATGTCGTTCTTCATGCACGTGAAG    5280   HAmCq^G8
----------------------------------G----------------------------   HAmCq^G0
----------------------------------A----------------------------   S-Lab
L  F  L  V  M  F  I  F  A  I  F  G  M  S  F  F  M  H  V  K
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GACAAGAGCGGGCTGGACGACGTGTACAACTTCAAGACGTTCGGCCAGAGCATGATCCTG    5340
D  K  S  G  L  D  D  V  Y  N  F  K  T  F  G  Q  S  M  I  L

CTGTTTCAGATGTCAACGTCTGCGGGGTGGGACGGTGTGCTGGATGGTATCATCAACGAG    5400
L  F  Q  M  S  T  S  A  G  W  D  G  V  L  D  G  I  I  N  E

GAGGACTGCCTGCCGCCGGATAACGACAAGGGTTACCCGGGGAACTGCGGGTCGGCGACG    5460    HAmCq^G8
----------------------------------------S---------------------    HAmCq^G0
----------------------------------------C---------------------    S-Lab
E  D  C  L  P  P  D  N  D  K  G  Y  P  G  N  C  G  S  A  T

ATCGGCATCACGTACCTGCTGGCATATCTGGTCATCAGTTTCCTGATCGTTATCAACATG    5520
I  G  I  T  Y  L  L  A  Y  L  V  I  S  F  L  I  V  I  N  M

TACATCGCTGTCATTCTCGAGAATTACTCGCAGGCCACGGAGGACGTGCAGGAGGGTCTG    5580
Y  I  A  V  I  L  E  N  Y  S  Q  A  T  E  D  V  Q  E  G  L

ACGGACGACGACTACGACATGTACTACGAGATCTGGCAGCAGTTCGATCCGGACGGTACG    5640
T  D  D  D  Y  D  M  Y  Y  E  I  W  Q  Q  F  D  P  D  G  T

CAGTACATCCGGTACGACCAGCTGTCGGACTTTTTGGACGTGCTGGAACCGCCGCTGCAG    5700
Q  Y  I  R  Y  D  Q  L  S  D  F  L  D  V  L  E  P  P  L  Q

ATTCACAAACCGAACAAGTACAAGATCATCTCGATGGACATTCCGATCTGTCGCGGCGAC    5760
I  H  K  P  N  K  Y  K  I  I  S  M  D  I  P  I  C  R  G  D

ATGATGTTCTGCGTGGACATTCTGGACGCGCTGACGAAGGACTTCTTCGCGCGGAAGGGC    5820
M  M  F  C  V  D  I  L  D  A  L  T  K  D  F  F  A  R  K  G

AACCCGATCGAGGACAGTGCCGAGATGGGTGAGGTCCAGCAGCGGCCGGACGAGGTCGGT    5880
N  P  I  E  D  S  A  E  M  G  E  V  Q  Q  R  P  D  E  V  G

TACGAGCCGGTTTCGTCGACGTTGTGGCGCCAACGGGAGGAGTACTGCGCGCGGTTGATA    5940
Y  E  P  V  S  S  T  L  W  R  Q  R  E  E  Y  C  A  R  L  I

CAGCACGCGTACCGGAACTTTAAGGAACGAGGCGGTGTTGGTGGCGGCGGCGGCGGTGGA    6000
Q  H  A  Y  R  N  F  K  E  R  G  G  V  G  G  G  G  G  G  G

GGTGGTGGAGGAGGAGGTGGTGGCGAAGGTGCCGGAGATGACACCGACGCCGATGCCTGT    6060
G  G  G  G  G  G  G  G  E  G  A  G  D  D  T  D  A  D  A  C

GATAACGAGCCCGGGATCGGGAGTCCCGGCGCGGTCAGCGGCGGTGGCGGCAGCATCGCC    6120
D  N  E  P  G  I  G  S  P  G  A  V  S  G  G  G  G  S  I  A

GGCGGAGGCTCCCAGGCTAACCTAGGGCCGCCGTCACCCAAAGAATCGCCCGATGGCAAT    6180
G  G  G  S  Q  A  N  L  G  P  P  S  P  K  E  S  P  D  G  N

AATGATCCTCAAGGTCGTCAAACGGCCGTCCTAGTAGAAAGTGATGGATTTGTAACTAAA    6240
N  D  P  Q  G  R  Q  T  A  V  L  V  E  S  D  G  F  V  T  K

AACGGTCACCGTGTCGTGATACACTCACGATCGCCAAGTATAACTTCACGATCGGCGGAT    6300
N  G  H  R  V  V  I  H  S  R  S  P  S  I  T  S  R  S  A  D

GTCTGAGCCAGGCCTCGCCCCCCTCCCTCTGATTCAGATTCAGAAGCACGACAGAAATAA    6360
V  *

TATTTTAAAGATTAAGAAAAATACTTAAAAAACAAAACCGAAAAAAAAAAAAAAAAAAAA    6420

AAAAAAAA
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Fig. 7.2

CHAPTER 8: THE FUTURE RESEARCH PERSPECTIVES

8.1 Research summary

My study has strongly indicated that high levels of pyrethroid resistance in *Cx. quinquefasciatus* are conferred by multiple mechanisms, such as gene overexpression and target gene mutations. It also suggests that the *kdr*-mediated mechanism is crucial for resistance, in which the transcriptional regulation of L-to-F *kdr* mutation of sodium channels through RNA allelic variation and RNA editing play an important role. It is the first study to reveal the transcriptional regulation in sodium channel gene expression as a molecular mechanism involved in the evolution of *kdr*-mediated pyrethroid resistance in insects, including mosquitoes, house flies, and German cockroaches. My study has discovered multiple sodium channel genes presented in both mosquitoes and house flies. The first full-length mosquito sodium channel gene has been cloned and sequenced and been compared between susceptible of resistant mosquitoes. Polymorphisms in the sodium channel gene between resistant and susceptible mosquitoes have been identified. Interestingly, I have found several silent mutations in sodium channels that correspond with levels of pyrethroid resistance in mosquitoes. How these silent mutations contribute to the sodium channel gene function remains to be further investigated. Results from my dissertation research indicate a complexity of the sodium channel gene expression and its role in pyrethroid resistance of insects. It provides a new framework to evaluate and

study the sodium channel gene expression regulation and its importance in insecticide resistance of mosquitoes.

8.2 Future research perspectives

8.2.1 Functional studies of single nucleotide polymorphisms (SNPs) of the sodium channel gene

Firstly, it will be very interesting to conduct functional studies to further characterize the role of single nucleotide polymorphisms (SNPs) that have been identified in mosquito sodium channel genes that confer insecticide resistance. Functional analysis of SNPs of the sodium channel gene in *Xenopus* oocytes, a sodium channel study model (Ingles et al. 1996*)*, will be indispensable to confirm the involvement of these mutations under insecticide selection pressure. The full-length mosquito sodium channel genes with mutations will be co-expressed with the *Drsophila melanogaster* tipE protein in *Xenopus* oocytes as described by Tan et al. (2002) and assayed by voltage-clamp. The results from the functional analysis will demonstrate whether these mutant genes indeed encode functional sodium channels or whether these mutations cause reduction in the sensitivity of sodium channels to pyrethroid insecticides. Secondly, comparison of the binding ability of pyrethroid insecticides with resistant and susceptible sodium channel proteins will be necessary to clarify an exact role of the sodium channel at the physiological level *in vitro*. In binding assays, mosquito sodium channel proteins will be expressed using prokaryotic and/or eukaryotic systems and pyrethroid insecticides will be radioactively labeled. The difference in binding affinity to pyrethroid insecticides between susceptible (wild type) and resistant (mutant) sodium channel proteins will be clarified. Thirdly,

studying the three-dimensional shape of sodium channel proteins by using nuclear magnetic resonance (NMR) and crystallography will be valuable for us to find out whether these SNPs change sodium channel tertiary structures, which, in turn, will affect their functions. For NMR spectroscopy technique (Akitt and Mann, 2000)**,** mosquito sodium channel proteins need to be expressed and labeled with isotopes. Then, resonances of sodium channel proteins are assigned, and restraints are generated. Finally, a structure of the sodium channel protein is calculated and validated. For crystallography method (Drenth, 1999), sodium channel proteins need to be expressed and crystallized. Once a crystal of the sodium channel protein is obtained, data can be collected using a beam of radiation. The mathematical methods are applied to analyze data, and a model will be predicted**.** This model structure will be compared to the actual pattern generated by the crystalline sample to determine the structure of the sodium channel protein. Using NMR and crystallography techniques, the tertiary structures of susceptible (wild type) and resistant (mutant) sodium channel protein can be achieved. Thus, whether the SNPs including silent mutations in sodium channel gene will cause tertiary structure change of sodium channels will be elucidated. Also, the whole sodium channel gene including promoter and intron sequences need to be cloned to clarify if there are *cis*-elements involved in the L-to-F *kdr* mutation transcription.

8.2.2 Investigation of genes involved in the transcriptional regulation and pyrethroid resistance

Investigating genes involved in transcriptional regulation and pyrethroid resistance will give us a considerable picture about how mosquito genes work together to deal with

the insecticide selection pressure. Genes differentially expressed between resistant and susceptible mosquitoes need to be identified using cDNA-AFLP and microarray techniques. For cDNA-AFLP method, mRNAs of susceptible and resistant mosquitoes need to be prepared, and then reverse-transcripted to single strand cDNAs. Single strand cDNAs will be used to synthesize double stand cDNAs, and then subjected to enzyme digestion. The products of enzyme digestion will be used as templates for AFLP analysis using different primer sets. For microarray method, normalized mosquito cDNA library needs to be constructed. Then, a number of PCR experiments will be carried out using cDNA library as templates. PCR products need to be arrayed on chips. These chips need to be hybridized with cDNA from insecticide susceptible and resistant mosquitoes that are labeled with two different fluorophores. Using this method, the differentially expressed genes between resistant and susceptible strains will be identified.

8.2.3 Study gene functions using mosquito transgenic and RNA interference techniques

Germline transformation of *Cx. quinquefasciatus* with the *piggyBac* transposable element carrying the green fluorescent protein (GFP) gene has recently been successfully established in our laboratory. Using this system, mutant genes can be introduced into mosquitoes and their functions will be studied *in vivo*. In addition, specific gene expressions also can be silenced using double stranded RNA (dsRNA)-mediated interference (RNAi) techniques to reveal functions of these genes. In summary, by conducting the future studies, a more comprehensive picture of genes involved in transcriptional regulation and pyrethroid resistance in mosquitoes will be obtained.

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