Multiple Mechanisms of Permethrin Resistance in the Southern House Mosquito, *Culex quinquefasciatus*

by

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Abstract

The inherited resistance to permethrin in *Culex quinquefasciatus*, which is the primary vector of encephalitis and lymphatic filariasis pathogens, resulted from the molecular basis of multiple mechanisms involved in changes of more than one resistant gene. Two major mechanisms of insecticide resistance in mosquito are P450-mediated detoxification via increased the expression level of P450 genes to metabolize the insecticide, and sodium channel insensitivity via modified mutations to change the channel structure and prevent the permethrin binding. In my current studies, I found 4 cytochrome P450 genes were up-regulated and inducible in the resistant mosquito strains indicating the importance of these P450 genes in permethrin resistance, and also synonymous and non-synonymous mutations present in sodium channel as well as multiple sodium channel variances caused the permethrin resistance in *Culex* mosquitoes. Furthermore, to identify the regulatory pathway of P450 gene expression in insecticide resistance of the *Culex* mosquito, my studies have revealed, for the first time, that among a total of 120 GPCR signaling-pathway-related genes, 5 of them were significantly up-regulated in the resistant *Culex* mosquitoes. Functional characterization of the 5 up-regulated-GPCR pathway-related genes in insecticide resistance using the double-stranded RNA-mediated gene interference (RNAi) and *Drosophila* transgenic techniques have proved that the function of the GPCR-related genes in insecticide resistance by regulating the P450 gene expression through the protein kinase A and AMP-dependent protein kinase pathways. My studies provide new information for the understanding of resistance development at a molecular level and a new

avenue for characterizing the regulation pathways in resistance development, and also provide new strategies for control mosquitoes, especially the resistant ones.

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Table of Contents

Abstract ii

Acknowledgments iv

List of Tables viii

List of Figures x

List of Abbreviations xiii

Chapter 1 Literature Review 1

References 27

Chapter 2 Research Goal and Objectives 52

References 54

Chapter 3 Genetics and Inheritance of Permethrin Resistance in the Mosquito *Culex quinquefasciatus* 56

References 77

Chapter 4 Larvicidal Activity of Mosquito Sterol Carrier Protein-2 Inhibitors to the Insecticide Resistant Mosquito *Culex quinquefasciatus* (Diptera: Culicidae) 82

References 95

Chapter 5 Multiple Cytochrome P450 Genes: Their Constitutive Overespression and Permethrin Induction in Insecticide Resistant Mosquitoes, *Culex quinquefasciatus* 98

References 116

Chapter 6 Multiple Mutations and Mutation Combinations in the Sodium Channel of Permethrin Resistant Mosquitoes, *Culex quinquefasciatus* 121

References 148

Chapter 7 Multiple Sodium Channel Variants in the Mosquito *Culex quinquefasciatus* 152

References 181

Chapter 8 A Rhodopsin-Like G-Protein-Coupled Receptor Involved the Regulation Pathway of Cytochrome P450 Gene Expression through Activation of Protein Kinase A in the Permethrin-Resistant Mosquito, *Culex quinquefasciatus* 189

References 217

Chapter 9 Characterization of G-Protein-Coupled Receptor Related Genes in Insecticide Resistance of Mosquitoes, *Culex quinquefasciatus* 226

References 262

Chapter 10 Future Study 271

References 273

List of Tables

Table 3.1 Toxicity of permethrin to MAmCq strains compared to the susceptible S-Lab strain of *Cx. quinquefasciatus* before and after permethrin selection in the laboratory 60

Table 3.2 Toxicity of permethrin to the larvae of *Cx. quinquefasciatus* 65

Table 3.3 Toxicity of permethrin to the adults of *Cx. quinquefasciatus* 67

Table 3.4 Toxicity of permethrin on susceptible (S-Lab), resistant ($HAmCq^{G8}$), reciprocal progenies (F_1SH and F_1HS), and self-cross (F_2 [F_1SH♂ x F_1SH♀] and F_2' [F_1HS♂ x F_1HS♀]) strains of *Cx. quinquefasciatus* 70

Table 3.5 The chi-square (χ^2) analysis for inheritance of permethrin resistance in *Cx. quinquefasciatus* 72

Table 4.1 Toxicity of SCPIs to 2nd instar larvae of mosquito strains of *Culex quinquefasciatus* 87

Table 4.2 Toxicity of permethrin with and without SCPI-1 and SCPI-2 to 2nd instar larvae of mosquito strains of *Culex quinquefasciatus* 89

Table 5.1 Permethrin treatment of the late 3rd instar larvae of *Culex* mosquitoes 102

Table 6.1 Non-synonymous and synonymous mutations in the sodium channel of *Cx. quinquefascaitus* 125

Table 6.2 Permethrin treatment of field and permethrin selected *Culex* mosquitoes 129

Table 6.3 Co-occurrence of the *kdr* mutations in the MAmCq groups with difference levels of tolerance to permethrin 131

Table 6.4 Pairwise Goeman's Bayesian score test values to check for correlations between SNP combination frequencies and permethrin resistance levels 137

Table 6.5 The 13 common mutation combinations of sodium channels in the mosquito populations of *Cx. quinquefasciatus* 140

Table 6.6 Oligonucleotide primers* used for amplifying the sodium channel cDNA

fragments and SNP (single nucleotide polymorphism) determination 141

Table 7.1 Oligonucleotide primers used in qRT-PCR reactions for amplifying sodium channel variants 156

Table 8.1 Oligonucleotide primers used in PCR and qRT-PCR reaction 194

Table 8.2 Permethrin treatment with dose range in the mosquito strains 196

Table 9.1 Oligonucleotide primers used in qRT-PCR reactions for amplifying the GPCR genes 231

Table 9.2 Oligonucleotide primers used in PCR and qRT-PCR reactions 246

Table 9.3 Summary of GPCRs in *Culex quinquefasciatus* 251

List of Figures

Figure 1.1 Diversity of G-protein-coupled receptor signaling 23

Figure 1.2 Double-stranded RNA pathways 26

Figure 2.1 Hypothetical mode of GPCR regulatory pathway involved in the insecticide resistance of mosquitoes, *Culex quinquefasciatus* 53

Figure 3.1 Log concentration-probit analysis of permethrin for S-Lab, a susceptible parental strain; $HAmCq^{G8}$, the 8th generation of permethrin-selected $HAmCq^{G0}$ offspring; and F_1 lines, F_1SH (S-Lab♂ x $HAmCq^{G8}$♀) and F_1HS (S-Lab♀ x $HAmCq^{G8}$♂), generated from reciprocal crosses of S-Lab and $HAmCq^{G8}$ 71

Figure 3.2 Log concentration-probit lines of permethrin for susceptible S-Lab, resistant $HAmCq^{G8}$, and back-cross progeny of BC_{1a} (F_1SH♂ x S-Lab♀). 73

Figure 3.3 Log concentration-probit lines of permethrin for susceptible S-Lab, resistant $HAmCq^{G8}$, and back-cross progeny of BC_{1b} (F_1SH ♂ x $HAmCq^{G8}$ ♀). 74

Figure 3.4 Log concentration-probit lines of permethrin for susceptible S-Lab, resistant $HAmCq^{G8}$, and self-bred progeny of F_{2b} (F_1HS♂ x F_1HS♀). 75

Figure 4.1 Inhibitor activity ratios (IRs) of SCPI-1 and SCPI-2 in S-Lab, $HAmCq^{G9}$, $MAmCq^{G2}$, and BAmCq strains of *Culex quinquefasciatus* 88

Figure 4.2 Permethrin resistance ratios (RRs) with and without the inhibitors SCPI-1 or SCPI-2 for the S-Lab, $HAmCq^{G9}$, $MAmCq^{G2}$, and BAmCq strains of *Culex quinquefasciatus* 90

Figure 5.1 Expression analysis of *CYP6AA7*, *CYP9J34*, *CYP9J40*, and *CYP9M10* in mosquitoes, *Cx. quinquefasciatus* 106

Figure 5.2 Expression of *CYP6AA7* in head, thorax, and abdomen tissue of adult *Culex* mosquitoes 108

Figure 5.3 Dose-dependent induction of P450 expression following treatment with permethrin 111

Figure 5.4 The duration of the gene expression following permethrin treatment at a

concentration of LC_{50} 113

Figure 6.1 Graphic representation of the locations of synonymous and nonsynonymous mutations in the *Cx. quinquefasciatus* sodium channel 124

Figure 6.2 Distribution of frequencies of alleles at each of the mutation sites in each of the mosquito groups that are sensitive to or tolerant of different concentrations of permethrin (LC_{10}, LC_{50}, and LC_{90}) in $MAmCq^{G0}$ field parental populations and their 6th generation permethrin selected offspring, $MAmCq^{G6}$ 130

Figure 6.3 Categorical plots of the sodium channel mutation combination patterns in mosquito groups that are sensitive to or tolerant of different concentrations of permethrin in $MAmCq^{G0}$ field parental populations and their 6th generation permethrin selected offspring, $MAmCq^{G6}$ 134

Figure 7.1 Polymerase chain reaction (PCR) amplification of *pa-ra*-type sodium channel transcripts from genomic RNAs of *Culex* mosquitoes 160

Figure 7.2 Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel transcripts (Cx-Na) in S-Lab *Culex* mosquitoes 162

Figure 7.3 Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel reanscripts (Cx-Na) in $HAmCq^{G0}$ *Culex* mosquitoes 166

Figure 7.4 Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel transcripts (Cx-Na) in $HAmCq^{G8}$ *Culex* mosquitoes 170

Figure 7.5 Alternative splicing of Cx-Nav from mosquitoes *Culex quinquefasciatus* 174

Figure 7.6 Expression of *Cx-Nav* in larvae and head+ thorax and abdomen tissues of 2-3 day-old female adult *Culex* mosquitoes 178

Figure 8.1 Expression of rhodopsin-like GPCR gene in *Culex* mosquito strains 202

Figure 8.2 Time and dose-dependent induction of the relative expression of the rhodopsin-like GPCR gene 203

Figure 8.3 Rhodopsin-like GPCR gene expression and bioassay resistance ratio in the mosquitoes with or without rhodopsin-like-dsRNA injection 205

Figure 8.4 Four P450 Gene Expression in the Mosquitoes with or without dsRNA-rhodpsin-like Injection 206

Figure 8.5 cAMP/PKA gene expression in mosquito with or without rhodopsin-like GPCR gene injection 208

Figure 8.6 Mosquito injection with or without PKA gene 209

Figure 8.7 Functional study of rhodopsin-kike GPCR in *Drosophila* transgenic and non-transgenic lines.. 211

Figure 9.1 Up-regulated GPCR-related gene expression in mosquito strains of *Cx. quinquefasciatus* 253

Figure 9.2 Down-regulated GPCR-related gene expression in mosquito strains of *Cx. quinquefasciatus* 255

Figure 9.3 Functional study of up-regulated CPIJ019111gene in permethrin resistance of HAmCq^{G8} strain.. 256

Figure 9.4 Functional study of up-regulated CPIJ007717gene in permethrin resistance of MAmCq^{G6} strain 257

Figure 9.5 Functional study of up-regulated CPIJ014419 gene in permethrin resistance of MAmCq^{G6} strain 258

Figure 9.6 Functional study of up-regulated CPIJ014334 gene in adult of permethrin resistance of MAmCq^{G6} strain 259

List of Abbreviations

RT-PCR: reverse transcription-mediated polymerase chain reaction;

3'-RACE: rapid amplification of 3' cDNA ends;

ANOVA: one-way analysis of variance;

BC_1: back-cross generation 1;

ORF: open reading frame;

PBO: piperonyl butoxide

CYP: Cytochrome P450

KDR: Knockdown-Resistance

GPCR: G Protein-Coupled Receptor

PKA: Protein Kinase A

AMPK: AMP-Activated Protein Kinase

Chapter 1 Literature Review

1. Insects and Insecticide Resistance

Three quarters of all species in the world are insects, which play important roles in economically and ecologically area. However, some of them act as agricultural pests, destroying a lot of our potential annual harvest, and can be primary vectors for major diseases such as malaria, dengue fever, yellow fever, elephantiasis, and sleeping sickness.

When the industrialization and mechanization of agriculture were rapidly developing in the eighteenth and nineteenth centuries, the large-scale production of naturally derived insecticides became the method of choice to control the pests. Different kinds of insecticides, such as organochloride, organophosphates, carbamate, pyrethroids, neonicotinoids, biological insecticides, as well as flubendiamide, diamide, and decaleside as example for current new insecticides, et al. are applied successfully and efficiently against insect pests in agriculture, medicine, industry and household. In the beginning of the twentieth century the production of synthetic insecticides such as DDT (introduced in the 1940s) and the synthetic pyrethroids (first commercialization in the 1970s) were used in common. However, since DDT (organochloride insecticide) was hard degraded in the environment and extremely accumulated in food-chain, it is no longer used worldwide. Organophosphate pesticides can be degraded rapidly by hydrolysis on exposure to sunlight, air and soil, and high efficient in pest control, thus they are widely used in agriculture. However, because of the high acute toxicity of organophosphate insecticide to human and animals, many of them were banned for using. The first carbamate pesticide, carbaryl, which was introduced in 1956, was used throughout the world, especially in lawn and

garden settings, because of its relatively low mammalian oral and dermal toxicity and broad control spectrum. Pyrethroids are the artificial compound based on the structure of natural pyrethrins, and it has high toxicity to insects but low toxicity to vertebrates. Thus, they are continually used to against agricultural, especially the medical pests by treating the bed net so far.

Unfortunately, the long-term and repeated use of large amount of insecticides bring on the development of insecticide resistance, which has had a substantial impact on modern agriculture and human health, and became a major obstruction to the control of agricultural (1-3) and medical pests (4).

2. Resistance Problem

A growing food-crisis in the world relates to insecticide control and the insecticide resistance has been a critical problem in more than 500 insect and mite species worldwide. Insect resistance was defined by the World Health Organization in 1957 as "the development of an ability in some individuals of a given organism to tolerate doses of a toxicant, which would prove lethal to a majority of individuals in a normal population of the same organism" (5). The insecticide problem is regarded as a major danger to control pest in agriculture and medicine areas. The insecticide resistance is inherited by carrying genetic traits for coping with the environment survive and reproduce, pass on these traits to their progeny (6-7). In addition, continued selection pressure exerted by the insecticide rapidly increases the frequency of the genetic trait of resistance in the population. Furthermore, some evidence indicated that the major factor influencing insecticide resistance development is the life cycle of the insect pest. For instances insects with long life cycles and the production of a very small numbers of young have no

development of insecticide resistance. In contrast, all the characteristics of mosquitoes, such as short day life cycle with huge progeny, results in rapid resistance development (8).

To date, hundreds of pests in agriculture and medicine have developed resistance to all kinds of insecticides, for instances, mosquito species (4, 9-15), house fly (16-19), German cockroach (20), horn fly (21), cotton bollworm (22), aphid species (23), whiteflies (24), rust red flour beetle (25), bed bug (26), and colorado potato beetle (27), et al.

Each insect resistance problem is potentially unique and may involve in a complex pattern of resistance to different type insecticide. Because of the insecticide resistance problems, the list of effective insecticide for the control of crop pests and disease vectors is rapidly shrinking. Although some natural biological tactics have been provided, the using of synthetic insecticides still plays a primary role in control management. However, fewer new insecticides are being introduced to the market, largely because of the high costs associated with research, development and registration, and the prediction of a limited effective lifespan of the new insecticide. Thus, understanding of the molecular basis of the insecticide resistance mechanisms will be an essential fundamental for designing new strategies to control the insects, especially, resistant ones (28-29).

3. Mechanisms of Insect Resistance

At the beginning, the resistance problems were detected by bioassay, which measures the overall result if the resistance genes present in a population. However, followed by the development of research on the biochemical and molecular bases of resistance, techniques characterizing each resistance gene in individuals become available (30-31).

In insects, there are many possible adaptations that permit an organism to survive under lethal doses of a toxicant and can be classified as either mechanisms of decreased exposure or

decreased response to the toxicant, including the behavioral avoidance resistance (32), reduced penetration or absorption (33), increased detoxification (alterations in the levels or activities of detoxification proteins) (34), or target site insensitivity (mutation in the target sites) (35), all contribute to decrease the dose of the pesticide, or a modification of target site number will contribute to rendering a dose of pesticide ineffective. These are achieved at the molecular level by: point mutation linked genetically to a sodium channel gene (target of DDT and pyrethroid insecticide) (36-37), the ion channel portion of a GABA receptor subunit (target of cyclodiene insecticide) (38), and the vicinity of the AChE active site (target of OP and carbamate insecticide) (39); amplification of esterase genes (target of OP and carbamate); and up-regulation of detoxification enzymes, such as cytochrome P450 and glutathione S-transferases (40).

All resistance genes, whether conferring a low or high level of resistance are selected as long as the selection pressure, are maintained because they confer a high fitness to their carriers. Increased detoxification is a common resistance mechanism that breaks down the insecticide to diminish or prevent the quality and quantity for target binding. It is due to either a modification of the enzyme catalytic property or to an increased enzyme production, or to the association of both. Increased enzyme production is in some cases either due to gene amplification or resistant gene up-regulations, such as esterase and P450s (41-42). Analyses of the mutations of resistance genes, such as AChE (43), GABA (38), and sodium channel gene (44), suggest that these mutations are involved in insecticide resistance by changing the target structure to prevent the insecticide binding. Thus, most studies are focus on the mutation related insecticide resistance. The rate of mutation plays an important role in insecticide resistance. The pool of mutant alleles represent at one or more loci, any of which could confer resistance for a given dose of toxicant (36-37). So we are considering that more polygenic is the basis of the resistance phenotype.

Development of resistance in field populations is due to the selection of resistance genes, which appear by increased probability of mutation in the treated populations or are imported by active or passive migration from other areas. However, as the result of insecticide-persistent selection in lab, the numerous mutations should be more common in lab-selected than in field-selected populations.

Genes responsible for an adaptation to a new environment stress (insecticide application) are usually assumed to have fitness cost (45). Some insecticide-resistant insects decreased other fitness-enhancing characters, resulting in an effect on the reproductive processes (46-47) and mating competition (48). Cost can be a determinant in the evolution of adaption since it can lead to force the resistant alleles in insecticide free condition (49). Pleiotropic effects associated with resistance are a consequence of the biochemical and physiological changes associated with the resistant phenotype. Therefore, understanding the molecular basis of insecticide resistance mechanisms will proved new angle to study the adaption and evolution of resistance in insects, in turn, explore new strategy to control the pests, especially, resistant ones.

3.1 Decreased absorption

To prevent the insecticide binding with the targets, insects develop the different mechanisms against insecticide. For example, the rate of absorption of insecticide by insect is a key determinant of insecticide toxicity. Early studies have indicated that the decrease in insecticide absorption was related to resistance was in house fly that was a resistant strain to pyrethroid and DDT. The nonpolar insecticides penetrate insects more rapidly than polar insecticides, probably because of their greater affinity for epicuticular grease (50). Autoradiographic with ^{14}C-dieldrin method has been completed, showing that insecticides applied topically to insects spread laterally within the integument and then reach the site of toxic action through the tracheal

integument (51). The decreased absorption as a resistance mechanism in mosquito has been hypothesized that the delayed penetration was controlled by the cuticle related gene (52). However, so far, how many genes are involved in penetration is still unknown.

3.2 Target site insensitivity

Target site insensitivity is one of the major mechanisms of insecticide resistance, and the three major insecticide target site genes have been cloned from the genetic model *Drosophila* melanogaster. They are: (1) the *para* sodium channel gene, the target site of DDT and pyrethroid (53); (2) the GABA receptor gene resistance to dieldrin or Rdl, the site of action of cyclodiene insecticides (54); and (3) acetylcholinesterase or AchE, the target for organoohosphrous(OP) and carbamate insecticides (55).

3.2.1 Sodium Channel

Sodium channel are integral membrane protein, which form for conducting sodium ions through cell's plasma membrane. There are two kinds of sodium channel based on their trigger, i. e. either a voltage-channel, or ligand-gated channel. The voltage-gated sodium channel affects channel gating and ion permeability during the initial rapidly rising phase of the action potential in excitable tissues. There are several groups of insecticides, including DDT and its analogues, pyrethroids, *N*-alkylamides, and dihydropyrazoles, all of which can be used to treat the sodium channel (56). The sodium channel is bound by insecticide and keep open, in turn, causes the action potential and paralysis of insect to death. However, the amino acid mutation(s) present in sodium channel results in the conformation or structure change of the channel to prevent the insecticide binding, in another word, called insecticide insensitivity of sodium channel resistance. Moreover, the recombination of mutation of nucleotide in same protein also caused different resistant patterns (36-37).

Pyrethroids are particularly suitable for veterinary and public health purposes because of their quick knockdown effects, high insecticidal potency, relatively low mammalian hazard at operational doses, and non-bioaccumulation (57). Furthermore, the pyrethroids account for approximately 25% of the world insecticide market. They are used as indoor residual house sprays to impregnate bednets, curtains and screens (58-59). However, the developed pyrethroid-resistance problem is increasing. Resistance to such insecticides is widespread in mosquitoes (60-61) and many other pests (60), causing operational problems for control programs. To date, the cross application of insecticide between carbamat and pyrethriod was used for mosquito control (62).

The term of "knockdown resistance" describes the cases of DDT and pyrethroid acting on the insect and other arthropods to reduce sensitivity of the nervous system that causes resistance development (63). The gene(s) conferring DDT and pyrethrins knockdown resistance (kdr) have long been known to be recessive in the chromosome III of house fly (64) and recessive in mosquitoes (65). Insensitivity of the sodium channels to insecticide inhibition was first recorded in house fly, *Musca domestica*. The para sodium channel of house flies contains 2108 amino acids, which fold into 4 hydrophobic repeat domains (I-IV) separated by hydrophilic linkers (66). The *kdr* resistance mechanisms include a leucine to phenylalanine substitution known as West *kdr* and a leucine to serine substitution known as East *kdr* (67). Moreover, super-kdr in house flies is associated with a second methionine to threonine substitution further upstream in the same domain. Either a single amino acid replacement associated with *kdr*, or an addition of a second replacement associated with an enhanced allele *super-kdr*, all involved in kdr resistance in insects. As an example, the first mutation to be characterized in *kdr* insects was a leucine to phenylalanine point mutation in the S6 transmembrane segment of domain II in the sodium

channel sequence of *M. domestica*, which produces 10- to 20-fold resistance to DDT and pyrethroids. In ‘‘*super-kdr*’’ house flies, this mutation also occurs with a second methionine to threonine substitution further upstream in the same domain, resulting in more than 500-fold resistance. The limited number of changes associated with *kdr*-type resistance may be constrained by the number of modifications that can influence pyrethroid/DDT binding to the sodium channels (68). However, multiple mutations in the same gene may result either from the accumulation of mutations, or from the recombination between genes carrying single mutations (36-37). The functional study of sodium channel gene in insecticide resistance demonstrated the mutation(s) in sodium channel prevent insecticide binding with the channel (69-70).

3.2.2 Acetylcholinesterase

Acetylcholinesterase (AChE) is an important enzyme located in the insect nervous system because it terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine on the post-synaptic nerve membrane. This enzyme is the target of insecticides of organophoorous and carbamate, which work on the AChE as nervous system inhibitors that cause insect paralysis to death.

In 1961, Smissaet described the first case of AchE with a reduced sensitivity to pesticides. So far, modified AChE has been involved in many insect resistance developments.

The first insect acetylcholinesterase gene (Ace) was cloned from Drosophila (71). Drosophila has only a single Ace gene, whereas mosquitoes have two different acetylcholinesterase proteins that are encoded by two different genes, *Ace-1* and *Ace-2*(72). Moreover, the mutation G119S of *Ace-1* gene is confirmed for high level of resistance in mosquitoes (73). In *Cydia pomonella*, one mutation of F399V may be involved in insecticide resistance (74). Multiple mutations of AchE could explain the highly resistant ratio in insects (75-77).

3.2.3 γ-Amino Butyric Acid (GABA) Receptors

Ligands-gated ion channel receive chemical signals, neurotransmiters, such as acetylcholine or γ-amino butyric acid (GABA), which they then convert into electrical signals via the opening of their integral ion channels. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both insects and vertebrates(78).

GABA receptors can be classified into two groups: GABAA and GABAB. Usually, the mutation of the GABAA receptor is responsible for cyclodienes resistance that blocks the chloride channel of mosquito species (61). In Drosophila, GABA-receptor-subunit gene was cloned and its mutant was involved in resistance to the cyclodiene insecticide (79). Moreover, according to recently studies, the mutant of chloride ion channel pore of the protein has been associated with resistance development.

To date, we may know that the para sodium channel gene homologue and the GABA receptor gene resistance to dieldrin map to the same genome regions as the DDT/pytrthroid and cyclodiene resistance loci, respectively. Although the acetylcholinesterase gene *Ace* dose not map to any known resistance locus, it maps very close to the sex-determining locus (80).

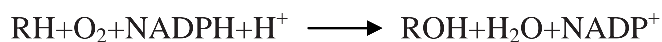
3.3 Increased Metabolism Resistance

Sequence amplifications, transcriptional enhancements, and coding mutations in genes of cytochrome P450 monooxygenases (P450s), esterases, and glutathione-*S*transferases (GSTs) have been identified as resistance mechanisms (81).

3.3.1 Cytochrome P450s

The Cytochrome P450 monoxygenases family is thus one of the oldest and largest gene superfamilies in organisms. They not only affect insect growth, development and reproduction, but also are involved in many cases of insect resistance to insecticides by metabolic reactions

(82). The cytochrome P450-dependent monooxygenases are a very essential enzymetic system involved in the metabolism of endogenous and exogenous. The overall reaction of P450 monooxygenase-mediated metabolism can be expressed as follows:



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

In the formula, the RH is the substrate, such as insecticides. P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into the substrate, which is water soluble for easy excretion. The nomenclature of the P450 superfamily is based on amino acid sequence homologies, with all families having the CYP prefix followed by a numeral for the family and a numeral for the individual gene. P450s are classified according to the degree of amino acid sequence identity, with P450s of the same family defined as having 40% identity, and P450s of the same subfamily having 55% identity.

Many insect species have obtained high levels of resistance to many insecticides by increased detoxification of P450s (40, 81, 83-84). Several approaches, including cross-strain comparisons of gene sequences, copy number, expression levels (genes and proteins), and substrate binding preferences, have been used to determine the molecular mechanisms for P450-mediated resistance. The P450 gene up-regulation is a primary mechanism involved in insecticide resistance. The insecticide resistance-related P450 genes almost belong to the families *CYP4*, *CYP6*, *CYP9*, and *CYP12*, which are overproduced via up-regulation in resistant insects (85-87). Usually, the over-production of genes is result from gene amplification by means which several copies of the same gene are found in the same genome. DNA amplification events create additional copies of chromosomal sequences (including functional genes), which can survive in either intra- or extra chromosomal forms. The amplified gene must be transcribed and translated (over-expressed) into functional protein products for expression of the resistance trait. Various

types of mutations can lead to changes in gene expression and these can occur in *cis* (for instance disruption or deletion of an upstream regulatory element of the gene, either this element is enhancing or repressing gene expression) or in *trans* (for instance disruption of a gene coding for a protein that binds to the above-mentioned *cis* elements).

Although gene up-regulation could theoretically result from increases in transcription, mRNA stability, and/or protein translation, it appears that in most cases increased expression is achieved through mutations and insertions/deletions (indels) in *cis*-acting promoter sequences and/or *trans*-acting regulatory loci. There was strong evidence to suggest that P450 monooxygenase-based resistance in *M. domestica* was mediated by mutations in *trans*-acting regulatory genes (89). Moreover, the Nrf2 orhtolog CncC is central regulator for resistant-related P450 gene expression (90). The duplication of P450 genes, *CYP6P9a* and *CYP6P9b* (located on 2R chromosome), in insecticide resistance have been reported in *Anopheles funestus* (91). The increased amount of evidence showed that the point mutation may play a secondary role in the P450-mediated insecticide resistance (92). Several evidences have showed that either identify mutations or indels in the promoter region of some P450 genes may cause gene up-regulation, such as the house fly *CYP6D3* (93), as well as fruit fly *CYP6A8* (94), *CYP12A4* (95), and *CYP6G1* (96). However, whether or how these mutations or indels result in the up-regulation of the corresponding resistance P450 genes need to be understood.

In *D. melanogaster*, a defective *Gypsy*-like long terminal repeat (LTR) retrotransposon known as *Accord* is inserted approximately 300 bp upstream from the transcription start site. The mechanisms by which these *Accord* and *Doc* insertions might cause *CYP6G1* upregulation encompass a wide range of options, from disrupting existing repressor elements, to introducing

enhancer elements, to altering the physical distance between regulatory elements and the transcriptional start site (96).

Study on the multi-gene enzyme systems of P450s, which detoxify a wide range of xenobiotics, suggested that the molecular basis of metabolic is complex and it is not easy to analyze the single gene function in vivo.

3.3.2 Esterase

The esterases are hydrolyzing the OPs, through an acylated cysteine in their active site, and are termed phosphoric triester hydrolases. Some insect species, such as mosquitoes (40), ticks (97), aphids (98) and cockroaches (99), have obtained resistance to organophosphorus and carbamate insecticide by over-production of carboxylesterases.

In mosquitoes, esterase was reported in organophosphate (OP) insecticide resistance, and secondary mechanism for carbamate resistance through gene amplification, up-regulation, coding sequence mutation, or a combination of these mechanisms. However, the overproduction of esterase by the Ester locus should cause alteration of some fitness related traits in order to generate the fitness cost of resistance gene. For instance, in some insects, the fitness traits have be changed and associated with several resistance genes, including a decrease of overwintering survival (100), small adult size, an increases predation and development time, and a decreased male reproductive success (48).

Many mosquito species have obtained the resistance to OP, such as *C. pipiens* complex, *C. tarsalis, C. tritaeniorhynchus, An. albimanus, An. stephensi,* and *Ae. aegypti.* In these mosquito species, elevated carboxylesterase activity involves rapid hydrolysis of the insecticide. Transposable elements or long interspersed repetitive elements (LINEs), which are capable of accelerating the frequency of gene mutation and gene amplification have been found in

association with the mosquito carboxylesterase genes. LINEs, designated as Juan-C, are closely associated with the amplified Est1 in the TEMR strain of *Cx. quinquefasciatus* (102). Moreover, the most common amplified esterase-based mechanism in *Culex* involves the co-amplification of two esterases, *est*a2*1* and *estb2*1 in *C. quinquefasciatus* and other members of the *C. pipiens* complex worldwide (103-104). The identical RFLP patterns of the *est*a2*1* and *estb2*1 loci in resistant *C. pipiens* complex populations worldwide suggest that the amplification of these alleles occurred once and has since spread by migration (105).

3.3.3 Glutathione S-Transferases (GSTs)

The glutathione s-transferases (GSTs) play a pivotal role in detoxification and cellular antioxidant defense against oxidation stress by conjugating reduced glutathione (GSH) to the electrophilic centers of natural and synthetic exogenous xenobiotics, including insecticides, allelochemicals, and endogenously activated compounds, such as unsaturated carbonyls, epoxides, organic hydroperoxides, lipid peroxidation products, and oxidized DNA bases (81). It prevents the insecticide to reach its site of action (106). The glutathione S-transferase (GST) based insecticide resistance mechanisms to organophosphorus and carbamte insecticides in many insects.

Both GST gene amplification and up-regulation are playing important role on resistance to OP in insect species. Multiple forms of these enzymes have been reported in several insect species (107). The GSTs in mosquito commonly confer resistance to the organochloride insecticide, DDT, and also can act as secondary OP resistance mechanism (61). In house fly, *Musca domestica, MdGSTD3* in OP-resistant strain is amplified (108). The remaining five GST genes implicated in insecticide resistance appear to be overexpressed in resistant insects, including *PxGSTE1* in OP-resistant *Plutella xylostella* (109), *AgGSTE2* in DDT-resistant *Anopheles*

gambiae (110-111), *DmGSTD1* in DDT-resistant *D. melanogaster* (112), and *AaGSTD1* and *AaGSTE2* in DDT-/pyrethroid-resistant *Aedes aegypti* (113).

3.4 Resistance to Insect Growth Regulators

Insect growth regulators are used for insect control several years (114), however, insect can obtain resistance against some growth regulators (such as juvenile hormone analogs and a substituted benzoylphenylurea) (115). Some insects resistant to insecticides as well as tolerant or resistant to JHAs, for example, house fly increased oxidative detoxifying activity is responsible for cross-resistance to JHAs. Mixed-function oxidases play an important role in both detoxifying JHAs and presumable in one OP-resistance strain of house fly.

4. Mosquito Problem

Vector-borne diseases are an increasing cause of death and suffering worldwide, such as mosquito, which is primary vector for many human and other animal diseases. Mosquitoes are attracted by the moisture, warmth, carbon dioxide, odor, and estrogen that surround human skin (116). Mosquito-borne human neuropathogen West Nile virus (WNV) which was first isolated in the West Nile region of Uganda in 1937 (117), then frequency outbreaks over the past decade result in millions of human and animal deaths. The vector of many pathogens (West Nile virus, Yellow fever virus, St. Louis encephalitis, and dengue fever), *Aedes albopictus*, called 'Asian tiger mosquito', which lead to serious outbreaks of arbovirus diseases worldwide from 20th century early to present (118-119). Japanese encephalitis virus (JEV), a mosquito-borne human disease, lead worldwide annual incidence of 45,000 human cases and 10,000 deaths and was largely transmitted by *Culex* mosquitoes (120). Malaria (transmitted by *Anopheles gambiae*) is one of the most serious public health problems worldwide and is the most important parasitic infection in humans, millions Africa people died each year as a result from it (121). Klinkenberg

reported that *Anopheles spp.* and *Cx. quinquefasciatus* outdoor biting rates is higher in areas around agricultural sites than in areas far from agriculture, and the agricultural area provides more opportunity for adults than larvae (122).

DDT was first introduced for mosquito control in 1946. Insecticides used for malaria control has included -BHC, organophosphorus, carbamate, and pyrethroid insecticides, with the latter now taking increasing market share for both indoor residual spraying and large-scale insecticide-impregnated bed net programs (123). Other insecticide groups, such as the benzylphenyl ureas and Bti, have had limited use against mosquitoes.

4.1 Life cycle of *Culex quinquefasciatus*

Culex quinquefasciatus, "Southern House Mosquito", belongs to *Diptera, Culicidea* as well as the major domestic pest in many urban areas. The female is medium-sized of brownish appearance; proboscis dark but often with some pale scaling midway on the underside; scutum with golden and bronzy narrow scales; veins with dark scaled; hind legs with femur pale almost to the tip except for dark scales along length dorsally, abdominal tergites dark scaled with pale basal bands and not merging with lateral patches except perhaps on terminal segments, sternites generally pale scaled but with a few to more dark scales scattered medially. Adults are generally active only during the warmer months; they usually attack humans towards the middle of the night indoors and outdoors, but are often more attracted to birds and are able to carry MVE virus. They usually bite in dusk and dawn and can not fly more than one mile. Each female can lay about 100 eggs in a life cycle. Larvae breed in septic ditches, artificial containers and other areas with foul water. The life cycle of this species is ~25-30 days at 25-29 °C.

4.2 Life cycle of *Aedes aegypti*

Aedes aegypti, the yellow fever mosquito belongs to the tribe *Aedini* of the *diptera* family culicidae. Female: a smallish, dark mosquito with conspicuous white markings and banded-legs; the proboscis is all black although the palps are white tipped; the scutum has a dorsal pattern of white scales in the form of a 'lyre' with curved lateral and 2 central stripes contrasting with the general covering of narrow dark scales; wings are dark scaled; hind legs with femur pale scaled for basal three-quarters with dark scales dorsally on apical two-thirds and ventrally on apical third, tibia dark but tarsi with pale basal bands on 1-4 and 5 all pale; abdominal tergites with median and lateral white scale patches or bands (possibly some white scales on apical margins), sternites predominantly pale scaled with subapical bands on distal segments. Adults are also found within or close-by human environments, often biting indoors or in sheltered areas near the house; biting is predominantly by day in shaded areas but may also occur early in the night.

5. Mosquito Resistance Problems

Mosquito as an important public health pest is controlled using a range of physical, biological and chemical methods, but the use of chemical pesticides has been the most effective way to control this pest and its associated diseases. However, prolonged use of insecticides can lead to the evolution of insecticide resistance that has been reported in all chemical classes of insecticides (61). Beginning with the extensive use of DDT(dichlorodiphenyltrichloroethane) in the 1950's and 1960's for controlling public health pests, and present due to their rapid mode of action and low mammalian toxicity, pyrethroid insecticides have proved to be particularly useful in vector control (124). However, the more recent dramatic expansion in use of pyrethroid and extensive agricultural where were around mosquito breeding habitats, have led to widespread insecticide resistance in mosquito vector populations (12, 38). Using malaria control as an example, *Anopheles gambiae* is the current major vector that has been studied in insecticide

resistance and the underlying resistance mechanisms area. Spraying house walls result in reducing malaria transmission and preventing vectors from entering or remaining inside long enough to bite (125). However, field studies in Africa, India, Brazil, and Mexico provide potent evidence for strong behavioral avoidance of DDT by the primary vector species, and pyrethroids also stimulate avoidance behaviors in insects. The problems for vector control created by use of insecticides in agriculture and the potential for management of resistance in both agriculture and vector-borne disease control are discussed.

Since then more than 100 mosquito species are reported as resistant to one or more insecticide. Organophosphorus insecticide resistance is widespread in all the major *Culex* vectors (126), and pyrethroid resistance occurs in *Cx. quinquefasciatus* (127). Pyrethroid resistance also has been noted in *An. albimanus* (38), *An. stephensi* (4) and *An. gambiae* (128) among others, while carbamate resistance is present in *An. sacharovi* and *An. albimanus* (129). The mosquito *Culex pipiens* resistance to OP has been studies more than 30 years (130).

As a result of constant insecticide pressure, mosquito populations have inevitably evolved resistance mechanisms that include target-site insensitivity and high levels of metabolic detoxification. Moreover, the multiple resistance mechanisms that contribute to permethrin have been reported (131). The metabolic genes probably influenced rang of xenobiotics on selecting for resistance in mosquitoes in different breeding sites (132). According to the recent studies, only three loci have developed major OP resistance alleles: *Est-2*, *Est-3* and *Ace.1*. These resistance alleles correspond to an esterase over-production, which binds or metabolizes the insecticide, relative to basal esterase production of susceptibility alleles. Pyrethroid resistance in *Anopheles gambiae* in East and West Africa appears to be linked to increased monooxygenase

titres, and also combined with an altered target-site mechanism. Mosquito species developed target site insensitivity causing the neuroactive insecticide resistance (4, 127).

Occasionally, it is possible to distinguish between heterozygous and homozygous resistant genotypes. This requires the prior establishment of a homozygous resistant strain, knowledge of the inheritance of resistance, and the assumption. These data could be useful to the population geneticist in the study of the development of resistance (133). We want to know if the resistance gene is dominant or recessive, we may also need to know which resistance mechanisms are involved, so we will know how the resistance provided by each resistance gene, which may combine together when they are in the same individual. For instance, the reduced penetration mechanism will combine multiplicatively with any other resistance mechanisms, meanwhile an increased detoxification and target insensitivity will combine additively. This variety of interaction indicates that understanding of gene interaction will help us to understand the physiological and molecular processes.

The gene function is required to predict gene interactions and understanding of what occurs in the metabolic pathways around the gene product is required for other types of interaction. Our understanding of how these genes are regulated will form another major advance in our understanding of the resistant systems, moving us closer to the goal of manipulating pest insect species with the aim of restoring insecticide susceptibility. In the insect's genome, one largest transmembrane receptor family, called G-protein coupled receptors (GPCRs), which sense the signal outside of cell and active intercellular signal transduction pathways. The GPCRs may be involved in insecticide resistance by regulating other resistant gene expression. In our previous study, one GPCR gene, called rhodopsin-like GPCR, has been identified up-regulation in resistant *Culex* mosquito strain (134). Moreover, GPCRs of *Ae. aegypti* have been sequenced

similarity to known drug targets and many reveal new opportunities for the development of novel insecticides (135).

6. G-protein and G-protein Coupled Receptors (GPCRs)

6.1 G-protein

G-protein (guanine nucleotide-binding proteins), the function as "molecular switches", are a family of protein to control intracellular messenger cascades and alternating between an inactive GDP and active GTP bound state, ultimately going on to regulate downstream cell processes. G proteins were discovered when Martin Rodbell and his colleagues tried to figure out how adrenaline stimulated cells. They found that when a hormone like adrenaline bound to a receptor, which did not stimulate adenylate directly. Instead, the receptor stimulated a G protein, which stimulated the adenylate cyclase to produce a second messenger, cyclic AMP. G-protein consists of α, β, γ subunits, which are involved in the regulation of intracellular signaling transduction (136). For this discovery they won the 1994 Nobel Prize in Physiology or Medicine.

G proteins belong to the larger group of enzymes called GTPases and are important signal transduction molecules in cells. In fact, diseases such as diabetes, blindness, allergies, depression, cardiovascular defects and certain forms of cancer, among other pathologies, are thought to arise due to derangement of G protein signaling. G protein can be separated to two distinct families of proteins. One is heterotrimeric G protein, sometimes referred to as the "large" G proteins that are activated by G protein-coupled receptors and made up of alpha (α), beta (β), and gamma (γ) subunits (137-138). Another is *"small" G proteins* (20-25kDa) that belong to the Ras superfamily of small GTPases, which are homologous to the alpha (α) subunit found in heterotrimers, and are in fact monomeric. However, they also bind GTP and GDP and are involved in signal transduction.

6.2 G protein coupled-receptor

They were named GPCRs because of their interaction with cellular G proteins. G protein-coupled receptors (GPCRs) represent >30% of the current drug targets (139). GPCRs, also known as 7 transmembrane domains, their sequences homology among all GPCRs is relatively low but they all have a seven-transmembrane (7TM) a-helical topology with an extracellular N-terminus and an intracellular C-terminus and active or inactive states (140). An outside of cell messenger, including biogenic amine, amino acid and ions, lipids, peptides and proteins, and light, odorant and nucleotides, binds to a specific GPCR. Thereby, the transducer, a G protein, composed of alpha-, beta- and gamma subunits, is activated. This, in turn, stimulates the amplifier, such as adenylate cyclase, which produces the second messenger, cyclic AMP, from ATP (adenosine- triphosphate). The cAMP could induce biological response in cell.

Because of the striking role of GPCRs and whole genome sequence determined in diversity species, 948 GPCRs in human that encode >2% of the total genes of human genome (141) and ~1000 olfactory receptors in mice (142) have been reported. The GPCRs are divided into different subfamilies based on their sequence homology and similar function. The largest class, family A, consists of light (rhodopsin) receptors and adrenaline (adrenergic) receptors with a highly conserved arginine in the Asp–Arg–Tyr (DRY in single-letter amino acid code) motif at the cytoplasmic side of the third transmembrane domain (TM3). The family B receptors are targets of peptide hormones and neuropeptides and comprise. Family C consists of GPCRs with an exceptionally large N-terminus, such as metabotropic glutamate receptors and g-amino butyric acid (GABA) receptors. In addition there are three smaller classes of yeast pheromone receptors (family D and E) and cAMP receptors (family F).

G protein-coupled receptors are found in eukaryotes, including yeast, plants, choanoflagellates, and animals. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. In all animals, GPCRs and their ligands steer important physiological processes such as development, reproduction, feeding, and behavior.

There are, at present, highly exciting development of next generation sequencing occurring within the field of insect research, the genome of fruitfly *Drosophila melanogaster* (143), the malaria mosquito *Anopheles gambiae* (144), and *Culex quinquefasciatus* (145) (Diptera) and that from the silkworm *Bombyx mori* (146) (Lepidoptera) have recently been sequenced. Especially, 276 GPCRs have been detected in *Anopheles gambiae* (147).

Olfaction plays a major role in host preference and blood feeding, integral behaviors for disease transmission by the malaria vector mosquito *Anopheles gambiae*. Insects are an attractive system in which to study olfaction because they display a rich repertoire of olfactory driven behaviours under the control of a nervous system that is much simpler than that of mammals. For example, the malaria mosquito, *Anopheles gambiae*, locates human hosts for blood feeding through its ability to detect multiple chemical cues emanating from our skin, including lactic acid and ammonia, and carbon dioxide in our breath. The literature contains contradictory claims that insect olfaction uses cAMP, cGMP, or IP3 as second messengers; that insect odorant receptors couple to Gas or Gaq pathways; and that insect odorant receptors are G-protein-coupled receptors or odor-gated ion channels. Here we consider all the evidence and offer a consensus model for a noncanonical mechanism of olfactory signal transduction in insects (149).

In insects, several GPCRs can regulate physiological pathways (150) and affect insect behavior (151), reproduction (152), development (153; 154), and metabolism (155). A large number of

neuropeptides has been identified in the brain of insects. Moreover, GPCRs also have been characterized in various insect species, revealing 276 GPCRs in *Anopheles gambiae* (156), 135 non-sensory and opsin GPCRs in *Aedes aegypti* (157). ~70 neurohormone GPCRs were found in the red flour beetle (158; 159). There are 56 neurohormone receptor genes in honey bee *Apis mellifera* and 69 in *Drosophila melanogaster* (153). The human body louse, *Pediculus humanus humanus*, has 107 GPCRs in whole genome (160). Pheromone biosynthesis in most moths is regulated by a peptide hormone produced in the subesophageal ganglion, located near the brain. This peptide, termed pheromone biosynthesis-activating neuropeptide (PBAN), which acts on pheromone glands to stimulate pheromone biosynthesis, was first identified as a 33-amino acid (aa) C-terminal amidated peptide. Action of PBAN requires binding to a receptor in pheromone gland cells causing an extracellular calcium influx that promotes the production of cAMP (161). The involvement of a G-protein was determined pharmacologically (162).

6.3 Hypothetical model of GPCR pathway

G protein-coupled receptors are activated by an external signal in the form of a ligand or other signal mediator, consequently, caused a conformational change in the receptor and activation of G protein. Further effect depends on the type of G protein. In the hypothetical model of GPCR pathway (Fig. 1.1) (163), different type of GPCR could receive the different ligands and active the different G-protein subunits, which could regulate the intracellular cascades, such as adenylyl cyclase, PKA, PKC, etc. All the G-protein subunits and their regulatory cascades could regulate the transcription factors and gene expression, eventually, produce the biological responses, such as proliferation, cell survival, differentiation, migration, etc.

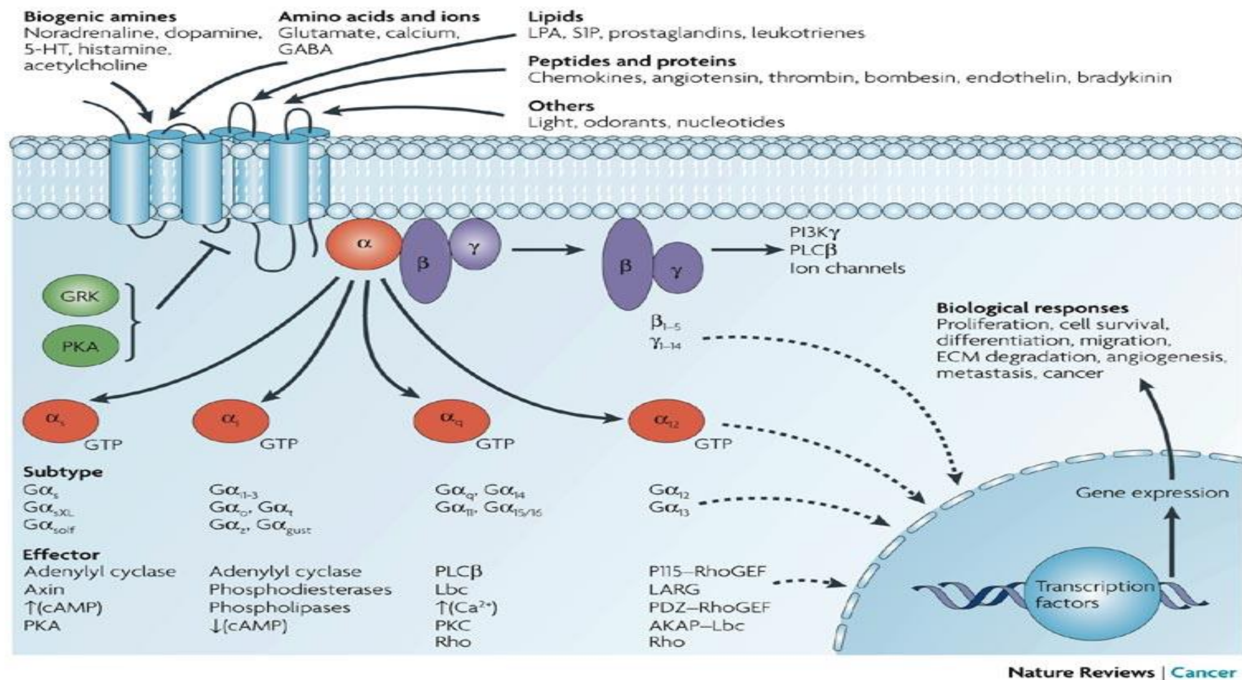


Fig. 1.1 Diversity of G-protein-coupled receptor signaling

However, some evidences began accumulating to suggest that some GPCRs are able to signal without G proteins, such as sensory organs. Sensory organs play primary role to explore the environment and transmit the information to the brain for processing. Both visual and olfactory systems are composed of sensory receptor epithelia with thousands of sensory receptor cell, which means each single sensory receptor gene has specific function (165-167).

Drosophila vision studies provide a model for characterizing the mechanisms underlying rapid responses to sensory stimulation. Compare human and Drosophila eyes different structure, there appear to be important parallels between the mechanisms underlying Drosophila retinal degeneration and human retinal dystrophies (168).

In the Drosophila retina, functional identity of photoreceptors depends on light-sensitive Rhodopsins (*Rhs*). The Drosophila compound eye is composed of approximately 750 simple ommatidia. Each ommatidium contains eight photoreceptor cells named R1–R8. It is widely accepted that individual Drosophila photoreceptors express a single rhodopsin gene: rh1 in R1–

R6, rh3 or rh4 in R7, and rh5 or rh6 in R8. However, present research identified that rh3 and rh4 co-express in R7 but maintain normal exclusion between rh5 and rh6 in R8 (169). Sprecher reported that there is switch mechanism between rh5 and rh6 in Drosophila developmental stages (170). The same rhodopsin fates play different roles in adult and larva stages (171). In current studies, the rhodopsin gene was up-regulated in resistant *Culex* mosquitoes (172, 173). These evidences indicated that the Rhodopsin may play very important role on insecticide resistance in *Cx.* mosquitoes by gene regulation and interaction with other resistance genes.

There are two arrestins expressed in photoreceptor cells, arrestin1 and arrestin2 (174). Otherwise, other genes, like ninaB and ninaD, are essential for rhodopsin, and may regulated to insect development (175). Interestingly, one arrestin, which was reported up-regulation in deltamethrin resistance strain of *Culex pipiens*, involved in the regulation of opsin gene and one P450 gene expression in cell line (176).

6.4 Protein kinases

Protein kinases are a big family of kinase enzymes, which could active or inhibit other proteins by phosphorylation. Protein kinases are involved in the GPCR-regulatory pathway. There are several different classes of protein kinases, however, I review protein kinase A (PKA), AMP-dependent protein kinase (AMPK), and protein kinase C (PKC) in this chapter, because of their regulatory function on P450 gene expression.

Protein kinase A, also known as cAMP-dependent protein kinase, is activated by increased level of cAMP, which is produced by adenylate cyclase (AC). In the signaling transduction pathway, AC is activated by Gs subunit (G protein alpha subunit s), in turn, it is regulated by GPCR(s). PKA is pleiotropic cellular regulators and could affect cell migration (177) and also could regulate the Ca^{+} channel and membrane potential (178). Moreover, because of the regulatory

function of PKA in gene expression on transcriptome level, such as endometrial stromal fibroblast genes (179), P450 gene expression (180-181), response to the cell pH stress in yeast (182), the PKA is developed as medicine target.

Protein kinase C is another family of protein kinase and involved in the GPCR signaling pathway with lipid pathway (183, 184). Moreover, Kamp et al. reported the PKC could regulate calcium channel status (185).

AMP is produced from ATP. So, AMP-dependent protein kinase (AMPK) is a sensor of cellular energy status by monitoring the concentrations of AMP and ATP. McBride A, et al. reviewed the function of AMPK as cellular energy sensor based on the structure of AMPK, indicting the AMP could be the sensor of glycogen for energy detection (186). It may be activated by drugs and xenobiotic, thus, activated AMPK work on the catabolic processes (187). Moreover, AMPK is involved in GPCR regulatory pathway (188), and could be the regulatory factors of the P450 expression (189, 190).

7. Functional Study for Resistance Genes

7.1 RNA interference

RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation or by hindering the transcription of specific genes. The first characterization of RNAi was identified by Fire and Mello in 1998. As the paper says, they tested the phenotypic effect of RNA injected into the worm *C. elegans, and also* they established that injection of double-stranded RNA (dsRNA) has more efficient than single-stranded RNA in loss of the target mRNA. It also indicated that the dsRNA had to correspond to the mature mRNA sequence (191). The targeted mRNA disappeared means that it was degraded and only a few dsRNA molecules per cell were sufficient to accomplish full silencing. The RNAi pathway is initiated by the

enzyme dicer that cleaves long dsRNA molecules into short fragments of 21–23 base pairs. One of the two strands of each fragment is then incorporated into the RNA-induced silencing complex (RISC) and pairs with complementary sequences (Fig. 1.2).

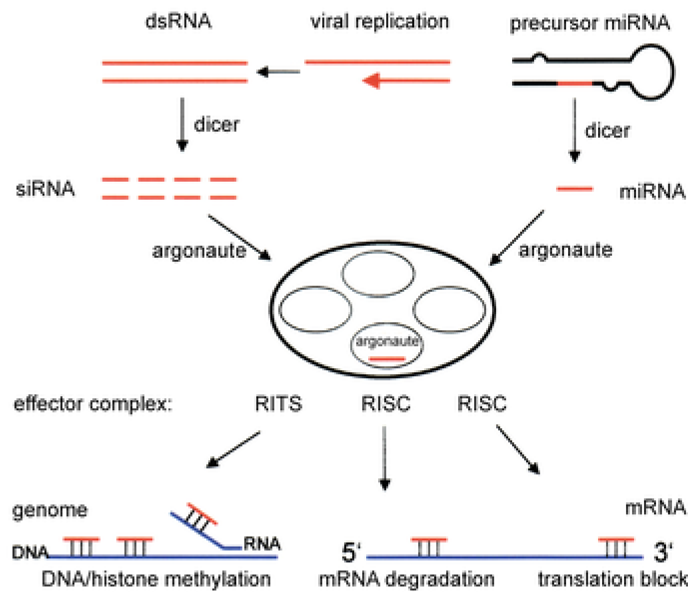


Fig. 1.2 Double-stranded RNA pathways

To date, RNAi has been a powerful tool to identify the gene function in a variety of organisms by knockdown of interested genes and changed phenotypes, such as protozoa, flies, nematodes, insects, parasites and mouse and human cell line. For insect studies, RNAi is a common method for gene functional study. Several studies used the injection as dsRNA uptake method to identify the gene function. dsRNA injection of RXR/USP gene in cockroach resulted in inhibition of molting process (192). Embryo injection of Pyralid moth with the dsRNA showed the loss of eye-color pigmentation (193). Bai, et al. injected the dsRNA of 111 GPCR genes in the larva of red flour beetle, showing 8 of them were involved in beetle development and metamorphosis (194). siRNA injection with Coo_2 gene in the adult of pea aphids showed the reduced life-span of aphid compared with the GPF-injection (195). In another way, scientists used other way to treat insect with dsRNA, such as feeding in light brown apple moth (196), termite (197), diamondback

moth (198), etc. Thus, knockdown of dsRNA in insect species caused the functional discovery of the new and old genes (199). Today, the RNAi has been applied for insect control by plant expression of dsRNA (200). The transgenic plant engineered to express the dsRNA caused reduced damage of plant by insect (201). In summary, the gene function identification by dsRNA treatment and developed dsRNA application for pest control by plant expression indicated the importance of RNAi as powerfully tool for new strategy of insect control (202).

7.2 Insecticide-Resistance and RNAi

To date, the RNAi technique has been used for studying the function of insecticide resistance related genes. Using the RNAi to knockdown of a resistance-related P450 gene, *CYP6BG1*, in larva of diamondback moth caused the decreased resistance to permethrin (198), and also knockdown of the overexpressed *CYP6BQ9* in red flour beetle resulted in the decreased tolerance to deltamethrin (203). NADPH cytochrome P450 reductase (CPR) is working on the P450 metabolism process, the study, knockdown of the CPR in Anopheles gambiae, showed us the decreased tolerance to permethrin (204). The RNA interference technique also was used to characterization of Drosophila melanogaster cytochrome P450 gene function (205).

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Chapter 2 Research Goal and Objectives

1. Brief introduction and background

The mosquitoes of *Culex quinquefasciatus* are a primary vector of pathogens, such as encephalitis, lymphatic filariasis (1). Insecticides are the major strategy to control mosquito vectors and their associated diseases. However, mosquito developed resistance to insecticides, especially pyrethroids, has caused the rise of mosquito-borne diseases worldwide (2). Multiple mechanisms of insecticide resistance have been characterized in mosquitoes, such as target site insensitivity of mutation-linked sodium channel gene (3) and increased cytochrome P450 mediated detoxification of insecticides, resulting from increased expression of P450 genes and gene products (4).

Previous studies showed that the *cis*- and *trans*-regulatory factors were associated with the elevated mRNA levels of P450s, such as *Cyp6d1* in house fly (5) and *Cyp6a8* in *Drosophila* (6). However, the specific regulatory factors responsible for insecticide resistance are largely undetermined in insects, especially in mosquitoes. G-protein coupled receptors (GPCRs) are the largest supergene family of transmembrane protein involved in signal transduction (7). The whole genome sequence of insects has released several GPCRs in *Cx. quinquefasciatus* (8), *Anopheles gambiae*, *Drosophila melanogaster*, *Bombyx mri*, and *Apis mellifera* (9). GPCRs are classified into different classes based on their sequence homology and function similarity. GPCRs in mosquito *A. aegyptin*, as an example, were classified into 4 classes, including Rhodopsin-like, Secretin-like, Metabotropic glutamate-like, and Atypical GPCRs (10). In

human, GPCRs and their ligands could be steering cell cascades and produce several biological responses, such as cell survival, migration, proliferation (11). In insects, GPCRs are involved in development, reproduction, metabolism, etc. Furthermore, the gene up-regulation of some GPCRs has been identified in insecticide resistance mosquitoes (12, 13). However, no more investigation shows their function in insecticide resistance. Protein kinases play critical role in GPCR-regulatory pathways (11), and also they are involved in the P450 gene expression in different organism species (14-15). Thus, we hypothesize that GPCRs are involved in the insecticide resistance by regulating the resistance-related P450 gene expression through the protein kinase regulation pathway (Fig. 2.1).

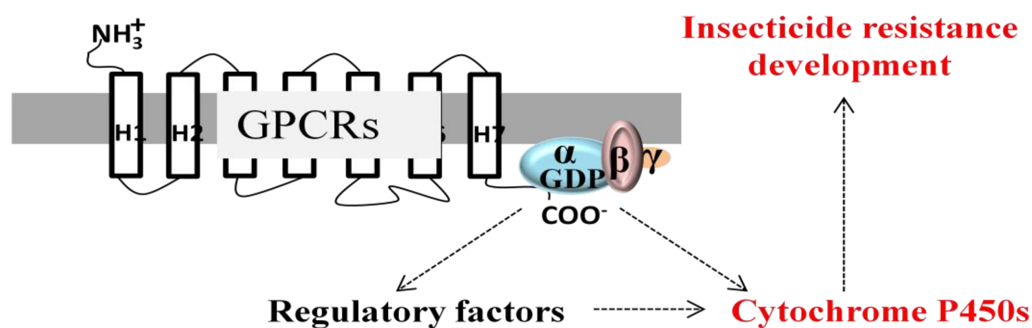

Fig. 2.1 Hypothetical mode of GPCR regulatory pathway involved in the insecticide resistance of mosquitoes, *Culex quinquefasciatus*

2. Project Long-term Goal and Objectives

To characterize the multiple mechanisms and the hypothesized GPCR functions in insecticide resistance, our long term goal of research is to determine the molecular basis of P450-mediated detoxification, sodium channel insensitivity, and regulatory function of GPCRs in the insecticide resistance of mosquitoes, *Culex quinquefasciatus*.

To achieve our long term goal, four objectives will be conducted in the current proposed project: 1) Determination of the inheritance of insecticide resistance in *Culex quinquefasciatus*; 2)

Characterization of the relationship between P450 gene expression and permethrin resistance in different *Culex* mosquito strains; 3) Identification the function of multiple mutations in the sodium channel of permethrin resistant mosquitoes; 4) Demonstration of the regulatory function of GPCR signaling pathways in resistance-related P450 gene expression through the protein kinase pathways in resistant mosquitoes.

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Chapter 3 Genetics and Inheritance of Permethrin Resistance in the Mosquito *Culex quinquefasciatus*

Abstract: The toxicity of permethrin to different life stages of *Culex quinquefasciatus* was examined in 3 field insecticide resistant strains, $HAmCq^{G0}$, $MAmCq^{G0}$ and $BAmCq^{G0}$, 2 permethrin selected strains, $HAmCq^{G8}$ and $MAmCq^{G6}$, and a laboratory susceptible strain, S-Lab. The levels of tolerance to permethrin differed among the developmental stages of *Culex* mosquito larvae with an ascending order from 1st to 4th instar, suggesting that physiological factors, such as size and weight, are involved in the sensitivity of mosquitoes to insecticides. A developmentally regulated pattern of permethrin resistance in *Culex* mosquito larvae was identified, once again increasing from 1st to 4th instar. Adult mosquitoes of $HAmCq^{G0}$, $MAmCq^{G0}$, $BAmCq^{G0}$, $HAmCq^{G8}$, and $MAmCq^{G6}$ had relatively lower levels of resistance in general compares with their larval stages, suggesting different mechanisms may be involved in the response to insecticide pressure for larvae and adult mosquitoes. Inheritance of resistance to permethrin was examined using reciprocal crosses between resistant $HAmCq^{G8}$ and susceptible S-Lab strains; the resulting logarithm (log) concentration-probit response curves for F_1 lines were similar to those for the susceptible strain, with degree of dominance values of -0.2 to -0.3 for F_1SH (S-Lab♂ x $HAmCq^{G8}$♀) and F_1HS (S-Lab♀ x $HAmCq^{G8}$♂), respectively, suggesting that the inheritance of permethrin resistance in *Cx. quinquefasciatus* is incompletely recessive. No significant difference was found in the values of LC_{50} and the slope of the log concentration-probit lines between F_1SH and F_1HS, indicating that the resistance to permethrin was inherited

autosomally. The chi-square (X^2) goodness-of-fit test and the log dose-probit line analysis on responses of backcross and self-bred progenies to permethrin indicated that permethrin resistance in the HAmCq^{G8} strain did not follow a monogenic gene inheritance model, revealing that permethrin resistance in *Cx. quinquefasciatus* is controlled by more than one gene.

Key Words Permethrin resistance, *Culex quinquefasciatus*, genetics, inheritance model

MOSQUITOES ARE KNOWN as vectors of parasites and pathogens of human and animal diseases and their control is an important part of the current global strategy to control mosquito-associated diseases (World Health Organization (WHO) 2000). Insecticides are the most important component in the vector-control effort, and pyrethroids are currently the most widely used insecticides for indoor spraying for mosquitoes worldwide (Zaim et al. 2000, Najera and Zaim 2001, McCarroll and Hemingway 2002). However, the widespread growth of resistance to insecticides in mosquitoes, especially to pyrethroids, is rapidly becoming a global problem, resulting in a rise in mosquito-borne diseases in many parts of the world (Phillips 2001, Hemingway and Vontas 2002, Liu 2008).

The mosquito *Culex quinquefasciatus* is a primary vector of West Nile encephalitis, Eastern equine encephalitis, Saint Louis encephalitis, and lymphatic filariasis pathogens (Sardelis et al. 2001, Jones et al. 2002, Reisen et al. 2005, WHO 2009). Insecticide applications play an important role in controlling *Culex* mosquitoes (Liu et al. 2004, 2009), but mosquitoes develop resistance to insecticides, such as pyrethroids, relatively quickly (Phillips 2001, Hemingway et al. 2002, Liu et al. 2004, 2005, 2009), thus reducing the effectiveness of those insecticides for mosquito control. Resistance is assumed to be a pre-adaptive phenomenon, in that prior to insecticide exposure rare individuals already exist who carry an altered genome that results in one or more possible mechanisms (factors) allowing survival from the selection

pressure of insecticides (Sawicki and Denholm 1984, Brattsten et al. 1986). The development of resistance in insects depends upon the level of genetic variability in a population (Liu and Scott 1995, Liu and Yue 2001). Efforts to characterize the genetics involved in the inheritance of insecticide resistance have therefore been fundamental in understanding the development of resistance and studying resistance mechanisms, as well as in practical applications such as designing novel strategies to prevent or minimize the spread and evolution of resistance development and control insect pests (Roush et al. 1990).

A mosquito strain of *Cx. quinquefasciatus*, $HAmCq^{G0}$, from Huntsville, Alabama, has been established in the laboratory (Liu et al. 2004b) and further selected with permethrin for 8 generations resulting in a strain $HAmCq^{G8}$ with a much higher level of resistance to permethrin than the parental strain, $HAmCq^{G0}$ (Xu et al. 2006, Li et al. 2009). In an effort to better understand the genetics involved and precisely how the pyrethroid resistance is inherited, and thus gain valuable insights into the development of permethrin resistance in *Culex* mosquitoes, in the study reported here we examined the genetic inheritance of permethrin resistance in *Culex* mosquitoes by crossing the resistant $HAmCq^{G8}$ strain with the susceptible S-Lab strain. The toxicity of permethrin was tested in mosquitoes at different stages of development, and in their F_1 and F_2 offspring, and backcross progeny.

Materials and Methods

Mosquitoes. Six strains of *Culex quinquefasciatus* were used in this study: three field strains, namely $HAmCq^{G0}$, $MAmCq^{G0}$ and $BAmCq^{G0}$, collected from Madison County, Mobile County, and Jefferson County, Alabama, respectively; $HAmCq^{G8}$, the 8th generation of permethrin-selected $HAmCq^{G0}$ offspring; $MAmCq^{G6}$, the 6th generation of permethrin-selected $MAmCq^{G0}$ offspring; and S-Lab, an insecticide susceptible strain from Dr. Laura Harrington

(Cornell University). All the mosquitoes were reared at 25±2°C under a photoperiod of 12:12 (L:D) h (Nayar and Knight 1999) and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

Bioassays. The stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) were prepared in acetone. The bioassay method for larvae was as described in our previous studies (Liu et al. 2004a, 2004b; Liu et al. 2009, Li et al. 2009); each bioassay consisted of 30 4th instar mosquito larvae in 6 oz. Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with regular tap water and 1% insecticide solution in acetone at the required concentration, with four or five concentrations that resulted in >0 and <100% mortality. Control groups received only 1% acetone. Mortality was assessed after 24 h.

For the adult bioassays, the topical application was performed as described by Liu and Yue (2001); a 0.5-μl drop of insecticide in acetone was delivered to the thoracic notum of each two-day-old mosquito adult with a 25- μl Hamilton gastight syringe (Fisher Scientific). Male and female adults, which were separated at <8-hr post-emergence and reared to 2 days old, from each *Cx. quinquifasciatus* mosquito strain were used in this study. Each bioassay consisted of 20 mosquitoes per dose and four or five doses that yielded >0 and <100% mortality. Control groups received acetone alone. Treated mosquitoes were reared in 6-oz Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with 10% sugar water on cotton. Mortality was assessed at 24 h post-treatment. The criterion for death was the mosquitoes' inability to move. All tests were run at 25°C and replicated at least three times.

Permethrin selection. The selection procedure was used as reported previously for HAmCq mosquitoes (Xu et al. 2006). Briefly, MAmCq^{G0} mosquitoes were selected with permethrin for six generations in the laboratory after collection, generating the MAmCq^{G6} strain.

The concentration (Table 3.1) for each selection was sufficient to kill ~60% of treated individuals in each generation after 24 h. Toxicity of permethrin was analyzed for each generation before and after selection.

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

Strains	Selected generation	Selecting concentration (ppm)	n[a]	Survival (%)	LC_{50}[b] (CI)[c] (ppm)	RR[d]	Slope (SE)
MAmCq	G0[e]				0.01 (0.01-0.02)	1.4	2.1 (0.3)
	G1	0.01	~4000	~30%	0.04 (0.02-0.08)	5.7	1.9 (0.2)
	G2	0.04	~4000	~30%	0.06 (0.02-0.1)	8.6	1.7 (0.2)
	G3	0.06	~4000	~30%	0.8 (0.5-1.1)	110	1.8 (0.2)
	G4	0.7	~4000	~30%	1.8 (1.3-2.6)	260	1.6 (0.2)
	G5	1.5	~4500	~20%	2.7 (1.5-4.2)	390	2.1 (0.3)
	G6	2.5	~4500	~30%	4.1 (3.0-5.6)	590	2.5 (0.4)
S-Lab					0.007 (0.005-0.08)	1	3.4 (0.6)

[a] Number of selected fourth instar larvae

[b] LC_{50} values in ppm

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

[d] RR: LC_{50} of the resistant strain/LC_{50} of the S-Lab strain

[e]Parental strain before permethrin selection

Bioassay data analysis. Bioassay data were pooled and analyzed by standard probit analysis, as described by Liu et al. (2004 a, b) with a computerized version of Raymond (1985). Statistical analysis of LC_{10} or LD_{10}, LC_{50} or LD_{50}, and LC_{90} or LD_{90} values was based on non-overlapping 95% confidence intervals (CI). Resistance ratios (RRs) were calculated by dividing the LC_{50} of the resistant field strains by the LC_{50} of the susceptible S-Lab strain.

Genetic crosses. For the genetic linkage study, reciprocal crosses were carried out between resistant $HAmCq^{G8}$ and susceptible S-Lab strains of *Cx. quinquefasciatus* as described by Liu and Yue (2001). The virgin female mosquitoes used in the crosses were isolated every 8 h. Two F_1 reciprocal cross lines, F_1SH (S-Lab♂ x $HAmCq^{G8}$♀) and F_1HS (S-Lab♀ x $HAmCq^{G8}$♂) were generated. Backcrosses of the reciprocal F_1 progenies of F_1SH and F_1HS to the parental strains of S-Lab and $HAmCq^{G8}$ were conducted and two BC_1 lines - BC_{1a} (F_1SH♂ x S-Lab♀) and BC_{1b} (F_1SH♂ x $HAmCq^{G8}$♀) were produced. The self-bred lines of the reciprocal progeny were F_{2a} (F_1SH ♂ x F_1SH ♀) and F_{2b} (F_1HS♂ x F_1HS♀).

The degree of dominance (D) levels of the permethrin resistance in the F_1s were calculated according to Stone (1968) and Liu and Scott (1997) using the logarithm of the LC_{50} values. The degree of dominance values ranged from - 1 to + 1, where $D = -1$ indicates the trait is completely recessive, $-1 < D < 0$ indicates it is incompletely recessive, $0 < D < 1$ indicates it is incompletely dominant, and $D = 1$ indicates it is completely dominant.

The number of genes that are potentially involved in the resistance of *Culex* mosquitoes to permethrin was estimated based on the responses of the backcross progeny, BC_{1a} (F_1SH♂ x S-Lab♀) and BC_{1b} (F_1SH♂ x $HAmCq^{G8}$♀), and self-breeding lines (F_2s) to permethrin. Two analysis methods were used in this study: the chi-square (χ^2) goodness-of-fit test and the log

dose-probit line analysis. In the χ^2 goodness-of-fit test, the null hypothesis of monogenic resistance was tested on the basis of chi-square goodness-of-fit between the observed and the theoretical expectation mortality by comparison of the expected and observed χ^2 values (Sokal and Rohlf 1981, Keena and Granett 1990, Preisler et al. 1990, Zhang et al. 2008). The χ^2-tests were performed using the equation (Preisler et al. 1990): $\chi_i^2 = (Oi - Ei)^2 / Ei + (Oi' - Ei')^2 / Ei'$, where Oi is the number of observed responses (mortality), Ei is the number of expected responses; Oi' is the number of observed null responses (survival) in all subjects and Ei' is the number of expected null responses. χ^2 is equal to $\sum_i^N \chi i^2$. In the χ^2 goodness-of-fit test, if the observed and expected χ^2 values for BC_1s or F_2s were not significantly different, the null hypothesis would be accepted, i.e., the resistance would be considered to be monogenic heredity, and if not, polygenic (Sokal and Rohlf 1981, Preisler et al. 1990, Zhang et al. 2008, He et al. 2009). In the log dose-probit line analysis, the null hypothesis of monogenic resistance was tested on the basis of the observed and the theoretically expected log dose-probit lines (Keena and Granett 1990). If a significant difference between the observed and expected log dose-probit curves for BC_1s or F_2s was detected, the null hypothesis of monogenic resistance would be rejected and more than one factor would be likely involved in resistance (Georghiou 1969, Keena and Granett 1990).

Results and Discussion

Permethrin Selection of the $MAmCq^{G0}$ Population. In previous selections of the $HAmCq^{G0}$ strain with permethrin in the laboratory for 8 generation, we found that resistance to permethrin in $HAmCq^{G0}$ develops rapidly (Xu et al. 2005). To investigate whether the rapid development of resistance is a general phenomenon in field *Culex* mosquito populations, we conducted a permethrin selection study on another field population of *Culex* mosquitoes,

MAmCq^{G0}, from Mobile County, Alabama. The level of resistance to permethrin in the field collected parental MAmCq^{G0} strain was 1.4-fold compared with the susceptible S-Lab strain (Table 3.2). After six generations of selection with permethrin, the level of resistance in MAmCq^{G6} increased to 590-fold compared with S-Lab (Table 3.2). The changes in permethrin resistance between the parental and selected mosquitoes suggest that resistance to permethrin in *Cx. quinquefasciatus* mosquitoes does indeed develop rapidly. The slope of the dose-response curve was also noticeably steeper for the MAmCq^{G6} strain after selection (Table 3.2), indicating that MAmCq^{G6} were much more homogeneous for resistance after selection compared with the parental strain MAmCq^{G0}. The lower level of resistance to permethrin and the gradual slope of the dose-response curve in the field populations of MAmCq^{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). Similar results have also been reported by Xu et al. (2005).

Toxicity of permethrin to *Culex quinquefasciatus*. We tested the response of mosquitoes at different life stages to permethrin treatments. First the toxicity of permethrin to the larvae of *Culex quinquefasciatus* was tested. The results revealed that the levels of tolerance to permethrin were different among the developmental stages of the mosquito larvae, with a ascending order of 1st instar < 2nd instar < 3rd instar < 4th instar, suggesting that physiological factors such as such as size and weight are involved in the sensitivity of mosquitoes to insecticides. However, there was also a developmentally regulated pattern of permethrin resistance in *Culex* mosquito larvae: the first instar larvae of the field *Culex* mosquito strains, HAmCq^{G0}, MAmCq^{G0} and BAmCq^{G0}, had no or relatively low levels of resistance to permethrin, with resistance ratios of 1, 4 and 10, respectively, to permethrin at LC_{50} compared with the susceptible S-Lab strain (Table 3.2). In

contrast, the first instar larvae of $HAmCq^{G8}$ and $MAmCq^{G6}$, the 8th and 6th generations of permethrin-selected $HAmCq^{G0}$ and $MAmCq^{G0}$ offspring, respectively, exhibited elevated levels of resistance compared to their parental strains, with resistance ratios of 100 and 40, respectively, to permethrin. The second instar larvae of $HAmCq^{G0}$, $MAmCq^{G0}$, and $BAmCq^{G0}$ showed increased levels of resistance, with resistance ratios of 3, 5 and 45 to permethrin, respectively; whereas $HAmCq^{G8}$ and $MAmCq^{G6}$ had 700- and 100-fold levels of resistance to permethrin. The third instar larvae of $HAmCq^{G0}$, $MAmCq^{G0}$ and $BAmCq^{G0}$ showed further increased levels of resistance with resistance ratios of 2.5, 5 and 50 to permethrin, while the permethrin selected strains of $HAmCq^{G8}$ and $MAmCq^{G6}$ had 850- and 250-fold levels of resistance, respectively. In the final (fourth) instar larvae, resistance to permethrin reach its maximum levels in all mosquito strains, at 10-, 10-, and 86-fold, respectively, in $HAmCq^{G0}$, $MAmCq^{G0}$ and $BAmCq^{G0}$, and 2700- and 570-fold, respectively, in $HAmCq^{G8}$ and $MAmCq^{G6}$ (Table 3.2). These results may suggest an optimum timing for the control of mosquitoes with insecticides, i.e., in the lower instar larval stages when resistance is at its lowest.

Table 3.2. Toxicity of permethrin to the larvae of *Cx. quinquefasciatus*

Stages	Strain	df	n[a]	χ^{2b}	LC_{50}[c] (CI)[d]	RR[e]	Slope (SE)
1st instar	S-Lab	4	180	2.2	0.001 (0.001-0.002)	1	1.7 (0.2)
	$HAmCq^{G0}$	4	180	6.4	0.001 (0.001-0.003)	1	1.6 (0.2)
	$HAmCq^{G8}$	5	220	3.5	0.1 (0.07-0.2)	100	1.7 (0.2)
	$MAmCq^{G0}$	4	180	0.8	0.004 (0.003-0.006)	4	1.7 (0.2)
	$MAmCq^{G6}$	3	150	6.8	0.04 (0.02-0.1)	40	1.6 (0.2)
	$BAmCq^{G0}$	4	180	3.8	0.01 (0.006-0.02)	10	1.1 (0.1)

2 nd instar	S-Lab	3	150	0.5	0.002 (0.002-0.003)	1	3.3 (0.5)
	$HAmCq^{G0}$	3	150	3.7	0.006 (0.004-0.01)	3	2.4 (0.4)
	$HAmCq^{G8}$	4	180	6.8	1.4 (0.7-2.7)	700	2.4 (0.2)
	$MAmCq^{G0}$	4	180	2.9	0.01 (0.009-0.02)	5	1.5 (0.2)
	$MAmCq^{G6}$	4	180	2.9	0.2 (0.09-0.2)	100	2.0 (0.2)
	$BAmCq^{G0}$	3	150	0.5	0.09 (0.07-0.1)	45	2.7 (0.4)
3rd instar	S-Lab	3	150	3.1	0.002 (0.001-0.003)	1	2.8 (0.5)
	$HAmCq^{G0}$	2	120	0.2	0.005 (0.003-0.007)	2.5	2.8 (0.4)
	$HAmCq^{G8}$	3	150	0.7	1.7 (1.0-2.0)	850	2.8 (0.4)
	$MAmCq^{G0}$	4	180	4.3	0.01 (0.008-0.02)	5	1.7 (0.2)
	$MAmCq^{G6}$	3	150	2.8	0.5 (0.3-0.6)	250	2.3 (0.3)
	$BAmCq^{G0}$	4	180	8.5	0.1 (0.04-0.3)	50	1.8 (0.2)
4th instar	S-Lab	3	150	1.0	0.007 (0.005-0.08)	1	3.4 (0.6)
	$HAmCq^{G0}$	3	150	1.3	0.07 (0.04-0.1)	10	2.4 (0.4)
	$HAmCq^{G8}$	3	150	5.9	19 (10-33)	2700	3.1 (0.5)
	$MAmCq^{G0}$	3	150	2.5	0.07 (0.04-0.1)	10	2.1 (0.3)
	$MAmCq^{G6}$	3	123	2.5	4.0 (3.0-5.6)	570	2.5 (0.4)
	$BAmCq^{G0}$	3	150	10	0.6 (0.2-1.2)	86	2.0 (0.3)

[a] Number of mosquito larvae tested

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

[c] LC_{50} values in ppm

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

[e] RR: LC_{50} of the resistant strain/LC_{50} of the S-Lab strain

We further tested the toxicity of permethrin to the adults of *Cx. quinquefasciatus* and found that female mosquitoes had 7.5- to10-fold less sensitivity to permethrin than their male counterparts in the mosquito strains tested (Table 3.3). Nevertheless, comparing the LD_{50} of both male and female adult mosquitoes of each of the 3 field strains with S-Lab mosquitoes of the corresponding gender revealed that both the males and females of each field mosquito strain had similar levels of resistance to permethrin (Table 3.3). Both males and females of $HAmCq^{G0}$, and $MAmCq^{G0}$ mosquitoes had 2.5- and 1.5-fold, respectively, elevated level of tolerance to permethrin compared with those of the S-Lab strain, while males and females of $BAmCq^{G0}$ had 3- and 4-fold, respectively, levels of tolerance to permethrin. Based on the results of bioassays on house flies and German cockroaches, Scott and Wen suggested that an insect could be referred to as cross-resistance when a resistance ratio was >4 (Scott and Wen 1997). Accordingly, the less than 4-fold lower sensitivity to permethrin in the adult mosquitoes of all 3 field population compared with S-Lab would be considered a tolerance to permethrin rather than resistance. In contrast, both males and females of permethrin selected $HAmCq^{G8}$ and $MAmCq^{G6}$ mosquito strains exhibited markedly greater levels of resistance to permethrin compared with their parental strains, $HAmCq^{G0}$ and $MAmCq^{G0}$, respectively. Both males and females of $HAmCq^{G8}$ had a 100-fold greater level of resistance, respectively, whereas, both males and females of $MAmCq^{G6}$ had a 15-fold greater level of resistance.

Adult mosquitoes of the three field populations, $HAmCq^{G0}$, $MAmCq^{G0}$ and $BAmCq^{G0}$, had relatively lower levels of resistance in general (Table 3.3). A similar result was also found in

permethrin selected mosquitoes, $HAmCq^{G8}$, $MAmCq^{G6}$, where again the adult mosquitoes showed relatively lower levels of resistance compared with the larval stages (Tables 3.2 and 3.3). The $HAmCq^{G8}$ and $MAmCq^{G6}$ strains were generated by selecting the 4th instar larvae of $HAmCq^{G0}$ and $MAmCq^{G0}$ with permethrin for 8 and 6 generations, respectively. It therefore seems possible that this phenomenon may arise not solely through exposure to different selection pressure, in this case the frequency and dose of insecticide, between larvae and adults in the field populations of mosquitoes, but also different mechanisms may be involved in the response to insecticide pressure for larvae and adult mosquitoes. Multiple mechanisms have been identified that may be involved in permethrin resistance in *Culex* mosquitoes (Liu et al. 2005, Xu et al. 2005). More recently, multiple genes were found to be overexpressed in resistant *Culex* mosquitoes (Liu et al. 2007), the expression of which are developmentally regulated (Li et al. unpublished data). To further test our hypothesis that multiple genes or mechanisms are involved in the development of insecticide resistance in *Culex* mosquitoes, the following genetic inheritance studies were conducted.

Table 3.3. Toxicity of permethrin to the adults of *Cx. quinquefasciatus*

Strain		df	n[a]	χ^{2b}	LD_{50}[c] (CI)[d]	F:M Ratio[e]	RR[f]	Slope (SE)
S-Lab	Female	5	200	2.9	0.002 (0.001-0.004)	10	1.0	1.8 (0.3)
	Male	3	180	0.5	0.0002 (0.0001-0.0004)	1	1 .0	2.4 (0.8)
$HAmCq^{G0}$	Female	4	220	2.9	0.005 (0.001-0.009)	10	2.5	1.9 (0.2)
	Male	3	160	2.9	0.0005 (0.0004-0.001)	1	2.5	1.9 (0.5)
$HAmCq^{G8}$	Female	5	240	2.3	0.2 (0.08-0.3)	10	100	1.4 (0.6)
	Male	3	180	1.0	0.02 (0.01-0.03)	1	100	1.9 (0.3)

$MAmCq^{G0}$	Female	3	150	0.9	0.003 (0.002-0.003)	10	1.5	1.8 (0.2)
	Male	4	180	2.4	0.0003 (0.0002-0.0005)	1	1.5	1.5 (0.2)
$MAmCq^{G6}$	Female	3	180	6.0	0.03 (0.007-0.04)	10	15	2.1 (0.3)
	Male	3	180	3.9	0.003 (0.001-0.005)	1	15	1.6 (0.2)
$BAmCq^{G0}$	Female	4	160	1.4	0.006 (0.003-0.007)	7.5	3.0	1.9 (0.3)
	Male	5	200	2.0	0.0008 (0.0004-0.001)	1	4.0	1.4 (0.2)

[a] Number of mosquitoes tested

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

[c] LD_{50} values in µg/mosquito

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

[e]F:M Ratio: LD_{50} of females /LD_{50} of males in each strain

[f] RR: LD_{50} of the resistant strains/LD_{50} of the S-Lab strain

Inheritance of resistance to permethrin in *Cx. quinquefasciatus*. The inheritance of resistance in *Culex* mosquitoes was characterized by logarithm (log) concentration response analyses of permethrin against the 4th instar larvae of F_1 progeny, F_1SH (S-Lab♂ x $HAmCq^{G8}$♀) and F_1HS (S-Lab♀ x $HAmCq^{G8}$♂), and their parental strains of S-Lab and $HAmCq^{G8}$. Comparison of the log concentration-response curves in F_1 progeny from reciprocal crosses with their parental strains of S-Lab and $HAmCq^{G8}$ revealed straight and parallel log concentration-probit lines with similar slopes between S-Lab and $HAmCq^{G8}$ (Fig. 3.1, Table 3.4). This indicates that the S-Lab and $HAmCq^{G8}$ strains were relatively homogeneous for susceptibility and resistance, respectively, to permethrin. The LC_{50} and slope values of permethrin in F_1 progeny (F_1SH and F_1HS) from reciprocal crosses of S-Lab and $HAmCq^{G8}$ were not significantly

different, indicating that the resistance to permethrin was autosomally inherited in the mosquito *Cx. quinquefasciatus*. These results are consistent with the findings of previous studies on pyrethroid resistant house flies (Scott et al. 1984, Liu and Scott 1995, Liu and Yue 2001, Zhang et al. 2008), in which the resistance to insecticides was inherited autosomally. However, a recent study on abamectin resistance in *Tetranychus cinnabarinus* indicated a maternal or cytoplasmic effect in the inheritance of resistance (He et al. 2009). The discrepancy between findings of pyrethroid resistance in mosquitoes and house flies and abamectin resistance in *Tetranychus cinnabarinus* may suggest either the involvement of different genetic mechanisms for insecticide resistance in these species or that the mechanisms of inheritance are different between different types of insecticides.

The degree of dominance (D) of resistance for each of heterozygous F_1s from the reciprocal crosses was calculated according to the index used by Stone (1968) and Liu and Scott (1997) (Table 3.4), where, as explained earlier, the dominance (D) was scored along a scale from 1.0 (completely dominant) to –1.0 (completely recessive). The D values for the F_1 progeny of F_1SH and F_1HS were -0.2 and -0.3, respectively, both within the range –1 to 0, indicating that the resistance of *Culex* mosquitoes to permethrin is incompletely recessive. This was further supported by the results of the concentration-probit response lines for F_1s from reciprocal crosses, which were more similar to those for S-Lab (Fig. 3.1) than to the line for HAmCq^{G0}. Such an incomplete recessive inheritance characteristic is in agreement with the previous reports on house flies (Liu and Scott 1995, Liu and Yue 2001, Zhang et al. 2008), mosquitoes (Priester et al. 1980, Halliday et al. 1985) and carmine spider mite (He et al. 2009).

Table 3.4. Toxicity of permethrin on susceptible (S-Lab), resistant ($HAmCq^{G8}$), reciprocal progenies (F_1SH and F_1HS), and self-cross (F_2 [F_1SH♂ x F_1SH♀] and F_2' [F_1HS♂ x F_1HS♀]) strains of *Cx. quinquefasciatus*

Cross	n[a]	LD_{50}[b] (CI)[c] (ppm)	RR[d]	Slope	Dominance Level (D)
S-Lab	150	0.007(0.005-0.08)	1	3.4 (0.6)	
$HAmCq^{G8}$	150	19 (10-33)	2700	3.1 (0.5)	
F_1SH	240	0.2 (0.1-0.2)	29	1.7 (0.2)	-0.2
F_1HS	240	0.1 (0.08-0.2)	14	1.7 (0.2)	-0.3

[a]n: Number of fourth instar larvae tested

[b] LC_{50} values in ppm

[c] 95% confidence interval, toxicity of insecticide is considered significantly different when the 95% CI fail to overlap

[d] RR: LC_{50} of the resistant strain/LC_{50} of the S-Lab strain

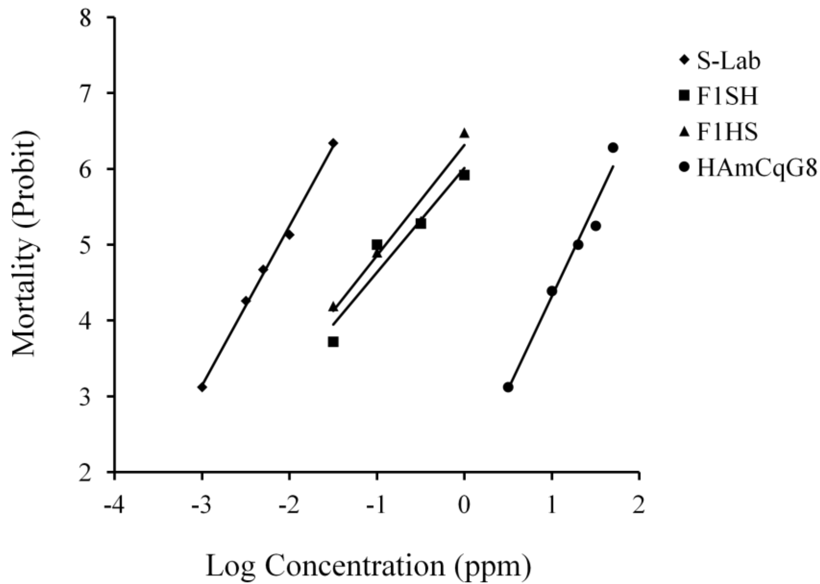

Figure 3.1. Log concentration-probit analysis of permethrin for S-Lab, a susceptible parental strain; HAmCq^{G8}, the 8th generation of permethrin-selected HAmCq^{G0} offspring; and F_1 lines, F_1SH (S-Lab♂ x HAmCq^{G8}♀) and F_1HS (S-Lab♀ x HAmCq^{G8}♂), generated from reciprocal crosses of S-Lab and HAmCq^{G8}.

Characterization of the possible number of genes involved in resistance. In order to characterize the number of genes involved in the development of resistance, permethrin toxicity bioassays were conducted on 4th instar larvae of backcross progeny of BC_{1a} (F_1SH♂ x S-Lab♀) and BC_{1b} (F_1SH ♂ x HAmCq^{G8} ♀) and self-bred lines of F_{2a} (F_1SH ♂ x F_1SH ♀) and F_{2b} (F_1HS♂ x F_1HS♀). The number of genes that are potentially involved in the resistance of *Culex* mosquitoes to permethrin was estimated based on the responses of the backcross progeny and self-bred lines to permethrin. The results of the goodness-of-fit chi-square test (Table 3.5) showed that the observed mortalities of BC_{1a} (F_1SH♂ x S-Lab♀) and BC_{1b} (F_1SH♂ x HAmCq^{G8} ♀) were significantly different from the expected mortalities; the observed χ^2 values were 41 and 34 for BC_{1a} and $BC_{1b,}$ respectively, which were higher than the expected χ^2 value of 9.5 in the

chi-square table (Ramsey and Schafer 2002). The self-cross progenies of F_{2a} and F_{2b} had LC_{50} values of 0.2 and 0.3 ppm, respectively (Table 3.5), and again showed significant differences between the actual and expected mortalities in the chi-square table with χ^2 values of 27 and 28, which were higher than the expected value of 9.5 in the chi-square table. Further, plots of the observed and expected concentration-response data for the backcross progenies showed that the expected log concentration-probit lines for BC_{1a} and BC_{1b}, i.e., BC_{1a}-E and BC_{1b}-E, were completely outside the 95% confidence limit of the observed lines of BC_{1a}-O and BC_{1b}-O (Figs. 3.2 and 3.3).

Table 3.5. The chi-square (χ^2) analysis for inheritance of permethrin resistance in *Cx. quinquefasciatus*

Back cross progenies	n[a]	LC_{50} (95%CL) (ppm)	RR[b]	Slope	Anticipant χ^2 (0.05, 4)	χ^{2c}	df
S-Lab	150	0.007(0.005-0.08)	1	3.4 (0.6)			
BC_{1a} (F_1SH♂ x S-Lab♀)	256	0.04 (0.02-0.1)	6	1.5 (0.23)	9.5	41	4
BC_{1b} (F_1SH♂ x HAmCq^{G8}♀)	264	0.6 (0.4-1.3)	86	1.2 (0.2)	9. 5	34	4
F_{2a} (F_1SH♂ x F_1SH♀)	180	0.2 (0.1-0.4)	29	1.6 (0.2)	9.5	27	4
F_{2b} (F_1HS♂ x F_1HS♀)	181	0.3 (0.1 -0.6)	43	1.1 (0.14)	9.5	28	4

[a] Number of the fourth instar larvae tested

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

[c] Show the value of X^2 calculated from Preisler's formula

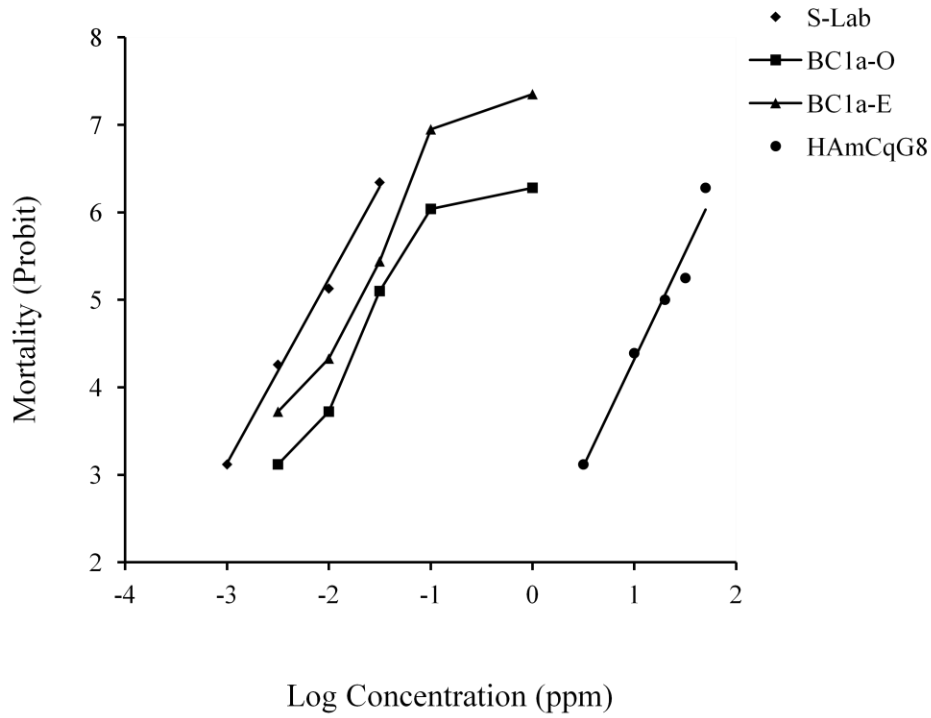

Figure 3.2. Log concentration-probit lines of permethrin for susceptible S-Lab, resistant HAmCq^{G8}, and back-cross progeny of BC_{1a} (F_1SH♂ x S-Lab♀). BC_{1a}-O: observed; BC_{1a}-E: expected.

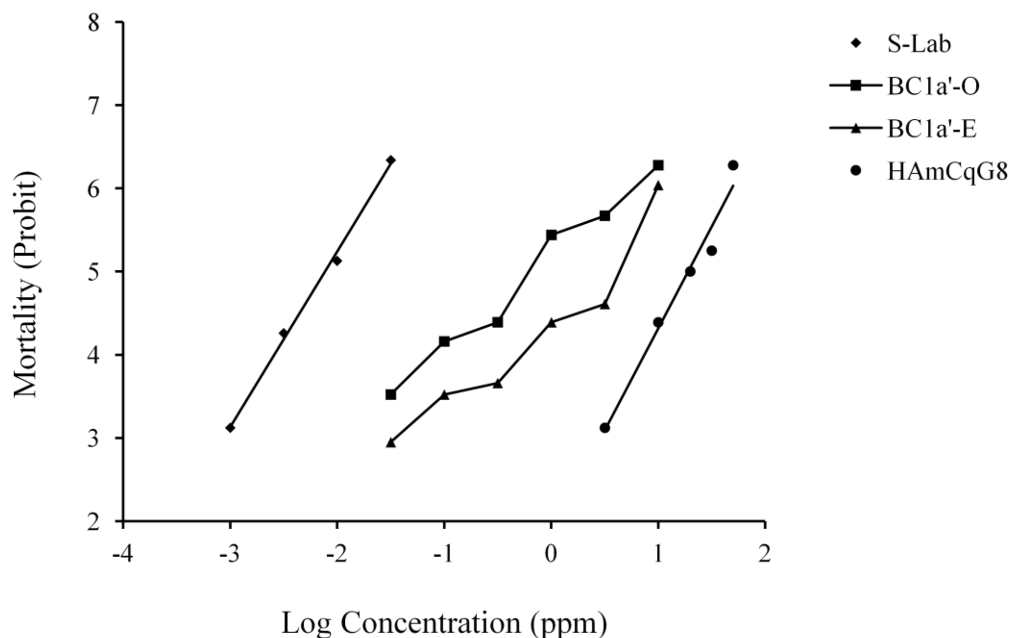

Figure 3.3. Log concentration-probit lines of permethrin for susceptible S-Lab, resistant HAmCq^{G8}, and back-cross progeny of BC_{1b} (F_1SH ♂ x HAmCq^{G8} ♀). BC_{1b}-O: observed; BC_{1b}-E: expected.

Furthermore, the expected log concentration-probit line for F_{2b} was also significantly separated from F_{2b}-O (Fig. 3.4). Both chi-square test and log concentration-probit analysis, therefore, strongly disagree with the monogenic inheritance model (Georghiou 1969, Tsukamoto 1983, Keena and Granett 1990), indicating the inheritance of permethrin resistance in HAmCq^{G8} is controlled by more than one gene.

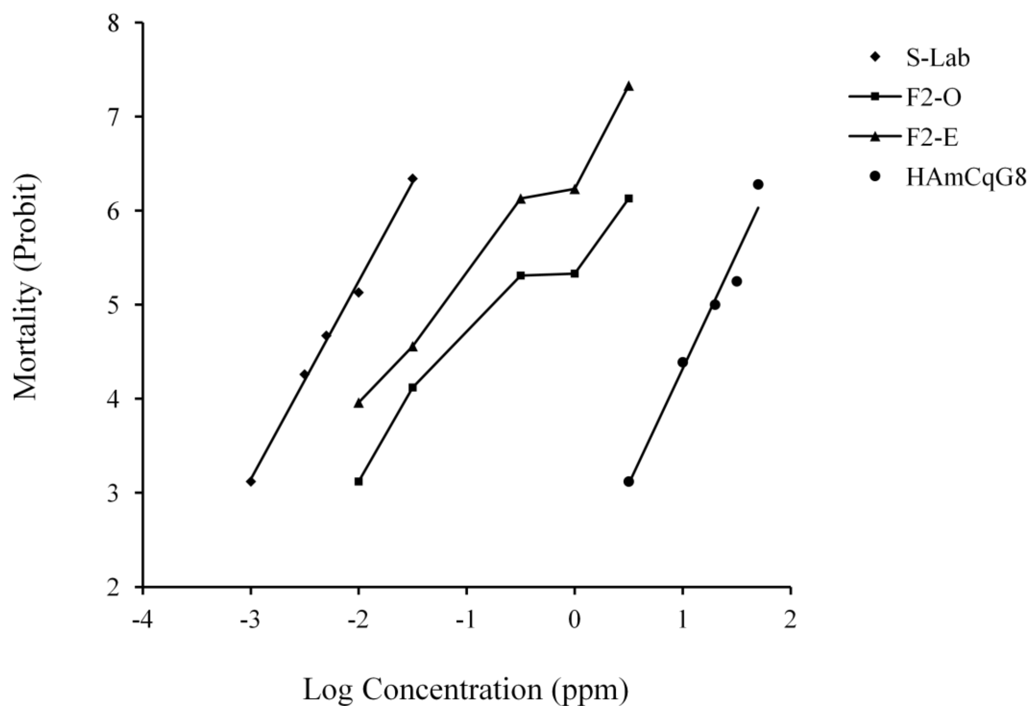

Figure 3.4. Log concentration-probit lines of permethrin for susceptible S-Lab, resistant HAmCq^{G8}, and self-bred progeny of F_{2b} (F_1HS♂ x F_1HS♀). F_{2b}-O: observed; F_{2b}-E: expected.

Multiple gene involvement in insecticide resistance have been reported in other mosquito strains (Priester et al. 1980, Halliday and Georghiou 1985), house flies, *Musca domestica* (Liu and Scott 1995, Liu and Yue 2001), German cockroaches, *Blatella germanica* (Pridgeon et al. 2002), diamondback moths, *Plutella xylostella* (Sayyed et al. 2005), and carmine spider mites, *Tetranychus cinnabarinus* (He et al. 2009). However, monofactorial inheritance in insecticide resistance has also been reported. For example, Davidson (1963) reported that inheritance of DDT resistance in *Aedes* and *Culex* mosquitoes appears to be controlled by a single incompletely recessive factor. Furthermore, a recent study by Zhang et al. (2008) indicated that the high level (4420-fold) of beta-cypermethrin resistance in the house fly, *Musca domestica* was inherited as a single, major, autosomal and incompletely recessive factor. Roush et al. (1986) reported that cypermethrin resistance appeared to be inherited as a single, autosomal, incompletely recessive

gene in the horn fly, *Haematobia irritans*, and permethrin resistance in tobacco budworms, *Heliothis virescens* has been shown to be inherited as a single, major, incompletely recessive autosomal factor (Gregory et al. 1988).

Recently, a synergism study on HAmCq mosquitoes with 3 synergists, piperonyl butoxide (PBO), S,S,S,-tributylphosphorotrithioate (DEF), and diethyl maleate (DEM), revealed that cytochrome P450s esterases and/or GSTs may be primarily involved in detoxifying permethrin and conferring permethrin resistance in HAmCq mosquitoes (Xu et al. 2005). A study on target site insensitivity of sodium channels in the permethrin resistance of HAmCq mosquitoes indicated a strong correlation between the frequency of the L to F (*kdr*) mutation and the level of resistance, suggesting that L to F (*kdr*) mutation-mediated target site insensitivity is an important mechanism in the development of permethrin resistance in HAmCq mosquitoes (Xu et al. 2006). A study comparing the gene expression profiles of resistant and susceptible *Culex* mosquitoes identified multiple genes that were overexpressed in the resistant HAmCq mosquitoes, suggesting the importance of these overexpressed genes in resistance. Taken together, the above findings suggest a multiple mechanism-interaction phenomenon is responsible for the development of permethrin resistance in *Culex* mosquitoes, which strongly agrees with the finding of our current genetic study, i.e. that the inheritance of permethrin resistance in HAmCq *Culex* mosquitoes is controlled by multiple genes.

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Chapter 4 Larvicidal Activity of Mosquito Sterol Carrier Protein-2 Inhibitors to the Insecticide Resistant Mosquito *Culex quinquefasciatus* (Diptera: Culicidae)

Abastract

AeSCP-2 inhibitors (SCPIs) compete with cholesterol for binding to a mosquito sterol carrier protein-2 (AeSCP-2) known to aid in the uptake of cholesterol in mosquito cells. The larvicidal activities of AeSCP-2 inhibitor-1 (SCPI-1) and inhibitor-2 (SCPI-2) against *Culex quinquefasciatus* were therefore examined in insecticide resistant *Culex* mosquitoes, $HAmCq^{G9}$, $MAmCq^{G2}$, and $BAmCq^{G0}$. All of the resistant *Culex* mosquito strains exhibited similar sensitivity to SCPI-1 and SCPI-2 inhibitors compared with a susceptible S-Lab strain. When an AeSCP-2 inhibitor was applied simultaneously with permethrin, the toxicity of permethrin to the 2nd instar larvae of all 4 strains of *Culex* mosquitoes increased, suggesting a synergistic effect of AeSCP-2 inhibitors on the toxicity of permethrin against *Culex* mosquitoes. Both SCPI-1 and SCPI-2 inhibitors caused a 2.4- to 3-fold reduction in the level of permethrin resistance in the highly resistant strain $HAmCq^{G9}$. This result suggests that the mode of action of the AeSCP-2 inhibitors, which reduces the uptake of cholesterol by inhibiting the function of AeSCP-2 in mosquito cells, may interfere with the mechanisms or ability that govern permethrin resistance in the $HAmCq^{G9}$ mosquito strain.

Key Words AeSCP-2 inhibitor, Insecticide resistance, Cross-resistance, *Culex quinquefasciatu*

Mosquito *Culex quinquefasciatus* S. is a nuisance species and 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 (SLE) in many urban settings (Jones et al. 2002). Current approaches to control mosquitoes rely primarily on source reduction and the application of insecticides for both larval and adult mosquitoes (Liu et al. 2004). This strategy has been followed for more than a decade, especially with the use of pyrethroids and organophosphates.

Three *Culex* strains, $HAmCq^{G0}$, $MAmCq^{G0}$, and $BAmCq^{G0}$ collected from Huntsville, Mobile, and Birmingham, Alabama, respectively, have demonstrated their ability to develop resistance and/or cross-resistance to pyrethroids and organophosphates (OPs), as well as relatively new insecticides such as fipronil and imidacloprid. $HAmCq^{G0}$ and $MAmCq^{G0}$ *Culex* strains were further selected by permethrin in the laboratory for 9 and 2 generations, respectively, to generate $HAmCq^{G9}$ and $MAmCq^{G2}$, both of which exhibited even higher levels of permethrin resistance than their parental strains (Xu et al. 2005, 2006). It seems likely that the ability to develop resistance and/or cross-resistance to insecticides, including pyrethroids and OPs, in *Culex quinquefasciatus* is a common phenomenon (Liu et al. 2004) and thus novel strategies for circumventing and/or delaying resistance development, controlling resistant mosquitoes, and reducing the prevalence of mosquito-borne diseases are urgently needed.

Lacking key enzymes in the cholesterol biosynthesis pathway, insects are unable to synthesize cholesterol *de novo* (Zdobnov et al. 2002). Consequently, obtaining cholesterol through dietary sources is a critical step in the physiological process of cholesterol absorption and translocation in insects, without which insects cannot grow and reproduce. Sterol carrier protein-2 (SCP-2) belongs to a family of proteins containing a sterol binding domain (SCP-2 domain) and a mosquito sterol carrier protein-2 (AeSCP-2) has been shown to aid in the uptake of cholesterol in mosquito cells (Blitzer et al. 2005). AeSCP-2 inhibitors (SCPIs) are known to

compete with cholesterol for binding to AeSCP-2 (Kim at al. 2005). SCPIs also exhibit larvicidal activity against mosquito species such as *Aedes aegypti* L., *Culex pipiens pipiens* L., *Anopheles gambiae* S.S., *Culex restuans* Theobald, and *Aedes vexans* M., and the tobacco hornworm, *Manduca sexta* L. (Kim et al. 2005, Larson et al. 2008). In the current study, we examined the larvicidal activities of AeSCP-2 inhibitor-1 (SCPI-1) and inhibitor-2 (SCPI-2) against *Culex quinquefasciatus* and tested for any synergistic effect of these inhibitors on the toxicity of permethrin against insecticide resistant *Culex* mosquitoes.

Materials and Methods

Cx. quinquefasciatus mosquito strains. The mosquitoes used were BAmCq^{G0} strains of *Cx. quinquefasciatus* collected from Birmingham, Alabama, USA in 2004; MAmCq^{G2}, the offspring of MAmCq^{G0} mosquitoes collected from Mobile, Alabama, USA in 2002 (Liu et al. 2004) that had been further selected with permethrin in laboratory for 2 generations after collection (Xu et al. 2006); HAmCq^{G9}, the offspring of HAmCq^{G0} mosquitoes collected from Huntsville, Alabama, USA in 2002 (Liu et al. 2004) that had been further selected with permethrin in laboratory for 9 generations (Xu et al. 2006); and S-Lab, a universal insecticide-susceptible strain 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).

Chemicals and insecticide. AeSCP-2 inhibitors SCPI-1 [*N*-(4-{[4-(3,4-dichlorophenyl)-1,3-thiazol-2-yl]amino}-phenyl)acetamidehydrobromide] and SCPI-2 [8-chloro-2-(3-methoxyphenyl)-4,4-dimethyl-4,5-dihydroisothiazolo[5,4-c]quinoline-1(2*H*)-thione] were purchased from ChemBridge Corporation (San Diego, CA), with at least 90% purity; and Permethrin (95.3%) was supplied by FMC Corp. (Princeton, NJ).

Bioassays. The AeSCP-2 inhibitor insecticidal activity assays were conducted with the AeSCP-2 inhibitors, SCPI-1 and SCPI-2, on 2nd instar larvae of *Cx. quinquefasciatus*. The inhibitors were diluted from a stock solution (100 mM in dimethyl sulfoxide [DMSO]) to various concentrations in double-distilled water as described by Larson et al. (2008). Each bioassay consisted of 30 2nd instar larvae of *Cx. Quinquefasciatus* in 6 oz. (~200 ml) Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with regular tap water and SCPI-1 or SCPI-2 solution, using three or four concentrations that resulted in >0 and <100% mortality. Control groups received correspondingly diluted DMSO.

The synergistic activity of the inhibitors on the toxicity of permethrin were assayed with or without SCPI inhibitors in serial dilutions of permethrin prepared in acetone, as described by Xu et al. (2005). Preliminary dose range and time course assays were performed with a corresponding dose range for each of the inhibitors to identify the maximum sublethal concentrations for SCPI-1 (0.1 μM) and SCPI-2 (0.01 μM). As before, each synergistic bioassay consisted of 30 2nd instar mosquito larvae in 6 oz. (~200 ml) Sweetheart ice cream cups with regular tap water and 1% permethrin solution in acetone at three or four concentrations that yielded >0 and <100% mortality. SCPI-1 or SCPI-2 was applied simultaneously with permethrin at the maximum sublethal concentration. Control groups received either 1% acetone alone or 1% acetone with an appropriate concentration of SCPI-1 (0.1 μM) and SCPI-2 (0.01 μM).

All tests were performed at 25°C and replicated at least 4 times on different days. Mortality was assessed after 24, 48, and 72 h. Bioassay data were pooled and probit analysis was conducted using Abbott's correction for control mortality (Abbott 1925). Statistical analysis of LC_{50}s was based on nonoverlapping 95% confidence intervals. Synergism ratios (SRs) were

calculated for each of the AeSCP-2 inhibitors (LC_{50} of insecticide alone divided by the LC_{50} of inhibitor + insecticide).

Results

Insecticidal activities of SCPIs in the insecticide resistant mosquito larvae, *Culex quinquefasciatus.* To evaluate the insecticidal activity of AeSCP-2 inhibitors, in this case SCPI-1 and SCPI-2, in the mosquito *Culex quinquefasciatus*, particularly with regard to their effects on the insecticide resistant strains, we applied the inhibitors to the 2nd instar larvae of 4 mosquito strains of *Culex quinquefasciatus* with different resistant phenotypes to insecticide resistance, ranging from susceptible (S-Lab), through moderate ($BAmCq^{G0}$ and $MAmCq^{G2}$), to highly resistant ($HAmCq^{G9}$). A previous study indicated different speeds of the toxic action of SCPIs for the mosquito *Ae. Aegyptia*, with SCPI-1 showing rapid toxic action and SCPI-2 being much slower to take effect, although no such difference was observed in the tobacco hornworm, *Manduca sexta*, under the same conditions (Kim et al. 2005). We therefore conducted preliminary concentration range and time course assays to examine the mortality after 24, 48, and 72 h treatment with serial concentrations of SCPI-1 and SCPI-2 and found no significant difference in the mortality over the time range (24, 48, and 72 h) for any of the test concentrations (data not shown). These preliminary results were consistent with the results reported for *Manduca sexta*, but differed from those for *Ae. Aegyptia*, suggesting different insect species may have different physiological responses to the inhibitors. Based on the preliminary results, a time point of 24 h after treatment was chosen for the evaluation of the motilities of treated mosquitoes.

The results revealed that all 4 *Culex* mosquito strains exhibited similar sensitivities to both the SCPI-1 and SCPI-2 inhibitors. The LC_{50} values of SCPI-1 were 1.7, 2.6, 1.3, and 2.7

μM of SCPI-1 for S-Lab, $HAmCq^{G9}$, $MAmCq^{G2}$, and $BAmCq^{G0}$, respectively, and the LC_{50} values for SCPI-2 were 0.3, 0.3, 0.4, and 1.3 μM, respectively (Table 4.1), indicating a relatively higher insecticidal activity of SCPI-2 in all tested *Culex* mosquito strains compared with SCPI-1. The finding that the inhibitors have a similar speed of insecticidal action in *Culex* mosquitoes, unlike in the study on *A. aegypti* larvae by Kim et al. (2005), supports the contention that different mosquito species exhibit different physiological responses to inhibitors. For example, Kim et al. (2005) argued that the different absorption rate or metabolic rate of each SCPI may lead to differences in the acute toxic action of SCPIs in different mosquito species. The overlap in the 95% confidence intervals of the LC_{50}s among the four mosquito strains tested (Table 4.1) for each of inhibitors suggests that there is no significant difference in the sensitivity of these mosquito strains towards the two inhibitors tested (Liu et al. 2004a, 2004b), although the $BAmCq^{G0}$ strain showed relatively less sensitivity to both SCPI-1 and SCPI-2 inhibitors compared to the other strains (Fig. 4.1).

Table 4.1. Toxicity of SCPIs to 2nd instar larvae of mosquito strains of *Culex quinquefasciatus*

Inhibitor	strain	df	n	χ^{2a}	$LC_{50}{}^{b}$ (CI)^{c}	Slope(SE)
SCPI-1	S-Lab	4	180	5.6	1.7 (0.8-3.8)	1.2 (0.2)
	$HAmCq^{G9}$	4	180	10	2.6 (0.7-15)	1.0 (0.1)
	$MAmCq^{G2}$	4	180	8.4	1.3 (0.3-5.2)	0.9(0.1)
	BAmCq	4	180	6.1	2.7 (1.3-6.2)	1.3 (0.2)
SCPI-2	S-Lab	4	160	1.2	0.3 (0.2-0.5)	1.7 (0.1)
	$HAmCq^{G9}$	4	160	2.1	0.3 (0.1-0.8)	1.2 (0.2)
	$MAmCq^{G2}$	4	160	2.0	0.4 (0.2-0.7)	1.1 (0.1)
	$BAmCq^{G0}$	4	160	2.0	1.3 (0.4-5.7)	1.0 (0.1)

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

[b] LC_{50} value in µM.

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

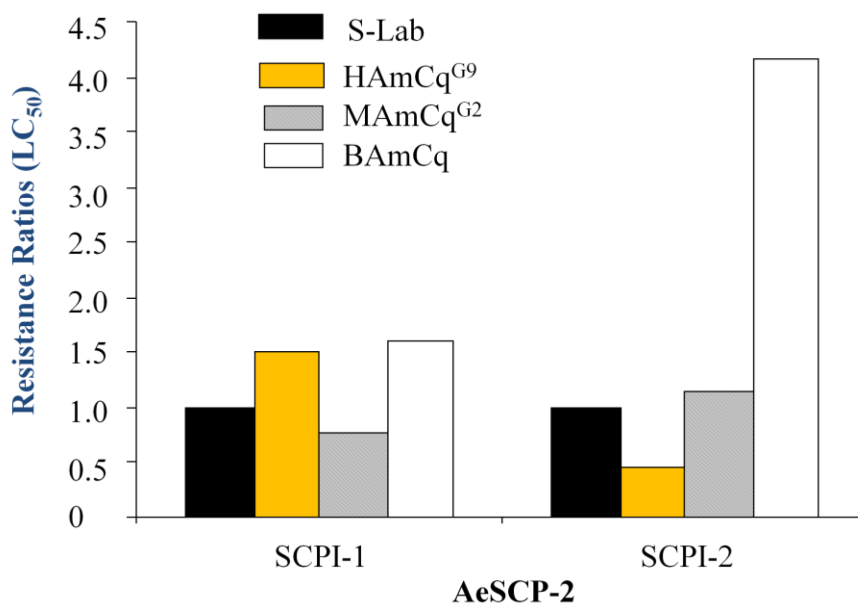

Figure 4.1. Inhibitor activity ratios (IRs) of SCPI-1 and SCPI-2 in S-Lab, HAmCq^{G9}, MAmCq^{G2}, and BAmCq strains of *Culex quinquefasciatus*. IRs were calculated by dividing LC_{50} of resistant strains by LC_{50} of S-Lab.

Synergism effects of SCPI-1 and SCPI-2 on permethrin against *Culex* mosquito larvae. In order to evaluate the synergistic effect of AeSCP-2 inhibitors on the toxicity of permethrin against *Culex* mosquitoes, we applied the maximum sublethal concentration of each of the AeSCP-2 inhibitors (0.1 µM SCPI-1 or 0.01 µM SCPI-2) in serial dilutions of permethrin solutions in which the 2nd instar larvae of each of BAmCq^{G0}, MAmCq^{G2}, HAmCq^{G9} and S-Lab strains were reared and the results were compared with those for permethrin alone, with no inhibitor present. The 2nd instar larvae of HAmCq^{G9}, MAmCq^{G2}, and BAmCq strains of *Cx.*

quinquefasciatus showed elevated levels of resistance to permethrin alone, with resistance ratios of 117, 7, and 14, respectively, at LC_{50} (Table 4.2, Fig. 4.2) compared to the susceptible S-Lab strain. These results were comparable to those in previous reports although the levels of resistance in $HAmCq^{G9}$ and $MAmCq^{G2}$ were relatively higher in the previous studies, largely because the 4th instar larvae of the mosquitoes were used (Xu et al. 2005, 2006). This variation in the results reported in different studies indicates that the sensitivity of mosquitoes to insecticides differ depending on the mosquito larvae stage, suggesting that mosquito control efforts should focus on the early instar larval stages.

Table 4.2. Toxicity of permethrin with and without SCPI-1 and SCPI-2 to 2nd instar larvae of mosquito strains of *Culex quinquefasciatus*

Chemicals	Strain	df	n	χ^{2a}	$LC_{50}{}^{b}$(CI)[c]	Slope(SE)	SR[d]
Permethrin	S-Lab	4	180	2.7	0.0006 (0.0004-0.0009)	1.7 (0.2)	
	$HAmCq^{G9}$	4	180	6.2	0.07 (0.03-0.15)	1.3 (0.2)	
	$MAmCq^{G2}$	5	200	3.1	0.004 (0.002-0.005)	1.3 (0.2)	
	BAmCq	5	190	5.8	0.008 (0.004-0.02)	1.1 (0.1)	
Permethrin	S-Lab	3	150	5.3	0.0001 (0.00004-0.0005)	1.3 (0.2)	6
+SCPI-1	$HAmCq^{G9}$	4	180	8.4	0.004 (0.001-0.01)	1.1 (0.1)	17
	$MAmCq^{G2}$	4	180	8.1	0.001 (0.0003-0.003)	1.0 (0.1)	4
	BAmCq	4	180	6.3	0.002 (0.001-0.006)	0.9 (0.1)	4
Permithrin	S-Lab	3	150	3.5	0.0002 (0.00008-0.0005)	1.5 (0.2)	3
+SCPI-2	$HAmCq^{G9}$	4	180	11	0.01 (0.002-0.09)	0.8 (0.1)	7

$MAmCq^{G2}$	4	180	6.8	0.002 (0.001-0.004)	1.6 (0.2)	2
BAmCq	4	180	5.8	0.005 (0.002-0.02)	1.0 (0.1)	1.6

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

[b] LC_{50} value in ppm.

[c] 95% confidence interval, toxicity of insecticide is considered significantly different when the 95% CI fail to overlap

[d] SR: Synergism ratios (SRs), LC_{50} of permethrin/LC_{50} of permethrin+inhibitors.

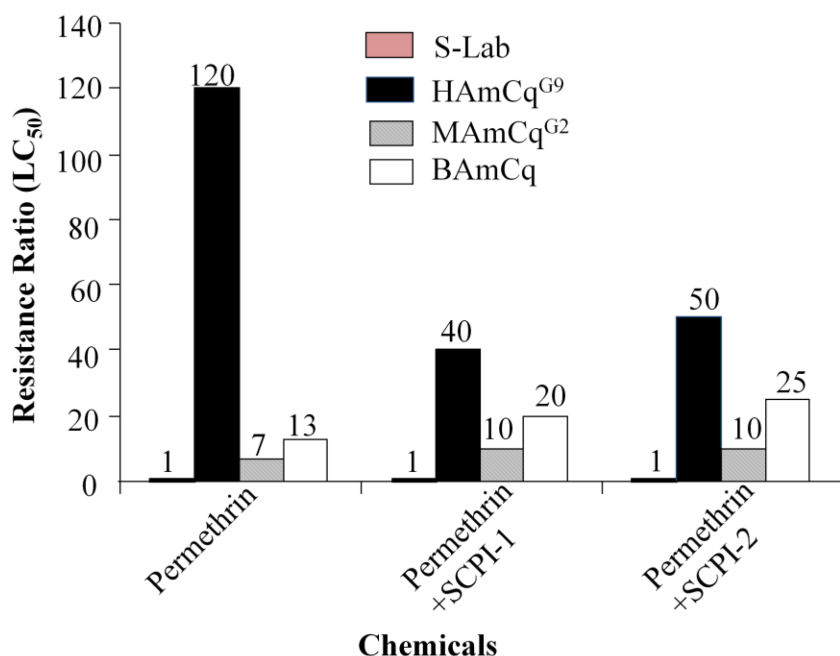

Figure 4.2. Permethrin resistance ratios (RRs) with and without the inhibitors SCPI-1 orSCPI-2 for the S-Lab, $HAmCq^{G9}$, $MAmCq^{G2}$, and BAmCq strains of *Culex quinquefasciatus*. RRs were calculated by dividing LC_{50} of resistant strains by LC_{50} of S-Lab.

When the maximum sublethal concentration of SCPI-1 (0.1 μM) was applied simultaneously with permethrin, the toxicity of permethrin to the 2nd instar larvae of all 4 strains of *Culex* mosquitoes increased markedly, with 5.3-, 17-, 4-, and 4-fold increases in the toxicity of permethrin to S-Lab, $HAmCq^{G9}$, $MAmCq^{G2}$, and BAmCq, respectively (Table 4.2). SCPI-2

(0.01 μM) caused 3-, 7-, 2-, and 1.6-fold increases in the toxicity of permethrin to S-Lab, HAmCq^{G9}, MAmCq^{G2}, and BAmCq, respectively (Table 4.2). These findings suggest that AeSCP-2 inhibitors do indeed exert a synergistic effect on the toxicity of permethrin against *Culex* mosquitoes. Both the AeSCP-2 inhibitors tested increased the toxicity of permethrin to *Culex* mosquitoes strains of S-lab, MAmCq^{G2}, and BAmCq^{G0} at the similar levels. However, SCPI-1 and SCPI-2 caused 17- and 7- fold synergism, respectively, for permethrin in HAmCq^{G9} (Table 4.2), , reducing the resistance ratio of permethrin from 120 to 40 and 50, a 2.4- to 3-fold reduction in the level of permethrin resistance (Fig. 4.2). These results suggest that the mode of action of the AeSCP-2 inhibitors, which reduce the uptake of cholesterol by inhibiting the expression of AeSCP-2 in mosquito cells, may interfere with the mechanisms or ability to develop permethrin resistance in the HAmCq^{G9} mosquito strain.

Discussion

Previous studies have indicated that AeSCP-2 inhibitors are toxic to the larvae of many different mosquito species, including *Ae. aegypti*, *An. gambiae*, and *Cx. pipiens pipiens*, *Cx. restuans* and *Ae. Vexans*, as well as the tobacco hornworm, *Manduca sexta* (Kim et al. 2005, Larson et al. 2008). It has also been reported that AeSCP-2 inhibitors are more effective against young larval instars, particularly the 1st and 2nd, than to those in the final growth period (Larson et al. 2008). We therefore chose to test the insecticidal activity of two AeSCP-2 inhibitors, SCPI-1 and SCPI-2, on the insecticide resistant mosquitoes *Culex quinquefasciatus* in this study. Previous work by our group has shown that the *Culex* mosquito strains MAmCq and HAmCq are resistant to a range of insecticides, including pyrethroids (permethrin and resmethrin) and organophosphates (OP; malathion) (Liu et al. 2004, Xu et al. 2005). These two strains have also demonstrated a considerable ability to develop cross-resistance to other insecticides, including

deltamethrin (a pyrethroid insecticide) chlorpyrifos (OP), fipronil (a phenylpyrazole insecticide), and imidacloprid (a nitroguaizidine analogue) (Liu et al. 2004) and this ability is likely to extend to other *Culex* mosquito populations. The current study found that even though they are resistant to conventional insecticides, $MAmCq^{G2}$, $HAmCq^{G9}$ and BAmCq mosquitoes exhibited a similar sensitivity to both SCPI-1 and SCPI-2 inhibitors to that of the susceptible S-LAB strain and no cross-resistance to the inhibitors was observed in these resistant mosquito strains. This is of interesting, because usually when a resistant strain is selected with an insecticide, resistance extends to other compounds of the same class of insecticides or to compounds with similar modes of action. The lack of cross-resistance to AeSCP-2 inhibitors observed in insecticide resistant $MAmCq^{G2}$, $BAmCq^{G0}$, and $HAmCq^{G9}$ mosquitoes therefore indicates that AeSCP-2 inhibitors have a different mode of action in mosquitoes than that of pyrethroids and organophosphates. Indeed, the studies by Kim et al. (2005) and Ryan et al. (2008) revealed that SCPI-1 suppresses cholesterol uptake in *Ae. aegypti* through the competitive binding of cholesterol to AeSCP-2 and suggested that one of the modes of action of SCPI-1 proceeds via a reduction in the cholesterol absorption in the mosquito's midgut.

Current approaches to control mosquitoes rely mainly on source reduction and the application of insecticides that target both larval and adult mosquitoes. This strategy has been followed for more than a decade, especially with pyrethroids, which are currently the most widely used insecticides for indoor spraying for mosquitoes worldwide and the only chemicals that are recommended to treat mosquito nets, the main tool for preventing malaria in Africa (Zaim et al. 2000). The downside of this heavy dependence on insecticides, including pyrethroids, is that this has resulted in the development of insecticide resistance in mosquitoes and the widespread resistance of mosquitoes to insecticides (Zaim and Guillet 2002), leading to a

resurgence in outbreaks of mosquito-related diseases (Hemingway et al. 2002). Due to the lack of new and/or alternative chemicals for public health uses (Zaim and Guillet 2002) and the economic limitations on investment for the development of new compounds (Rose 2001), a better understanding of the effectiveness of the available classes of insecticides for controlling mosquitoes has become a priority. Consequently, the search for new solutions or alternatives for controlling mosquitoes is now receiving considerable attention.

Synergists, chemicals that can enhance the toxicity of current insecticides (Scott 1990), are used to overcome resistance and extend the life spans of current insecticides, as well as enabling them to be used efficiently and economically, have the potential to serve as an important new option in mosquito management programs. Many studies have demonstrated the efficacy of insecticidal synergists in the control of insect pests and prolonging the usefulness of insecticides, especially those where the target insect has developed resistance (Georghiou 1983, Scott 1990, Clark et al. 1995). This study has revealed potentially valuable synergistic effects of both the AeSCP-2 inhibitors tested on permethrin insecticidal toxicity. These synergistic effects significantly improved the effectiveness of the permethrin against *Culex* mosquitoes by markedly decreasing the LC50s of permethrin to mosquitoes. However, the question of whether this synergistic relationship between AeSCP-2 inhibitors and permethrin poison extends to insects other than *Culex* mosquitoes and to different insecticides is open to further investigation.

Synergism studies are an important initial step in characterizing the mechanisms of insecticide resistance, as the synergist can be used to establish a causal link between its role and a resistance mechanism. One of the best examples of this is the use of inhibitors of metabolic detoxification gene families, such as piperonyl butoxide (PBO, the inhibitor of cytochrome P450s), S,S,S,-tributylphosphorotrithioate (DEF, the inhibitor of esterases), and diethyl maleate

(DEM, inhibitors of cytochrome P450 monooxygenases, hydrolases, and glutathione S-transferases [GST]), as synergists to help identify the possible roles of metabolic detoxification in resistance (Georghiou 1983, Clark et al. 1995, Liu and Yue 2000, Pridgeon et al. 2002, Xu et al. 2005). Our previous synergism study with PBO, DEF, and DEM revealed that P450s, esterases, and GSTs may be primary enzymes involved in detoxifying permethrin and conferring permethrin resistance in HAmCq mosquitoes (Xu et al. 2005). In the current study, significantly higher synergism ratios were obtained for both SCPI-1 and SCPI-2 in HAmCq^{G9}, resulting in 2.4- to 3-fold reduction in levels of permethrin resistance in the strain. This result suggests that the AeSCP-2 inhibitors reduce the uptake of cholesterol by inhibiting the function of AeSCP-2 in mosquito cells, thus interacting with or influencing the ability of HAmCq^{G9} mosquito larvae to develop resistance to permethrin. However, the function of the AeSCP-2, which is known to aid in the uptake of cholesterol in mosquito cells (Blitzer et al. 2005), has not been reported to play a role in resistance, although multiple mechanisms and genes have been identified in the HAmCq mosquitoes (Xu et al. 2006, Liu et al. 2007). Interestingly, Larson et al. (2009) reported that a SCPI suppresses esterase activity in 24 hour-treated *Aedes* larvae, which may contribute to the synergistic effect of the SCPI to temephose toxicity. Therefore, the synergistic effect of SCPIs on permethrin against HAmG9 might be results from SCPI's ability to spurress esterase activity indirectly. The fact that SCPIs can suppress the resistance of permethrin in HAmCq^{G9} by inhibiting the function of sterol carrier protein-2, and consequently the uptake of cholesterol, reflects the complex process involved in the development of insecticide resistance. It is therefore possible that cholesterol metabolism may be linked in some way to the pathways that influence to some extent the ability of mosquitoes to develop resistance. The relationship with these pathways remains unclear and deserves attention in future research.

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Chapter 5 Multiple Cytochrome P450 Genes: Their Constitutive Overexpression and Permethrin Induction in Insecticide Resistant Mosquitoes, *Culex quinquefasciatus*

Abstract

Four P450 cDNAs, *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*, were isolated from mosquitoes, *Culex quinquefasciatus*. P450 gene expression and induction by permethrin were compared for three different mosquito populations bearing different resistance phenotypes, ranging from susceptible (S-Lab), through intermediate ($HAmCq^{G0}$, the field parental population) to highly resistant ($HAmCq^{G8}$, 8th generations of permethrin selected offspring of $HAmCq^{G0}$). A strong correlation was found for P450 gene expression with the levels of resistance and following permethrin selection at the larval stage of mosquitoes, with the highest expression levels identified in $HAmCq^{G8}$, suggesting the importance of *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10* in the permethrin resistance of larva mosquitoes. Only *CYP6AA7* showed a significant overexpression in $HAmCq^{G8}$ adult mosquitoes. Other P450 genes had similar expression levels among the mosquito populations tested, suggesting different P450 genes may be involved in the response to insecticide pressure in different developmental stages. The expression of *CYP6AA7*, *CYP9J34*, and *CYP9M10* was further induced by permethrin in resistant mosquitoes. Taken together, these results indicate that multiple P450 genes are up-regulated in insecticide resistant mosquitoes through both constitutively overexpression and induction mechanisms, thus increasing their overall expression levels of P450 genes.

Key Words: Cytochrome P450s; Up-regulation; Induction; Permethrin; Resistance

Introduction

Cytochrome P450s have long been of particular interest because they are critical for the detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens. They are also involved in metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Basal and up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their pharmacological/toxicological effects (Pavek and Dvorak, 2008). Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides (Scott, 1999; Feyereisen, 2005) and plant toxins (Berenbaum, 1991; Schuler, 1996). A significant characteristic of insect P450s associated with the enhanced metabolic detoxification of insecticides is the increase in the levels of P450 proteins and P450 activity that results from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects, which has been implicated in the development of resistance to insecticides (Carino et al., 1994; Liu and Scott, 1997; 1998; Kasai et al., 2000; Feyereisen, 2005, Zhu and Liu, 2008; Zhu et al., 2008a) and tolerance to plant toxins (Li et al., 2002; Wen et al., 2003). Another feature of some insect P450 genes is that their expression can be induced by both exogenous and endogenous compounds (Feyereisen, 2005), a phenomenon known as induction. It has been suggested that the induction of P450s and their activities in insects is involved in the adaptation of insects to their environment and, hence, the development of insecticide resistance (Terriere, 1983, 1984; Zhu et al., 2008b).

While all insects probably possess some capacity to detoxify insecticides and xenobiotics, the degree to which they can metabolize and detoxify these toxic chemicals is of considerable importance to their survival in a chemically unfriendly environment (Terriere, 1984) and to the

development of resistance. The constitutively increased expression and induction of P450s are both thought to arise in response to increased levels of detoxification of insecticides (Zhu et al., 2008a; 2008b). It has been suggested that many chemical inducers act as substrates for P450s and that the induction or modulation of P450s by such substrates will, in turn, reduce the effects of the substrates by enhancing substrate metabolism (Okey, 1990b; Zhu et al., 2008b). The modulation of gene expression may therefore reflect a compromise between the insect's need to conserve energy and its ability to adjust to a rapidly changing environment by enhancing the activity of the detoxification system only when a chemical stimulus occurs (Depardieu et al., 2007).

The primary goal of our study was to investigate whether insecticide resistant insects may be uniquely resistant to insecticides due to their ability to mount an adequate cellular response when challenged with insecticides by up-regulating the production of P450s, which may, in turn, significantly diminish the toxicological effects of the insecticides on these insects (Pavek and Dvorak, 2008). In a previous study we used a combination of subtractive hybridization and cDNA array techniques to identify several P450 EST sequences overexpressed in resistant mosquitoes, *Culex quinquefasciatus* (Liu et al., 2007). The current study focused on isolating the full-length cDNA sequences of those P450 ESTs, characterizing the expression profiles of these P450 genes from mosquito populations of *Culex quinquefasciatu*s bearing different phenotypes in response to permethrin (susceptible, intermediate and highly resistant), and determining the response of these P450 genes to permethrin treatment among the three mosquito populations.

Materials and Methods

Mosquito strains

Three strains of mosquito *Cx. quinquefasciatus* were studied: $HAmCq^{G0}$, a field resistant strain collected from Huntsville, Alabama, USA (Liu et al., 2004); $HAmCq^{G8}$, the 8th generation of permethrin-selected $HAmCq^{G0}$ offspring; and S-Lab, an insecticide susceptible strain provided by Dr. Laura Harrington (Cornell University). All the mosquitoes were reared at 25±2°C under a photoperiod of 12:12 (L:D) h (Nayar and Knight, 1999) and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

Permethrin Treatment

Preliminary dose range, time course, and P450 gene induction assays were performed on late 3rd instar larvae using a range of concentrations (LC_{10}, LC_{50} and LC_{90}) and a time course of 12, 24, 48, and 72h. Results of the pilot experiment, in which the induction of P450s in both resistant HAmCq mosquito populations showed a clear concentration (LC_{50})- and time (24 h)-dependent response. Based on these preliminary results, two different permethrin treatment experiments were conducted: 1) ~1000 late 3^{dr} instar larvae of each of the three *Culex* mosquito strains were treated with permethrin at their respective LC_{50} concentration (0.007ppm, 0.07ppm, and 20ppm for the S-Lab, $HAmCq^{G0}$, and $HAmCq^{G8}$ strains, respectively) and the expression of the P450 genes examined 12, 24, 48, and 72h after the permethrin treatment; and 2) mosquito strains were treated with their corresponding LC_{10}, LC_{50} and LC_{90} concentrations of permethrin (Table 5.1) and the surviving mosquitoes were collected for RNA extraction 24 h after permethrin challenge. Control mosquitoes that had not received the permethrin treatment (treated with acetone alone) were collected at the same timepoints as their permethrin treated counterparts. The experiments were repeated three times.

Table 5.1. Permethrin treatment of the late 3rd instar larvae of *Culex* mosquitoes

		Permethrin Treatment*		
Strain	n‡	LC_{10} Treatment†	LC_{50} Treatment†	LC_{90} Treatment†
S-Lab	~1000	0.003 ppm	0.007 ppm	0.02 ppm
$HAmCq^{G0}$	~1000	0.02 ppm	0.07 ppm	0.2 ppm
$HAmCq^{G8}$	~1000	10 ppm	20 ppm	30 ppm

*Each treatment was repeated 3 times

† The concentrations of permethrin for these mosquitoes have been identified previously (Xu et al. 2006, Li et al. 2010)

† The number of late 3rd instar mosquito larvae used at the beginning of each permethrin treatment

RNA extraction, cDNA preparation, and the 3' and 5' race

Larvae and adults of each mosquito population had their RNA extracted for each experiment. using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997). mRNA was isolated with oligotex-dT suspension (QIAGEN). Three replications were performed, each on a different day. Rapid amplification of 3' and 5'cDNA ends (3'and 5'-RACE) was carried out using the Marathon™ cDNA Amplification Kit (Clontech) (Liu and Zhang, 2002). The first strand cDNAs were synthesized with AMV reverse transcriptase using mosquito mRNAs as templates. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of the double strand cDNA as described by the manufacturer. The double strand cDNAs were amplified by PCR with the primers designed according to our previous EST sequences (Liu et al., 2007) and AP1 primer (based on the sequence of the adaptor). The PCR products were cloned into PCR™

2.1 Original TA cloning vector (Invitrogen) and sequenced. The full length of the P450 cDNAs was generated by RT-PCR using specific primer pairs according to the 5'and 3'end sequences of the putative P450 genes. Cloning and sequence analyses of the P450 cDNA fragments were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

Quantitative Real-time PCR (qRT-PCR)

Total RNA samples (0.5 μg/sample) from larval mosquitoes were reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR. qRT-PCR was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and a P450 gene specific primer pair designed according to each of the P450 gene sequences at a final concentration of 3-5 μM. A 'no-template' negative control and all samples were performed in triplicate. The reaction cycle consisted of a melting step of 50ºC for 2 min then 95°C for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction using Dissociation Curves software (Wittwer et al., 1997). Relative expression levels for the sodium channel gene were calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software (Livak and Schmittgen, 2001). The β-actin gene, an endogenous control, was used to normalize the expression of target genes (Aerts et al., 2004, Zhu et al., 2008a, 2008b). Preliminary qRT-PCR experiments with primer pairs of Actin S1 (5' AGGCGAATCGCGAGAAGATG 3') and Actin AS1 (5' TCAGATCACGACCAGCCAGATC 3') designed according to the sequence of the *β*-actin cDNA had revealed that the *β*-actin gene expression remained constant among all 3

mosquito strains, so the *β*-actin gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated three times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

Results

P450 genes and their expression profiles in *Culex quinquefasciatus*

Four full lengths of P450 cDNAs were isolated from *Culex quinquefasciatus* with 3' and 5' RACE using the specific primers designed from our previous P450 EST sequences (Liu et al., 2007). The full lengths of these P450 cDNA sequences were assigned the names *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10* (accession numbers: JF501089, JF501091, JF501092, JF501093, respectively) by the P450 nomenclature committee (Dr. D. Nelson, personal communication). The putative protein sequences of CYP6AA7, CYP9J40, CYP9J34, and CYP9M10 deduced from the cDNA sequences shared 99, 97, 100, and 99% identity with *Culex quinquefasciatus CPIJ005959*, *CPIJ010543*, *CPIJ010546*, and *CYP9M10*, respectively (Dr. D. Nelson, personal communication); apart from *CYP9M10*, none have yet been reported to be involved in insecticide resistance.

Diversity in the developmental expression and regulation of insect P450s is well established, so expression patterns of 4 P450 genes, *CYP6AA7*, *CYP9J34*, *CYP9J40* and *CYP9M10*, were examined in larval and adult mosquitoes of *Culex quinquefasciatus*. Quantitative real-time PCR (qRT-PCR) analysis was performed to compare expression levels of the 4 P450 genes for larvae and adults among three different mosquito populations bearing different resistance phenotypes in response to permethrin, ranging from susceptible (S-Lab),

through intermediate resistant (HAmCq^{G0}, field parental population) to highly resistant (HAmCq^{G8} 8th generation permethrin selected offspring of HAmCq^{G0}). Our results showed that besides *CYP9M10*, the expression of which was developmentally regulated and specifically overexpressed in the larval stage (4th larval instar) compared with the adults, the expression of the other three P450 genes, *CYP6AA7*, *CYP9J34*, *CYP9J40,* were at similar levels in the larval and adult stages (Fig. 5.1). Significant differences in the expression of 4 P450 genes in the larval stage were identified among susceptible S-Lab, intermediate resistant HAmCq^{G0} and highly resistant HAmCq^{G8} mosquito populations (Fig. 5.1). The expression of *CYP6AA7* was overexpressed (~2-fold) in the 4th instar of field parental populations HAmCq^{G0} compared with susceptible S-Lab mosquitoes, increasing to ~5-fold in HAmCq^{G8} after permethrin selection (Fig. 5.1A). A similar expression pattern was also found for *CYP9M10*; the expression of the gene was ~4-fold higher in the 4rd instar of HAmCq^{G0} compared with S-Lab, increasing to ~11-fold in HAmCq^{G8} (Fig. 5.1D). Although the expression of *CYP9J34* in HAmCq^{G0} was similar to that in the S-Lab strain, the expression was significantly increased after permethrin selection (Fig. 5.1B). The correlation of the gene expression with the levels of resistance developed following permethrin selection suggests the importance of *CYP6AA7, CYP9M10* and *CYP9J34* in permethrin resistance in *Culex* mosquitoes.

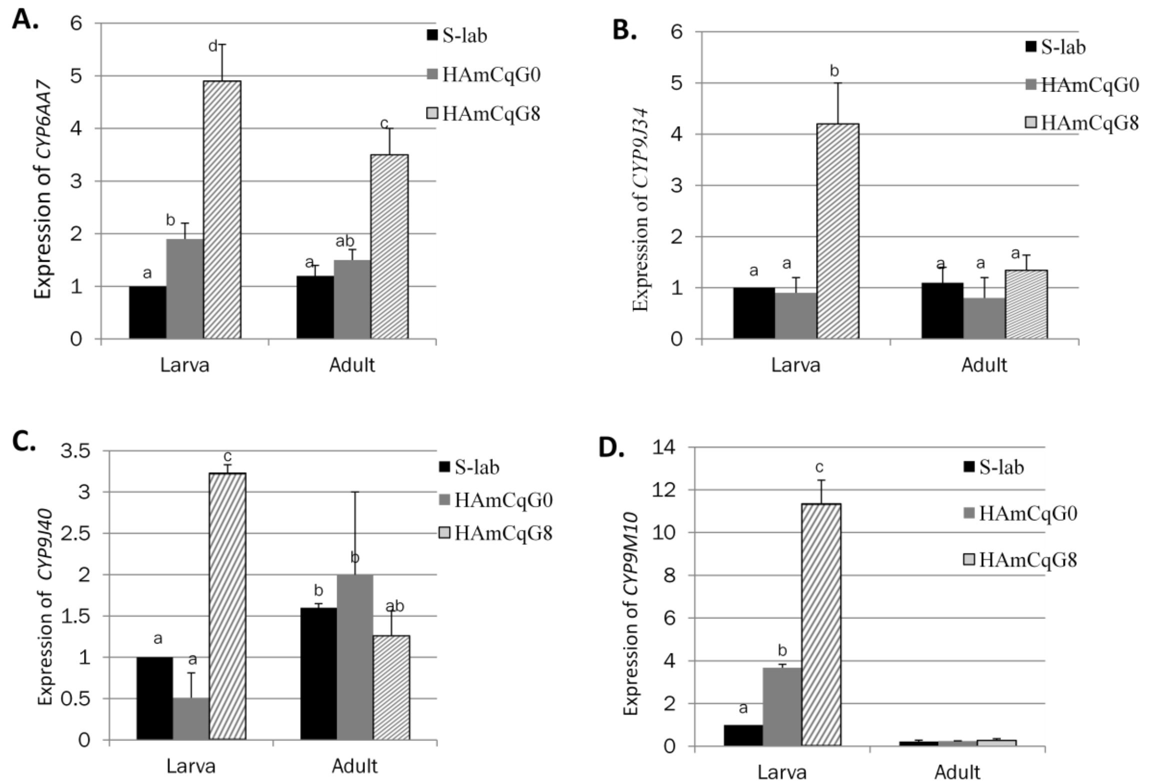

Figure 5.1. Expression analysis of *CYP6AA7*, *CYP9J34*, *CYP9J40*, and *CYP9M10* in mosquitoes, *Cx. quinquefasciatus*. The relative level of gene expression shown along the Y axis represents the ratio of the gene expression in each mosquito strain compared with that in the susceptible S-lab strain. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 gene expression among samples with the same alphabetic letter (i.e., a, b, or c). A. Relative *CYP6AA7* RNA levels. B. Relative *CYP9J34* RNA levels. C. Relative *CYP9J40* RNA levels. D. Relative *CYP9M10* RNA levels.

Comparison of the gene expression among these 4 P450 genes in the adult stage of mosquitoes revealed that among the three P450 genes *CYP6AA7*, *CYP9J34*, *CYP9J40,* whose expression levels were similar for the larva and adult stages, only *CYP6AA7* showed a significant overexpression in $HAmCq^{G8}$ mosquitoes following permethrin selection (~3.5-fold, Fig. 5.1A). No significant difference was found in the expression of *CYP9J34* and *CYP9J40* at the adult

stage among susceptible S-Lab, field population HAmCq^{G0} and permethrin selected highly resistant HAmCq^{G8} mosquitoes (Figs. 5.1B and 5.1C). These results strongly suggest that *CYP9J34* and *CYP9J40* play no role in the development of resistance in adult HAmCq mosquitoes. These results further suggest that different mechanisms and/or P450 genes may be involved in the response to insecticide pressure for different developmental stages of mosquitoes and different populations of mosquitoes (Li and Liu, 2010).

Tissue specific overexpression of *CYP6AA7* in resistant and susceptible mosquitoes

Insect P450s may also vary as to the tissues where they are expressed in response to physiological and environmental stimulators. In insects, the midgut and fat body tissue are generally considered to be the primary detoxification organs where most insect detoxification P450s are expressed (Scott et al., 1998). Nevertheless, other tissue, such as the brain (Zhu et al., 2010) and nervous system (Korytko and Scott, 1998) may also be important for P450 gene expression and response to insecticide resistance. Our study found that *CYP6AA7* was overexpressed not only in larvae of resistant HAmCq^{G8} mosquitoes, but also in adults of the same strain. To further characterize whether the overexpression of *CYP6AA7* is detoxification tissue specific, RNAs from the head, thorax, and abdomen of S-Lab, HAmCq^{G0} and HAmCq^{G8} mosquitoes were therefore subjected to qRT-PCR analyses. Comparison of the levels of *CYP6AA7* expression among the three tissues indicated that it was lower in the head, increased in thorax tissue and reached its highest concentration in the abdomen tissue of all three mosquito strains (Fig. 5.2). As midgut and most fat body components are located in the abdomen of insects and are known to be of primary importance in detoxification-related functions, the relatively high levels of *CYP6AA7* in the abdomens of all three mosquito strains suggest the importance of the gene in the detoxification of insecticides in mosquitoes. However, because midgut and fat body

tissues are not exclusively found in the abdomen, further dissection of detoxification-related tissue (such as midgut and fat body) is needed to pinpoint the precise location for the overexpression of *CYP6AA7*. Significant overexpression was particularly evident in the permethrin selected HAmCq^{G8} population in all three types of tissue (Fig. 5.2) and were closely correlated with each strain's level of insecticide resistance. The field parental HAmCq^{G0} strain, with a relatively lower level of resistance, showed no significant difference in the *CYP6AA7* expression in the head tissues compared with the susceptible S-Lab strain but increased expression (2-fold) of *CYP6AA7* in both the thorax and abdomen tissues (Fig. 5.2).

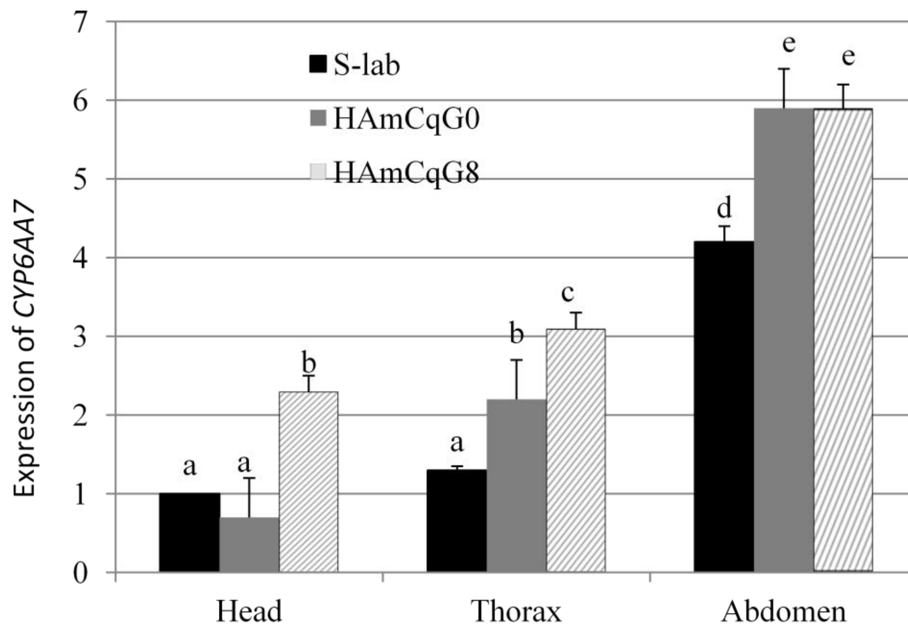

Figure 5.2. Expression of *CYP6AA7* in head, thorax, and abdomen tissue of adult *Culex* mosquitoes. The relative level of gene expression shown along the Y axis represents the ratio of the gene expression in each tissue of each mosquito strain compared to that in the head of the susceptible S-lab strain (=1). The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of *CYP6AA7* expression among samples with the same alphabetic letter (i.e., a, b, or c).

Response of P450 genes to permethrin challenge in resistant and susceptible mosquitoes

It has been proposed that many chemical inducers act as substrates for the P450s that they induce and that the induction of the P450s by the substrates will, in turn, reduce the effects of the substrates by enhancing substrate metabolism (Okey, 1990). We thus hypothesized that insecticide resistant mosquitoes may be uniquely resistant to insecticides due to their ability to mount an adequate cellular response, for example the ability to up-regulate their production of P450s, when challenged with insecticides. We therefore compared the inducibility of expression of the four P450 genes, *CYP6AA7*, *CYP9J34*, *CYP9J40* and *CYP9M10*, among susceptible S-Lab, intermediate resistant HAmCq^{G0} (field parental population), and highly resistant HAmCq^{G8} (permethrin selected offspring of HAmCq^{G0}) mosquitoes.

To examine the effect of permethrin on induction of the 4 P450 genes, we measured the expression of the genes in mosquitoes challenged with permethrin at corresponding dose ranges (LC_{10}, LC_{50}, and LC_{90} for each strain) for various durations (Table 5.1). Our preliminary results showed that although no significant induction was detected in the susceptible S-Lab mosquitoes for the dose range and time intervals tested (data not shown), permethrin induced all three P450 genes in resistant HAmCq^{G8} mosquitoes with varying levels in a clear concentration dose- and time-dependent manner. Based on these data, a permethrin concentration of LC_{50} for each mosquito strain and a time interval of 24 h were chosen for the further induction studies (Figs 5.3 and 5.4) and the expression of 4 P450 genes in response to permethrin challenge in each of three mosquito populations was characterized. The duration of the P450 gene expression following permethrin treatment at the LC_{50} concentration and the expression of the genes 24 h after permethrin treatment over a concentration range of LC_{10}, LC_{50}, and LC_{70} were investigated. No significant induction in the expression of *CYP6AA7* was detected in susceptible S-Lab and field

parental strain $HAmCq^{G0}$ that had been treated with either acetone alone (control) or with any of the three concentrations of permethrin solution in acetone at 24 h after treatment (Fig. 5.3A). However, in the permethrin selected $HAmCq^{G8}$ strain, an initial induction of *CYP6AA7* (~1.5-fold) was found in mosquitoes that had been treated with the LC_{10} of permethrin and a marked induction (~4.5-fold) in the mosquitoes treated with the permethrin at a concentration of LC_{50}. No significant induction was detected in the mosquitoes with a permethrin concentration of LC_{70}. Although no induction of *CYP9J34* was detected in the susceptible S-Lab strain, elevated levels of *CYP9J34* expression were detected in $HAmCq^{G0}$ mosquitoes treated with permethrin compared with the corresponding no-permethrin treated control. The levels of *CYP9J34* RNA in $HAmCq^{G0}$ were readily induced by LC_{10} permethrin concentration, induced to a maximum (~1.7-fold) by LC_{50} permethrin concentration, with no further significant induction up to LC70 of permethrin concentration (Fig. 5.3B). Nevertheless, significant induction of *CYP9J34* was more evident in the $HAmCq^{G8}$ strain than in their parental $HAmCq^{G0}$, with an induction peak of ~2.7-fold at a permethrin concentration of LC_{50} (Fig. 5.3B). A similar induction pattern was also found for *CYP9M10* RNA (Fig. 5.3D) in $HAmCq^{G0}$ and $HAmCq^{G8}$. However, no significant induction of CYP9J40 was identified in any of the three mosquito strains tested (Fig. 5.3D). The significant induction of the P450 genes only in the field resistant and/or permethrin selected highly resistant mosquito strains suggests their importance in the resistant mosquitoes' response to permethrin treatment.

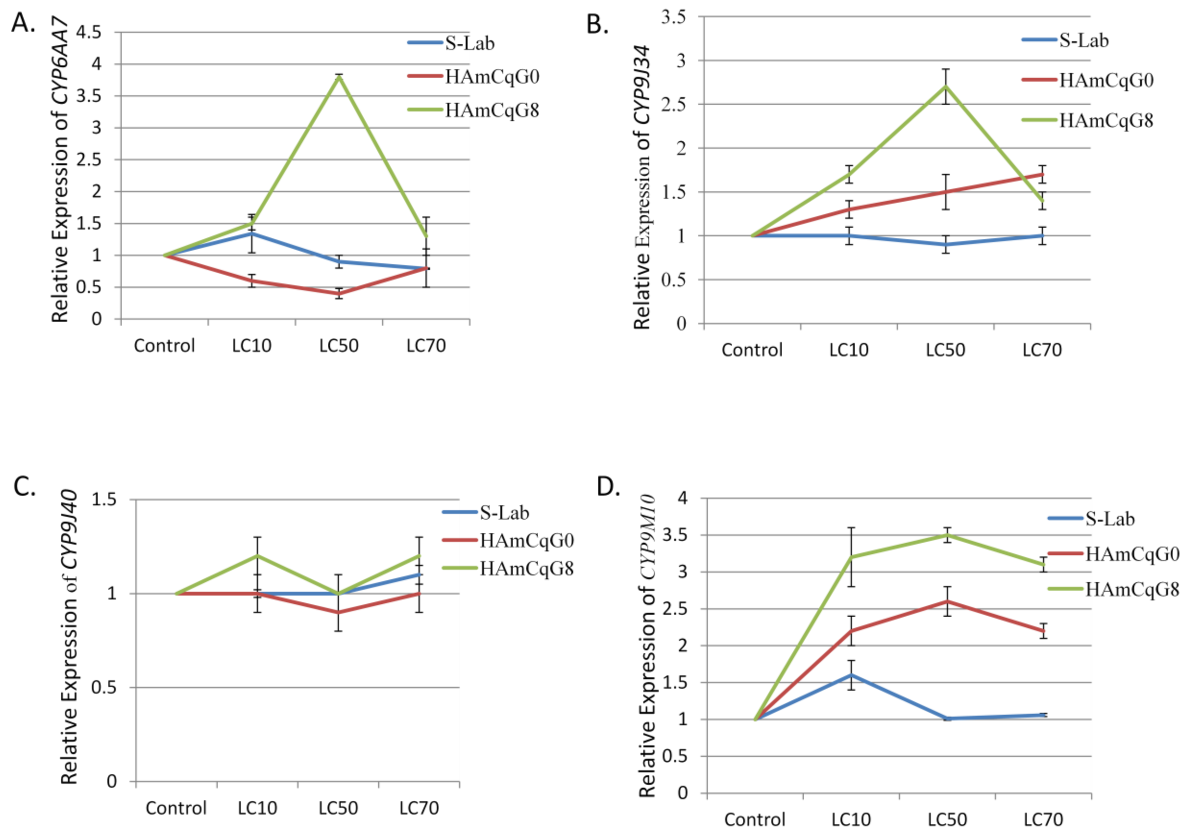

Figure 5.3. Dose-dependent induction of P450 expression following treatment with permethrin. The expression of *CYP6AA7*, *CYP9J34*, *CYP9J40*, and *CYP9M10* in *Cx. quinquefasciatus* in each of the mosquito populations 24 h after permethrin treatment with a corresponding concentration range of LC_{10}, LC_{50}, and LC_{90} (Table 5.1) was analyzed by qRT-PCR as described in Section 2.4, Materials and Methods. The relative level of gene expression shown along the Y axis represents the ratio of the gene expression in each treatment compared with that in acetone treated control mosquitoes. The experiments were repeated three times. The results are shown as the mean ± S.E.

Examining the durations of P450 gene induction with LC_{50} permethrin concentration treatment revealed no significant induction in the expression of *CYP6AA7* in susceptible S-Lab

and field parental strain $HAmCq^{G0}$ at any time after the treatment (Fig. 5.4A). However, in the $HAmCq^{G8}$ strain, the initial induction (~2.5-fold) was found 12 h after LC_{50} permethrin concentration (20 ppm) treatment, reaching a peak at 24 h after permethrin treatment with an induction level of 4.5-fold, and declining dramatically 48 h after treatment (Fig. 5.4A). While we did not detect the induction of either *CYP9J34* or *CYP9M10* in the susceptible S-Lab strain at any time after the mosquitoes were treated with LC_{50} permethrin concentration (0.007 ppm), elevated levels of *CYP9J34* and *CYP9M10* expression were detected in $HAmCq^{G0}$ mosquitoes treated with permethrin at the LC_{50} concentration (0.07 ppm) compared with their no-permethrin treated controls (Figs. 5.4B and 5.4D). The induction for both *CYP9J34* and *CYP9M10* in $HAmCq^{G0}$ reached a maximum (~1.9- or 2.3-fold, respectively) for both genes 24 h after permethrin LC_{50} concentration treatment. The induction levels of both genes then declined by 48 h after treatment, with no significant induction (*$p \leq 0.05$) detected after 72 h treatment compared with untreated or acetone treated mosquitoes (Figs. 5.4B and 5.4D). Similarly, the induction of both *CYP9J34* and *CYP9M10* in $HAmCq^{G8}$ reached a maximum (~2.7- or 3.7-fold, respectively) for both genes at 24 h after permethrin LC_{50} concentration treatment, declining after 48 h treatment (Figs. 5.4B and 5.4D). No significant induction of CYP9J40 was identified in any of the three mosquito strains at any time after the mosquitoes had been treated by permethrin at their corresponding LC_{50} concentrations (Fig. 5.4D). The significant induction of the P450 genes only in field resistant and/or permethrin selected highly resistant mosquito strains suggests their importance in the response to permethrin treatment of resistant mosquitoes.

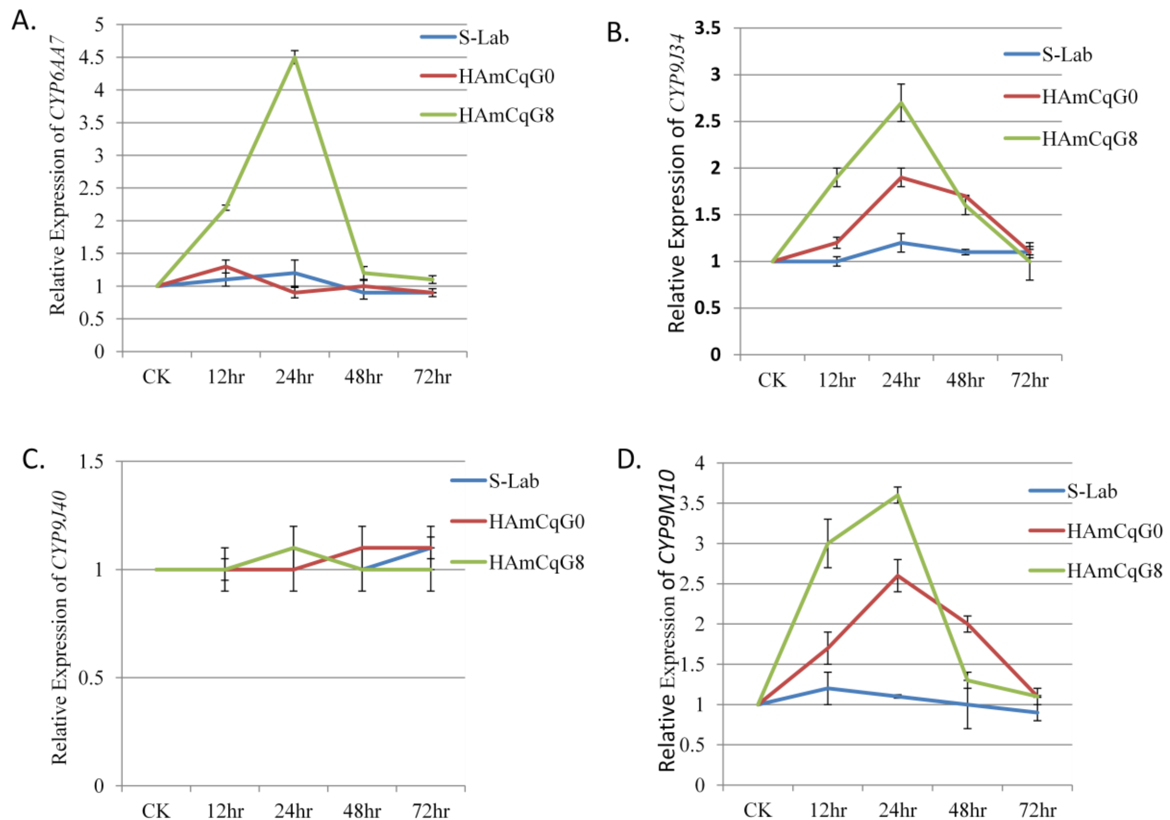

Figure 5.4. The duration of the gene expression following permethrin treatment at a concentration of LC_{50}. The expression of *CYP6AA7*, *CYP9J34*, *CYP9J40*, and *CYP9M10* in *Cx. quinquefasciatus* following treatment with permethrin at the respective LC_{50} concentrations (0.007ppm, 0.07ppm, and 20ppm for the S-Lab, $HAmCq^{G0}$, and $HAmCq^{G8}$ strains, respectively) were analyzed 12, 24, 48, and 72h after the permethrin treatment. The relative level of gene expression shown along the Y axis represents the ratio of the gene expression in each treatment in comparison with that in acetone treated control mosquitoes. The experiments were repeated three times. The results are shown as the mean ± S.E.

Discussion

In many cases, increased levels of P450 gene expression (i.e., the overexpression of P450 genes) are known to result in increased levels of total P450s and the P450 activities that are

responsible for insecticide resistance (Carino et al., 1992; Liu and Scott, 1997; Festucci-Buselli et al., 2005; Feyereisen, 2005; Zhu et al., 2008). Both constitutively increased expression (overexpression) and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides (Pavek and Dvorak, 2008). Multiple P450 genes that are induced in insects in response to host plant allelochemicals or secondary products have been extensively studied and are fairly well documented in terms of their function in the adaptation of insects in "animal-plant warfare" (Gonzalez and Nebert, 1990) and in the co-evolution of insects and plants (Li et al., 2004). In contrast, P450 gene induction in response to insecticide resistance is less well understood.

Our previous research has indicated that resistance in HAmCq, the *Culex quinquefasciatus* mosquito strain used in this research, could be partially suppressed by piperonyl butoxide (PBO), an inhibitor of cytochrome P450s (Xu et al., 2005). Further study identified several P450 EST sequences that were overexpressed in resistant HAmCq mosquitoes (Liu et al., 2007). Nevertheless, until now no individual P450 genes have been isolated and characterized in the HAmCq mosquitoes as being responsible for resistance. In the current study, we isolated and sequenced 4 P450 cDNAs, *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*, from mosquitoes . Besides *CYP9M10*, the overexpression of which has been reported in a resistant *Culex* mosquito strain in Japan (Komagata et al., 2010), the other three P450 genes have not previously been reported in *Culex* mosquitoes in terms of insecticide resistance.

In this study, both the constitutive overexpression of these P450 genes and the induction of the P450 genes in response to a challenge with insecticides in resistant mosquitoes, *Culex quinquefasciatus* were characterized. Clear correlations were found between the levels of P450 gene expression or induction and the levels of permethrin resistance or susceptibility among the

susceptible S-Lab strain, the field parental (low resistant) strain HAmCq^{G0}, and the highly resistant HAmCq^{G8} strain, the permethrin selected offspring of HAmCq^{G0}. Because insecticide resistance is generally assumed to be a pre-adaptive phenomenon, where prior to insecticide exposure rare individuals carrying an altered (varied) genome already exist thus allowing the survival of those carrying the genetic variance after insecticide selection, we expected that the number of individuals carrying the resistance genes or alleles should increase in a population following selection and become predominate in the population. The approach adopted for this study, which compared P450 gene expression and induction among different mosquito populations and between the parental field population, HAmCq^{G0}, and its permethrin selected offspring, HAmCq^{G8}, for different levels of insecticide resistance highlighted the importance of P450 genes in resistance by detecting changes in their expression within each population following permethrin selection. We restricted the induction response to permethrin treatment because it is the insecticide that these mosquitoes are resistant to. We found that the overexpression levels of 4 P450 genes (*CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*) in all three mosquito populations was closely correlated to their levels of resistance and were higher in 8th generation permethrin-selected mosquitoes, HAmCq^{G8}, compared to the parent strain HAmCq^{G0}. Furthermore, we also found that the induction levels of *CYP6AA7*, *CYP9J34*, and *CYP9M10*, but not *CYP9J40*, in the mosquito populations correlated with their levels of resistance and were again higher in HAmCq^{G8} compared to HAmCq^{G0}. Our study strongly indicates that the overexpressed P450 genes are more strongly induced when the mosquitoes are exposed to insecticides, which, in turn, increase the overall expression levels of multiple P450 genes in resistant mosquitoes. We also observed that P450 gene induction in mosquitoes follows a resistance-specific pattern; similar results have been reported in *Drosophila melanogaster* [42],

where the expression of *CYP6g1* and *CYP12d1* were induced in the DDT resistant strains post-exposure to DDT. Recent studies by Zhu et al. (2008a; 2008b) indicated that several P450 genes were up-regulated in insecticide resistant house flies through a similar induction mechanism, Taken together, these studies strongly suggest a common mechanism for P450 induction in response to detoxification-mediated insecticide resistance in a number of different insect species.

Conclusions

This study provides direct evidence that four P450 genes, *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10*, are up-regulated in insecticide resistant mosquitoes through constitutive overexpression and/or induction mechanisms. As this was found only in resistant mosquitoes, and was markedly higher in the permethrin selected highly resistant mosquitoes, this strongly suggests the functional importance of these four P450 genes in the increased detoxification of insecticides in resistant *Culex* mosquitoes. Both P450 induction and constitutive overexpression may be co-responsible for detoxification of insecticides, evolutionary insecticide selection, and the ability of insects to adapt to changing environments.

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Chapter 6 Multiple Mutations and Mutation Combinations in the Sodium Channel of Permethrin Resistant Mosquitoes, *Culex quinquefasciatus*

Abstract

A previous study identified 3 nonsynonymous and 6 synonymous mutations in the entire mosquito sodium channel of *Culex quinquefasciatus*, the prevalence of which were strongly correlated with levels of resistance and increased dramatically following insecticide selection. However, it is unclear whether this is unique to this specific resistant population or is a common mechanism in field mosquito populations in response to insecticide pressure. The current study therefore further characterized these mutations and their combinations in other field and permethrin selected *Culex* mosquitoes, finding that the co-existence of all 9 mutations was indeed correlated with the high levels of permethrin resistance in mosquitoes. Comparison of mutation combinations revealed several common mutation combinations presented across different filed and permethrin selected populations in response to high levels of insecticide resistance, demonstrating that the co-existence of multiple mutations is a common event in response to insecticide resistance across different *Cx. quinquefasciatus* mosquito populations.
Key words: Mosquito vector; nonsynonymous/synonymous mutations; evolution, insecticide resistance; sodium channel; target protein

Vector control of mosquitoes with insecticides is an important part of the current global strategy to control mosquito-associated diseases. However, the widespread growth of resistance to insecticides in mosquitoes, especially to pyrethroids, is rapidly becoming a global problem [1]. The voltage gated sodium channels in the insect's nervous system are the primary

target of both pyrethroids and DDT, but modifications in the structure of the sodium channels due to point mutations or substitutions resulting from single nucleotide polymorphisms [SNP] results in insensitivity to both these insecticides in the sodium channels via a reduction in or an elimination of the binding affinity of the insecticides to proteins, thus diminishing the toxic effects of the insecticides and resulting in the development of insecticide resistance [2-5]. Among these *kdr* mutations, the substitution of leucine by phenylalanine [L to F], histidine [L to H], or serine [L to S] in the 6th segment of domain II (IIS6) has been clearly associated with resistance to pyrethroids and DDT in many insect species, including mosquitoes [6-11], while other *kdr* mutations appeared to be unique to specific species [3-5]. Systematic *in vitro* site-directed mutagenesis in insect sodium channel genes has revealed multiple regions in the sodium channels that contribute to the binding and action of pyrethroids [12,13], suggesting that the interactions of multiple mutations may play a role in the response of an insect's sodium channels to insecticides.

A recent analysis of all the naturally occurring mutations, both nonsynonymous and synonymous mutations, and the mutation combinations in the entire *Culex quinquefasciatus* sodium channel of the field parental population and its permethrin selected offspring has revealed, for the first time, the co-existence of multiple sodium channel mutations. Both nonsynonymous and synonymous mutations were observed in resistant mosquitoes and found to be important factors contributing to high levels of resistance [14], with the prevalence of mutations in the resistant mosquito sodium channels increasing dramatically following permethrin selection. However, it is unclear whether this is unique to this specific resistant population or if it is common to *Cx. quinquefasciatus* field populations subjected to insecticide pressure and hence the development of insecticide resistance. The current study therefore sought

to further investigate these mutations and their combination in other field collected and permethrin selected *Cx. quinquefasciatus* mosquito populations. The co-occurrence of both nonsynonymous and synonymous mutations in insecticide-resistant mosquitoes and their inheritance following insecticide selection were characterized and the specific thresholds for the insecticide concentrations at which particular mutations or mutation combinations occur in different mosquito populations or groups were tested. The study provides valuable information confirming that the co-existence of all 9 mutations, both nonsynonymous and synonymous, were indeed presented in resistant mosquitoes across different populations.

Results

Expression frequency of 9 mutations in pyrethroid resistant mosquitoes *Cx. quinquefasciatus*

We investigated the expression frequency of all 9 mutations, 3 nonsynonymous ($A^{109}S$, $L^{982}F$, and $W^{1573}R$) and 6 synonymous (L^{852}, G^{891}, A^{1241}, D^{1245}, P^{1249}, and G^{1733}), identified in an earlier study involving a different *Cx. quinquefasciatus* population (Fig. 6.1, [14]) in the sodium channels of the field parental population $MAmCq^{G0}$ and its 6th generation permethrin selected highly resistant offspring $MAmCq^{G6}$. The SNPs at the mutation sites were examined in 60 adult individuals from each of the $MAmCq^{G0}$ and $MAmCq^{G6}$ mosquito populations. All tested individuals in both populations showed expression of the polymorphic T325 allele at the codon $A^{109}S$ (Table 6.1), resulting in the substitution alanine to serine ($A^{109}S$). Interestingly, in the susceptible S-Lab population, 65% of the tested individuals expressed the susceptible allele G325, generating a codon encoding alanine, 35% expressed both the G325 and T325 alleles, and none expressed the polymorphic T325 allele (Table 6.1). A strong correlation between the prevalence of polymorphic allelic expression of A2946T and T4717C at the codons $L^{982}F$ and $W^{1573}R$, respectively, and the levels of pyrethroid resistance in the *Culex* mosquitoes were

identified. While all tested individuals in the susceptible S-Lab population expressed the susceptible alleles A2946 and T4717, producing codons encoding leucine (L^{982}) and tryptophan (W^{1573}), respectively (Table 6.1), all tested individuals in the highly resistant $MAmCq^{G6}$ population expressed polymorphic allele T2946, producing a substitution codon encoding phenylalanine (F^{982}), and 92% also expressed polymorphic allele C4717, generating a substitute codon encoding arginine (R^{1573}). The intermediate resistance population, $MAmCq^{G0}$, showed an intermediate level of allelic expression for SNPs of T2946A and T4717C (Table 6.1). These results suggest that the $L^{982}F$ and $W^{1573}R$ mutations are highly likely to be involved in the mosquitoes' elevated levels of pyrethroid resistance, and that individual mosquitoes with these polymorphic alleles are indeed selected by permethrin application. This result confirms the findings reported by Xu et al. [14] in their study of sodium channel mutations in another resistant *Cx. quinquefasciatus* mosquito population.

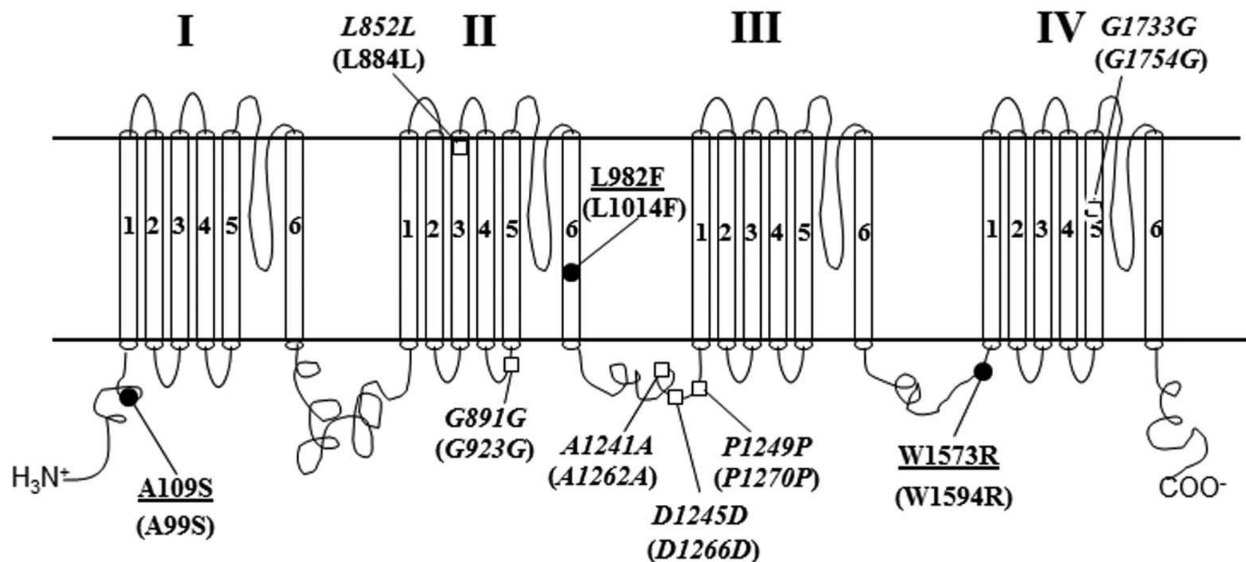

Figure 6.1. Graphic representation of the locations of synonymous and nonsynonymous mutations in the *Cx. quinquefasciatus* sodium channel. Nonsynonymous mutations are indicated by solid dots and their locations are underlined. Synonymous mutations are indicated by open

tetragons and their locations are in italics. Positions of the mutations are numbered according to amino acid sequences of *Cx. quinquefasciatus* (accession numbers: JN695777, JN695778, JN695779); the corresponding positions in the house fly Vssc1 sodium channel protein are shown in parentheses. The domain locations of the mutations are assigned according to the sodium channel amino acid sequences in house flies[7, 22].

Table 6.1. Non-synonymous and synonymous mutations in the sodium channel of *Cx. quinquefasciatus*

Mutation	Strain	n*	Phenotype†	Codons‡ (Frequency [%] ± SE)		
A109S[§]	S-Lab	60	Susceptible	GCA (65±5.0)	G/TCA(35±5.0)	TCA (0)
	MAmCq^{G0}	60	10-fold	GCA (0)	G/TCA (0)	TCA (100)
	MAmCq^{G6}	60	570-fold	GCA (0)	G/TCA (0)	TCA (100)
L982F[§]	S-Lab	60	Susceptible	TTA (100)	TTA/T (0)	TTT (0)
	MAmCq^{G0}	60	10-fold	TTA (22± 3.0)	TTA/T (52 ±6.0)	TTT (26 ±7.5)
	MAmCq^{G6}	60	570-fold	TTA (0)	TTA/T (0)	TTT (100)
W1573R[§]	S-Lab	60	Susceptible	TGG (100)	T/CGG (0)	CGG (0)
	MAmCq^{G0}	60	10-fold	TGG (72± 10.5)	T/CGG (25 ±8.5)	CGG (3.0 ±3.0)
	MAmCq^{G6}	60	570-fold	TGG (0)	T/CGG (8 ±5.5)	CGG (92 ±6.0)
L852L[#]	S-Lab	60	Susceptible	CTG (100)	CTG/A (0)	CTA (0)
	MAmCq^{G0}	60	10-fold resistance	CTG (27±10)	CTG/A (38±7.5)	CTA (35±5)
	MAmCq^{G6}	60	570-fold resistance	CTG (0)	CTG/A (6.5±2.8)	CTA (93.5±2.9)
G891G[#]	S-Lab	60	Susceptible	GGC (100)	GGC/A (0)	GGA (0)
	MAmCq^{G0}	60	10-fold resistance	GGC (28±10)	GGC/A (42±7.5)	CTA (30±10)

	MAmCq G6	60	570-fold	GGC (0)	GGC/A (5±5)	CTA (95±5)
A1241A#	S-Lab	60	Susceptible	GCA (100)	GCA/G (0)	GCG (0)
	MAmCq G0	60	10-fold resistance	GCA (2±3)	GCA/G (18±2.9)	GCG (80±5.5)
	MAmCq G6	60	570-fold	GCA (0)	GCA/G (0)	GCG (100)
D1245D#	S-Lab	60	Susceptible	GAC (100)	GAC/T (0)	GAT (0)
	MAmCq G0	60	10-fold resistance	GAC (38±7.5)	GAC/T (45±8.5)	GAT (17±5.5)
	MAmCq G6	60	570-fold	GAC (0)	GAC/T (8±5.5)	GAT (92±5.5)
P1249P#	S-Lab	60	Susceptible	CCG (100)	CCG/A (0)	CCA (0)
	MAmCq G0	60	10-fold resistance	CCG (37±5.5)	CCG/A (42±5.5)	CCA (21±5.5)
	MAmCq G6	60	570-fold	CCG (0)	CCG/A (5.0±5.0)	CCA (95±5.0)
G1733G#	S-Lab	60	Susceptible	GGA (48±12.5)	GGA/G (52±12.5)	GGG (0)
	MAmCq G0	60	10-fold resistance	GGA (0)	GGA/G (5±5.0)	GGG (95±5.0)
	MAmCq G6	60	570-fold resistance	GGA (0)	GGA/G (0)	GGG (100)

G0 represents the parental insects collected directly from the field; G6 represents the 6th generation of permethrin-selected MAmCq G0 offspring; Values represent mean ± SE for the three replications of frequency (%) analyses for each mutation.

* The total number of tested insects (three replicates for each of 10 males and 10 females)

† Data from [28]

‡The nucleotide polymorphisms are underlined

§ Non-synonymous mutations

Synonymous mutations

The SNP determination also revealed strong correlations between the frequency of polymorphic expression at the 6 synonymous codon sites and the levels of susceptibility and

resistance in *Cx. quinquefasciatus* (Table 6.1). All the synonymous nucleotide polymorphisms, as with the nonsynonymous polymorphisms, showed a strong association between the prevalence of polymorphic codon usage and the evolution of permethrin selection (Table 6.1). Non nucleotide substitutions at the synonymous codon sites, besides $G^{1733}G$, were detected in S-lab mosquitoes; higher frequencies of the polymorphic expression were detected in $MAmCq^{G6}$; and relatively low frequencies were detected in $MAmCq^{G0}$ (Table 6.1). Only the polymorphisms of A3723G and A5199G at the codons $A^{1241}A$ and $G^{1733}G$ showed relatively high frequencies (80% and 95%, respectively) of the polymorphic expression in $MAmCq^{G0}$ (Table 6.1*),* suggesting that synonymous polymorphisms A3723G at the codon $A^{1241}A$ and A5199G at the codon $G^{1733}G$ may evolve in the earliest stage of permethrin selection.

Correlation of polymorphic allele frequencies with the tolerance of mosquitoes to permethrin

To examine whether the mutation frequency/occurrence is related to increased levels of resistance or increased levels of tolerance of mosquitoes to certain concentrations of permethrin, and to characterize the permethrin concentration threshold that causes a particular mutation to occur in the mosquitoes and/or the differences in the timing of the occurrence of nonsynonymous and synonymous mutations, we examined the prevalence of each sodium channel mutation and correlated the results with the mosquitoes' tolerance to certain concentrations of permethrin in $MAmCq^{G0}$ and its permethrin selected offspring $MAmCq^{G6}$. We treated mosquito larvae of each population with different concentration of permethrin (Table 6.2) and assembled them into four groups (1 to 4) of each mosquito strain based on their similar levels of tolerance to permethrin (low to high, respectively). The results showed that all individuals in all tested groups across the field parental and permethrin selected offspring populations were homozygous for polymorphic allele T325 at the codon $A^{109}S$ (Fig. 6.2, Table 6.3). In addition, with the exception of groups 1

and 2 in $MAmCq^{G0}$, which had the lowest levels of tolerance to permethrin and showed heterozygous individuals for polymorphic allele G5199 at codon $G^{1733}G$, all individuals in the tested groups across both the field parental and permethrin selected offspring populations were homozygous for the mutation, which is consistent with the suggestion that $A^{109}S$ and $G^{1733}G$ may evolve in the earliest stage of permethrin resistance. A significantly different distribution of the frequency of polymorphisms for the remainder of the 7 nonsynonymous and synonymous mutations was found among different groups of mosquito populations (Figs. 6.2 and 6.3). Correlating the mutation prevalence with the level of tolerance to permethrin revealed the direct relevance of these 7 mutations to permethrin selection and resistance evolution. Homozygous polymorphic alleles A^{2556}, A^{2673}, T^{2946}, G^{3723}, T^{3735}, and A^{3747} began to appear in group 2 of $MAmCq^{G0}$, with a tolerance to permethrin concentrations between 0.003 and 0.01 ppm (Table 6.2), suggesting that these polymorphisms may be responsible for the initiation of moderate levels of permethrin resistance. The most noticeable mutation is the nonsynonymous C^{4717}, which emerged starting from group 4 of $MAmCq^{G0}$ and exhibited tolerance to permethrin concentrations of more than LC_{90} (>0.1 ppm), suggesting that this polymorphism may be the most important for the initiation of high levels of resistance.

Table 6.2. Permethrin treatment of field and permethrin selected *Culex* mosquitoes

Strains	Permethrin Treatments*									
	LC_{10} Treatment			LC_{50} Treatment			LC_{90} Treatment			
	n‡	†LC_{10} PPM	1st Group (collect dead mosquitoes)	n§	†LC_{50} PPM	2nd Groups (collect dead mosquitoes)	n¶	† LC_{90} PPM	3rd Groups (collect dead mosquitoes)	4th Groups (collect alive mosquitoes)
$MAmCq^{G0}$	~1500	0.003	$MAmCq^{G0}$-$<LC_{10}$	~1300	0.01	$MAmCq^{G0}$-LC_{10-50}	~800	0.1	$MAmCq^{G0}$-LC_{50-90}	$MAmCq^{G0}$-$>LC_{90}$
$MAmCq^{G6}$	~1500	0.3	$MAmCq^{G6}$-$<LC_{10}$	~1300	1	$MAmCq^{G6}$-LC_{10-50}	~800	10	$MAmCq^{G6}$-LC_{50-90}	$MAmCq^{G6}$-$>LC_{90}$

*Each treatment was performed 3 times

†The concentrations of permethrin administered to these mosquitoes was as identified previously [28]

‡The number of early 4th instar larvae used at the beginning of the permethrin treatment with LC_{10}

§The mosquitoes surviving permethrin treatment with LC_{10} 10 h after treatment

¶The mosquitoes surviving permethrin treatment with LC_{50} 10 h after treatment

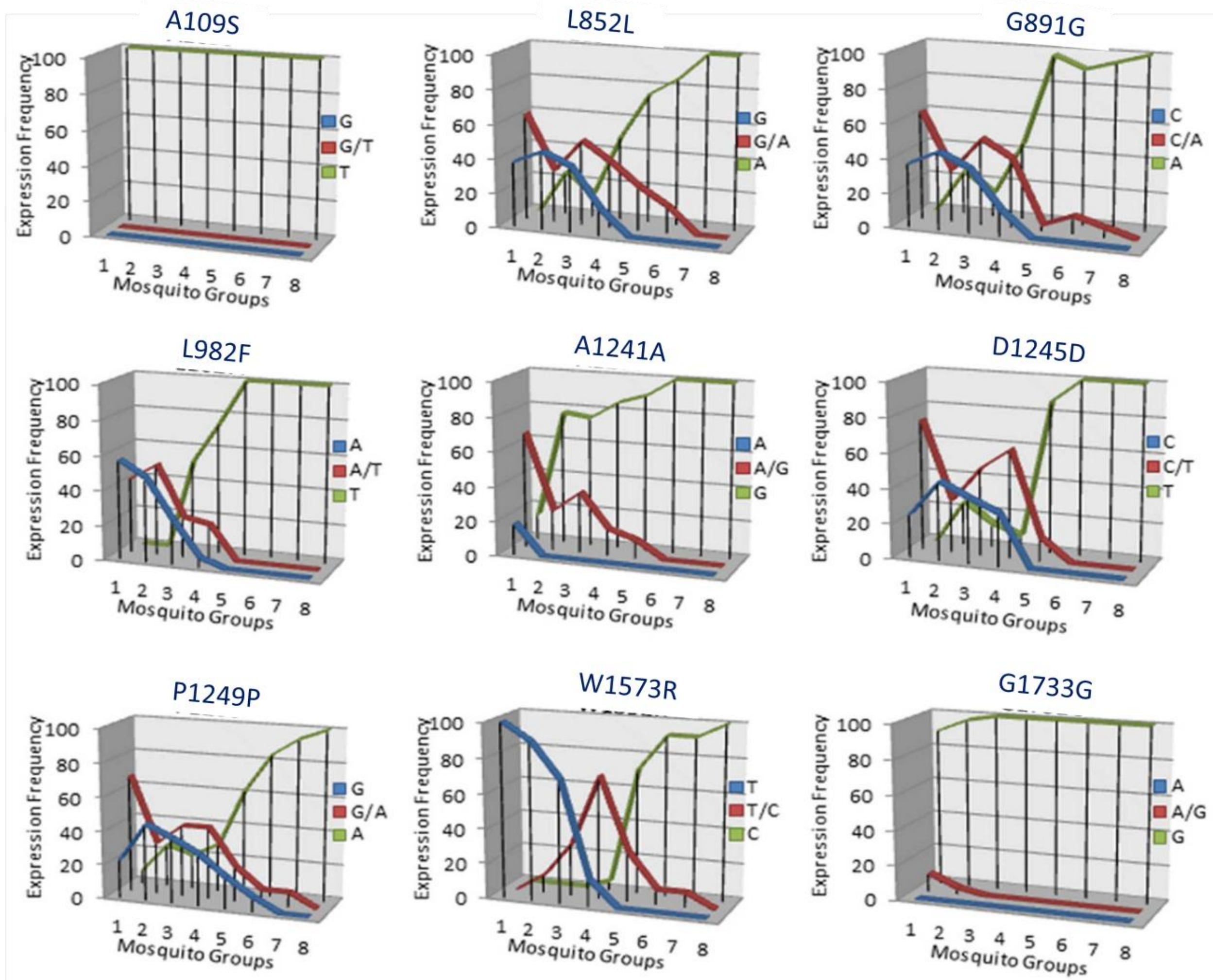

Figure 6.2. Distribution of frequencies of alleles at each of the mutation sites in each of the mosquito groups that are sensitive to or tolerant of different concentrations of permethrin (LC_{10}, LC_{50}, and LC_{90}) in $MAmCq^{G0}$ field parental populations and their 6th generation permethrin selected offspring, $MAmCq^{G6}$. The frequency of allele expression shown along the Y axis is the percentage of the mosquitoes (n = 40) carrying the homozygous or heterozygous allele(s) of the mutation. Mosquito groups are shown along the X axis; 1, 2, 3, and 4 represent the groups in $MAmCq^{G0}$ that were dead under LC_{10}concentration treatment, between LC_{10} and LC_{50}, between LC_{50} and LC_{90} and alive above LC_{90}, respectively; and 5, 6, 7, and 8 represent the groups in

MAmCq^{G6} that are dead under LC_{10}, between LC_{10} and LC_{50}, between LC_{50} and LC_{90}, and alive above LC_{90} concentration treatment, respectively.

Table 6.3. Co-occurrence of the *kdr* mutations in the MAmCq groups with difference levels of tolerance to permethrin

				Polymorphisms at Amino Acid Mutation Sites								
				A109S	L852L	G891G	L982F	A1241A	D1245D	P1249P	W1573R	G1733G
*Mosquito Groups		†N	‡F % (SE)	G to T	G to A	C to A	A to T	A to G	C to T	G to A	T to C	A to G
MAmCqG^{0}	1	1	10 (7)	T	G	C	A	A	C	G	T	A/G
		3	7.5 (3.5)	T	G	C	A	A	C	G	T	G
		4	5 (0)	T	G	C	A	A/G	C	G	T	G
		8	7.5 (3.5)	T	G	C	A	A/G	C/T	G/A	T	G
		10	7.5 (3.5)	T	G	C	A	G	C/T	G/A	T	G
		12	20 (7)	T	G/A	C/A	A	A/G	C/T	G/A	T	G
		13	35 (7)	T	G/A	C/A	A/T	A/G	C/T	G/A	T	G
		19	7.5 (3.5)	T	G/A	C/A	A/T	G	C/T	A	T	G
	2	2	2.5 (3.5)	T	G	C	A	G	C	G	T	A/G
		4	7.5 (3.5)	T	G	C	A	A/G	C	G	T	G
		5	12.5 (3.5)	T	G	C	A/T	A/G	C	G	T	G
		6	12.5 93.5)	T	G	C	A	G	C	G	T	G
		9	10 (0)	T	G	C	A/T	G	C	G	T/C	G
		13	2.5 (3.5)	T	G/A	C/A	A/T	A/G	C/T	G/A	T	G

		14	27.5 (3.5)	T	G/A	C/A	A/T	G	C/T	G/A	T	G
		27	25 (0)	T	A	A	A	G	T	A	T	G
	3	4	12.5 (3.5)	T	G	C	A	A/G	C	G	T	G
		5	10 (0)	T	G	C	A/T	A/G	C	G	T	G
		7	15 (7)	T	G	C	T	A/G	C	G	T	G
		15	17.5 (3.5)	T	G/A	C/A	A/T	G	C/T	G/A	T/C	G
		17	12.5 (3.5)	T	G/A	C/A	T	G	C/T	G/A	T	G
		18	12.5 (3.5)	T	G/A	C/A	T	G	C/T	G/A	T/C	G
		21	10 (7)	T	G/A	C/A	T	G	C/T	A	T	G
		27	10 (0)	T	A	A	A	G	T	A	T	G
	4	7	5 (0)	T	G	C	A	A/G	C	G	T	G
		11	10 (7)	T	G	C	T	G	C	G	T	G
		15	20 (7)	T	G/A	C/A	A/T	G	C/T	G/A	T/C	G
		16	15 (0)	T	G/A	A	T	G	C	G	T/C	G
		20	10 (0)	T	A	A	T	A/G	C/T	G/A	T/C	G
		22	20 (7)	T	G/A	C/A	T	G	C/T	A	T/C	G
		23	15 (7)	T	A	A	T	G	C/T	G/A	T/C	G
		31	5 (0)	T	A	A	T	G	T	A	C	G
MAmCqG[6]	1	20	10 (7)	T	A	A	T	A/G	C/T	G/A	T/C	G
		22	2.5 (3.5)	T	G/A	C/A	T	G	C/T	A	T/C	G
		24	10 (0)	T	G/A	A	T	G	T	G/A	T/C	G
		25	17.5 (3.5)	T	A	A	T	G	T	G	C	G
		29	12.5 (3.5)	T	G/A	A	T	G	T	A	C	G

	30	5 (0)	T	A	A	T	G	T	A	T/C	G
	31	42.5 (10.5)	T	A	A	T	G	T	A	C	G
2	24	7.5 (3.5)	T	G/A	A	T	G	T	G/A	T/C	G
	25	7.5 (3.5)	T	A	A	T	G	T	G	C	G
	28	15 (7)	T	A	C/A	T	G	T	A	C	G
	29	10 (0)	T	G/A	A	T	G	T	A	C	G
	31	60 (7)	T	A	A	T	G	T	A	C	G
3	26	7.5 (3.5)	T	A	A	T	G	T	G/A	T/C	G
	28	5 (0)	T	A	C/A	T	G	T	A	C	G
	31	87.5 (3.5)	T	A	A	T	G	T	A	C	G
4	31	100 (0)	T	A	A	T	G	T	A	C	G

*Group 1 mosquitoes tolerated permethrin concentration of $<LC_{10}$ (i.e., MAmCq^{G0}-$<LC_{10}$, and MAmCq^{G6}-$<LC_{10}$); group 2 mosquitoes tolerated permethrin concentrations of between LC_{10} - LC_{50} (i.e., MAmCq^{G0}-LC_{10-50}, and MAmCq^{G6}-LC_{10-50}); group 3 mosquitoes tolerated permethrin concentrations between LC_{50} - LC_{90} (i.e., MAmCq^{G0}-LC_{50-90}, and MAmCq^{G6}-LC_{50-90}); and group 4 mosquitoes tolerated permethrin concentrations $>LC_{90}$ (i.e., MAmCq^{G0-}$>LC_{90}$, and MAmCq^{G6}-$>LC_{90}$) (Table 6.2).

†N: The numbers indicate different combinations of the mutations and were assigned by weighing the numbers of the homozygous susceptible alleles, heterozygous, and homozygous resistance alleles in that combination, so higher numbers indicate higher incidences of heterozygous and homozygous resistance alleles.

‡F: the frequency (%) with which each of the mutation combinations occurred in each group. A total of 40 individuals (two replicates for each of 20 4th instar larvae) with all ten mutations in their sodium channel cDNAs was analyzed.

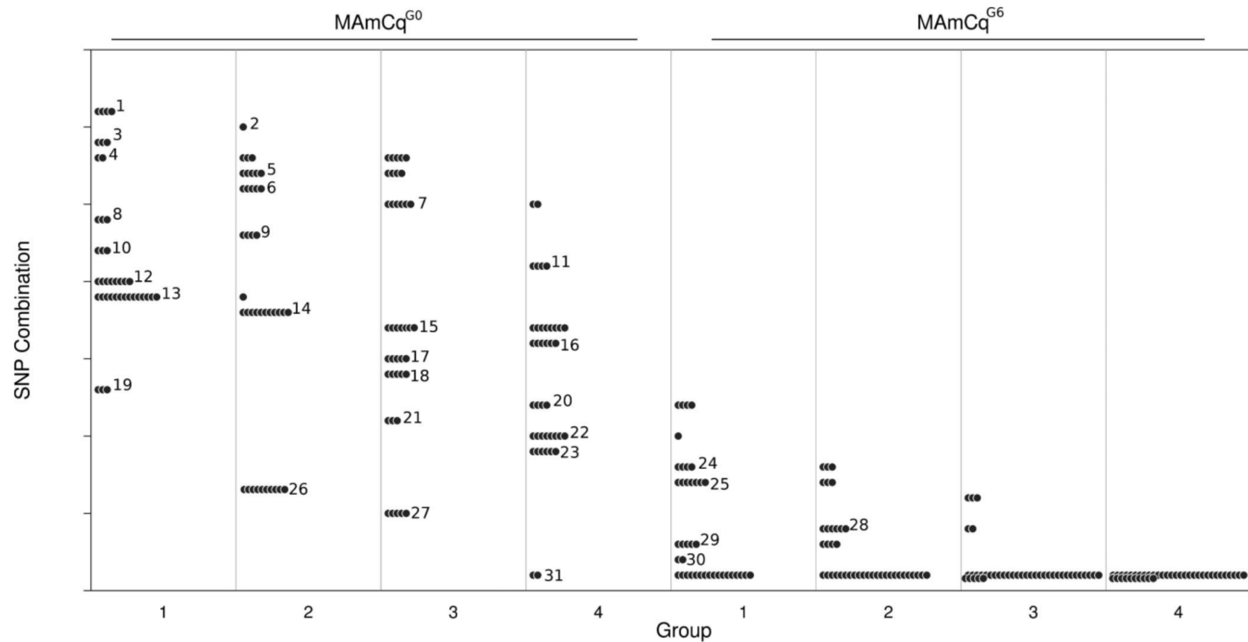

Figure 6.3. Categorical plots of the sodium channel mutation combination patterns in mosquito groups that are sensitive to or tolerant of different concentrations of permethrin in MAmCq^{G0} field parental populations and their 6th generation permethrin selected offspring, MAmCq^{G6}. The Y axes depict categories of mutation combinations (indicated by the numbers correspond to categories in Table 6.4) presented in each group (n = 40) of mosquitoes. On the X axes, mosquito groups are shown with the numbers 1-8 representing the same groups of MAmCq^{G0} and MAmCq^{G6} as in Fig. 6.2.

Mutation combinations of the mosquito sodium channel in response to permethrin application. To investigate the effects of different mutation combinations in mosquitoes' response to permethrin and the specific thresholds of permethrin concentrations at which particular mutations or mutation combinations occur, we examined the frequency of particular synonymous and/or nonsynonymous mutations that co-occur in the mosquito groups across different populations. The sodium channel mutations were analyzed in a total of 40 individuals,

which had all 9 mutations present in their full length sodium channel, in each of the mosquito groups. A total of 31 mutation combinations were identified across the mosquito populations and groups (Table 6.3, Fig. 6.3). Category #13 (double homozygous mutations and quintuple heterozygous mutations; T^{325}, g/a^{2556}, c/a^{2673}, a/g^{3723}, c/t^{3735}, g/a^{3747}, G^{5199}) was the predominant mutation combination in group 1 (the group with the lowest tolerance to permethrin) of $MAmCq^{G0}$. Categories #14 (triple homozygous mutations and quintuple heterozygous mutations; T^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{2735}, g/a^{3747}, G^{5199}) were the dominant combinations in group 2 of $MAmCq^{G0}$. The differences between categories #13 and #14 was the changes from susceptible homozygous A^{2946} to heterozygous a/t^{2946} and from heterozygous a/g^{3723} to polymorphic homozygous G^{3723}. A similar transition pattern was identified in the dominant mutation combinations of the consecutive mosquito groups with increased levels of tolerance to permethrin. Category #15, for example, was the predominant mutation combination in groups 3 and 4 (triple homozygous mutations and sextuple heterozygous mutations; T^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{2735}, g/a^{3747}, t/c^{4717}, G^{5199}), which showed a single change from heterozygous a/t^{2946} to polymorphic homozygous T^{2946} compared to category #14 in group 2. The occurrence of category #31 (nonuple homozygous mutations, T^{325}, A^{2556}, A^{2673}, T^{2946}, G^{3723}, T^{2735}, A^{3747}, C^{4717} and G^{5199}) emerged in the group 4 mosquitoes of $MAmCq^{G0}$ with a low frequency of 5%, suggesting that permethrin concentrations at 0.1 ppm may represent the threshold at which the particular #31 nonuple homozygous mutations combination occurs in the field mosquito population $MAmCq^{G0}$.

Comparing mutation combinations in permethrin selected offspring $MAmCq^{G6}$ with those in their field parental mosquitoes $MAmCq^{G0}$ revealed a clear shift in the mutation combinations in these populations from the majority being heterozygous mutation combinations,

for example categories #14 and #15 in $MAmCq^{G0}$, to the majority being resistant homozygous combinations like category #31 in $MAmCq^{G6}$ (Table 6.3, Fig. 6.3). Pairwise Goeman's Bayesian scores [15] tested using the AssotesteR software package in R [16,17] revealed the significant correlation between resistance levels of mosquito groups and their SNP combination frequencies (Table 6.4). A significant ($P \leq 0.05$) transition in the prevalence of the nonuple homozygous mutation combinations (category #31) was observed between the field parental strain and its permethrin selected offspring (Table 6.4, Fig. 6.3). Nevertheless, in place of the combination transition pattern for the predominant mutation combinations identified in the field mosquito population $MAmCq^{G0}$, category #31 (nonuple homozygous mutations) was the predominant mutation combination across all four groups of the permethrin selected offspring $MAmCq^{G6}$. A significant shift in the prevalence of this mutation combination was also observed in $MAmCq^{G6}$, rising from 42.5% in group 1, the lowest level of tolerance to permethrin treatment, to 100% in group 4, the highest level.

Table 6.4. Pairwise Goeman's Bayesian score test values to check for correlations between SNP combination frequencies and permethrin resistance levels

		$MAmCq^{G0}$				$MAmCq^{G6}$			
	Group	1	2	3	4	1	2	3	4
$MAmCq^{G0}$	1	-							
	2	120^{*}	-						
	3	22^{**}	90^{*}	-					
	4	760^{**}	450^{**}	200^{*}	-				
$MAmCq^{G6}$	1	2200^{**}	1700^{**}	1300^{**}	600^{**}	-			
	2	2500^{**}	2000^{**}	1600^{**}	800^{**}	30^{*}	-		
	3	2700^{**}	2200^{**}	1800^{**}	300^{**}	60^{**}	8.3^{*}	-	
	4	2800^{**}	2300^{**}	1900^{**}	1000^{**}	90^{**}	18^{**}	1.8^{**}	-

$^{*}P<0.05$; $^{**}P<0.001$

[†]Goeman's Bayesian score test value based on 500 permutations. Goeman's Bayesian scores represent a relative value for the comparison of paired samples. The higher the score, the more significant the correlation between resistance level and the SNP combination frequencies for the paired samples.

Discussion

Xu et al [14] characterized the mutations and mutation combinations over the entire sodium channel of individual resistant *Culex* mosquitoes $HAmCq^{G0}$ and their 8th generation permethrin selected offspring $HAmCq^{G8}$, identifying a total of 9 mutations, 3 of which were nonsynonymous and 6 synonymous. The prevalence of these corresponded closely to the mosquitoes' level of permethrin selection, permethrin treatment, and resistance to permethrin.

However, it is unclear whether Xu et al's results represent the unique case of this specific resistant population or whether this is a common response in field populations of resistant mosquitoes exposed to insecticide pressure. Our current study therefore further investigated all 9 of the mutations reported by Xu et al and their combinations in individual mosquitoes of a field population of *Cx. quinquefasciatus* mosquitoes MAmCq^{G0}, collected from Mobile, Alabama, ~600 km away from the location where the original HAmCq^{G0} mosquitoes studied by Xu et al. were collected. The *kdr* mutations over the entire mosquito sodium channel were analyzed and the mutation combinations in different mosquito groups categorized in terms of their levels of tolerance to a range of permethrin concentrations within and among the populations of the field parental strains and their permethrin selected offspring. The current study not only demonstrated that the co-existence of all 9 mutations, both nonsynonymous and synonymous, was presented in the resistant mosquitoes cross the different field populations but also identified common mutation combinations that corresponded to high levels of insecticide resistance among the mosquito populations studied. Interestingly, our results also suggest that the co-existence of multiple mutations is a common feature in insecticide resistant mosquitoes.

Our study found a similar allelic expression pattern of the 9 mutations cross the mosquito populations tested to those reported by Xu et al. [14]. A clear shift of mutation combinations was again detected from those with primarily homozygous susceptible alleles, through those with mostly heterozygous alleles, to those with all or nearly all homozygous polymorphic alleles at the mutation sites, corresponding to the increasing tolerance of the mosquito groups to permethrin treatments in both field mosquito populations and their permethrin selected offspring. Although both HAmCq and MAmCq exhibited their own specific mutation combinations, with a total of 20 mutation combinations identified in the HAmCq mosquitoes [14] and 31 mutation

combinations in the MAmCq mosquitoes, these two *Culex* populations shared 13 categories of mutation combinations (Table 6.5), the majority of which were the predominant mutation combinations in the mosquito groups in either or both HAmCq and MAmCq mosquito populations in response to certain concentration(s) of permethrin treatments. For example, combination category F - T^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{2735}, g/a^{3747}, G^{5199} (Table 6.5) - was the predominant mutation combination in group 2 of both the field parental mosquito populations of $HAmCq^{G0}$ (category #8, [14]) and $MAmCq^{G0}$ (category #14). Interestingly, this combination was also the dominant mutation combination in groups 3 and 4 of $HAmCq^{G0}$, whereas combination category G - T^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{2735}, g/a^{3747}, t/c^{4717}, G^{5199} - was the dominant combination in groups 3 and 4 of $MAmCq^{G0}$. The only difference between mutation combination categories F and G is a switch from the susceptible homozygous T^{4717} to the heterozygous t/c^{4717} (Table 6.5). The first occurrence of combination category F was in the group 2 mosquitoes of both $HAmCq^{G0}$ and $MAmCq^{G0}$, both of which have a tolerance to permethrin concentrations of between 0.003 and 0.05 ppm, suggesting that the concentration range of 0.003 to 0.05 ppm represents a threshold, at which the T^{325}, g/a^{2556}, c/a^{2673}, a/t^{2946}, G^{3723}, c/t^{2735}, g/a^{3747}, G^{5199} mutation combination occurs in field mosquito populations. Mutation combination category M (nonuple homozygous mutations, T^{325}, A^{2556}, A^{2673}, T^{2946}, G^{3723}, T^{2735}, A^{3747}, C^{4717} and G^{5199}) emerged in the group 4 mosquitoes of both $HAmCq^{G0}$ and $MAmCq^{G0}$ with very low frequencies of 2.5 and 5%, respectively, suggesting that permethrin concentrations between 0.1 and 0.2 ppm may represent the threshold at which the particular individuals with the mutation combination of T^{325}, A^{2556}, A^{2673}, T^{2946}, G^{3723}, T^{2735}, A^{3747}, C^{4717} and G^{5199} could be selected from in field mosquito populations. These results strongly suggest that the same or similar mutation combinations are present in different field populations of *Cx. quinquefasciatus* mosquitoes and

are responsible for similar levels of resistance, revealing the importance and common features of these combinations in the development of insecticide resistance in field mosquito populations.

Table 6.5. The 13 common mutation combinations of sodium channels in the mosquito populations of *Cx. quinquefasciatus*

Mutation Combination			Polymorphisms at Amino Acid Mutation Sites								
			A109S	L852L	G891G	L982F	A1241A	D1245D	P1249P	W1573R	G1733G
Category	N[1]	N[2]	G to T	G to A	C to A	A to T	A to G	C to T	G to A	T to C	A to G
A	1	2	T	G	C	A	G	C	G	T	A/G
B	2	6	T	G	C	A	G	C	G	T	G
C	3	8	T	G	C	A	A/G	C/T	G/A	T	G
D	4	10	T	G	C	A	G	C/T	G/A	T	G
E	5	13	T	G/A	C/A	A/T	A/G	C/T	G/A	T	G
F	8	14	T	G/A	C/A	A/T	G	C/T	G/A	T	G
G	9	15	T	G/A	C/A	A/T	G	C/T	G/A	T/C	G
H	13	24	T	G/A	A	T	G	T	G/A	T/C	G
I	14	25	T	A	A	T	G	T	G	C	G
G	15	26	T	A	A	T	G	T	G/A	T/C	G
K	18	29	T	G/A	A	T	G	T	A	C	G
L	19	30	T	A	A	T	G	T	A	T/C	G
M	20	31	T	A	A	T	G	T	A	C	G

N[1]: The numeral indicates the category of mutation combination(s) in the HAmCq mosquitoes [14]

N[2]: The numeral indicates the category of mutation combination(s) in the MAmCq mosquitoes.

Table 6.6. Oligonucleotide primers[*] used for amplifying the sodium channel cDNA fragments and SNP (single nucleotide polymorphism) determination.

Primer name	Function	Primer sequence (5' to 3')	Primer Location (nt)
KDR S16	cDNA fragment 1 and full length amplification	TGTTGGCCATATAGACAATGACCGA	-17 to 8
KDR AS34	cDNA fragment 1 amplification and 5' RACE	GTAATACTGACAATCCCTGAACGC	2584 to 1561
PG_KDR S4	cDNA fragment 2 amplification	GCGGTAACTACTTCTTCACGGC	2414 to 2435
KDR AS02	cDNA fragment 2 amplification	CCAKCCYTTRAAKGTGGCYACTTG	4411 to 4434
KDR S03	cDNA fragment 3 amplification and 3'RACE	TGAACTTYGACCACGTGGGG	4370 to 4389
KDR AS09	cDNA fragment 3 and full length amplification	GCTTCTGAATCTGAATCAGAGGGAG	6290 to 6266
Cx_SNP 2	SNaP determination	GCCACCGTAGTGATAGGAAATTT	2923 to 2945
Cx_SNP 4	SNaP determination	CTCGAGGATATTGACGCTTTTTAC	301 to 324
Cx_SNP 6	SNaP determination	TGAAGGCCATTCCGCGGCCCAAG	4693 to 4716
Cx_SNP 12	SNaP determination	CTTTCGCTGCTCGAGCTCGGTCT	3532 to 2555

Cx_SNP 13	SNaP determination	TCCATCATGGGCCGAACGATGGG	2649 to 2672
Cx_SNP 14	SNaP determination	AACTGCTACAAGCGGTTCCCGGC	3699 to 3722
Cx_SNP 15	SNaP determination	GGTTCCCGGCRCTGGCCGGCGA	3713 to 3734
Cx_SNP 16	SNaP determination	TGGCCGGCGAYGACGACGCGCC	3725 to 3746
Cx_SNP 18	SNaP determination	ATGTTCATCTTCGCCATCTTCGG	5176 to 5198

Comparing mutation combinations in the permethrin selected offspring HAmCq^{G8} [14] and MAmCq^{G6} with those of their field parental mosquitoes HAmCq^{G0} and MAmCq^{G0} revealed a clear shift from the majority being heterozygous mutation combinations, for example mutation combination categories F and/or G (Table 6.5), in HAmCq^{G0} and MAmCq^{G0}, to the majority being homozygous mutation combinations, such as mutation combination category M in both HAmCq^{G8} [14] and MAmCq^{G6}. This clear-cut pattern of mutation combination was observed following permethrin selection across all the different mosquito populations. Although mutation combination category M was the major mutation combination in all 4 groups of both HAmCq^{G8} and MAmCq^{G6}, a significant shift in the prevalence of this mutation combination was also observed, rising from 12.5% in group 1 with the lowest level of tolerance to permethrin treatment, to 62.5% in group 4 with the highest level of tolerance, in HAmCq^{G8} [14] but from 42.5% in group 1 to 100% in group 4 mosquitoes of MAmCq^{G6}. The strong correlation between the frequency of the mutation combination and its association with permethrin selection and tolerance to permethrin treatment confirmed that not only are these mutations co-selected by permethrin, but the combination of all 9 mutations is also involved in the high levels of resistance.

Insecticide resistance is generally assumed to be a pre-adaptive phenomenon in which prior to insecticide exposure rare individuals carrying an altered (varied) genome already exist, allowing those carrying the genetic variance to survive insecticide selection [18]. Accordingly, the proportion of individuals carrying the resistance genes, polymorphisms or alleles should increase in a population following selection through inheritance and eventually become predominate in a population subjected to prolonged exposure to insecticides. Indeed, both this study and the previous work by Xu et al. [14] show a clear permethrin selection force favoring individuals carrying the polymorphic alleles. For instance, 0.6 to 1.3% individuals carrying all nine mutations were present in the field populations of both $HAmCq^{G0}$ and $MAmCq^{G0}$, but after a few generation of permethrin selection in the laboratory individuals carrying all 9 mutations increased to 34.4% and 72.5% in the populations of $HAmCq^{G8}$ and $MAmCq^{G6}$, respectively.

The synergistic effects of the co-existence of insect sodium channel mutations on insecticide resistance have been previously reported by several research groups. Possibly the most notable of these is the co-presence of the methionine (M) to threonine (T) mutation (M918T), termed a *super-kdr* mutation, in the linker connecting IIS4 and IIS5, with the L to F (L1014F) mutation in IIS6 of the sodium channel in *super-kdr* house flies, which exhibit higher levels of resistance to DDT and pyrethroids than *kdr* house flies, where only the L1014F mutation is observed [3,4,7]. Besides the co-existence of the L1014F and M918T mutations in *super-kdr* house flies, the same combination of M-to-T and L-to-F mutations has also been observed in other insect species, namely *Haematobia irritans* [19], *Thrips tabaci* [20], and *Myzus persicae* [21], all of which have been found to exhibit relatively high-level resistance to pyrethroids. However, these three species plus mosquitoes are the only ones where the *super kdr* mutation has been reported. In other insect species, rather than the M-to-T *super kdr* mutation,

there is some evidence to suggest that additional sodium channel mutations that co-exist with the L-to-F mutation are associated with high levels of resistance [3-5]. Although the M to T *super kdr* mutation in the linker connecting IIS4 and IIS5 was not identified in the sodium channel sequences of any of the individual mosquitoes in either the current study or the earlier study by Xu et al. [14], the synonymous polymorphism of C2673A at codon $G^{891}G$ (corresponding to G^{923} of the house fly Vssc1 sodium channel protein) resulting from a single nucleotide polymorphism (SNP) of cytosine to adenine at nt 2673 (C2673A) was found in all the field parental and permethrin selected mosquito individuals tested. The synonymous codon $G^{891}G$ is located in the linker connecting IIS4 and IIS5, five amino acids downstream from the methionine residue (corresponding to the position of the M918T mutation in the house fly Vssc1 sodium channel protein (Fig. 6.1, [7,22]). Our results also showed that not only was the C2673A synonymous polymorphism almost always linked with the $L^{982}F$ mutation (corresponding to the position of the L1014F mutation in house fly Vssc1) in resistant *Culex* mosquitoes, but also that they co-presented together with other mutations in resistant mosquitoes.

Conclusion

Our data, taken together with the data reported previously by Xu et al. [14], combine to make a strong case linking the incidence of these 9 synonymous and nonsynonymous mutations with the levels of permethrin resistance in *Cx. quinquefasciatus* mosquito populations. Future research should focus on investigating the function and functional interaction of these mutations in the sodium channel in terms of how they may affect the channel's structure and proteins, particularly with regard to its gating properties and the binding configurations of the sodium channel to insecticides. The precise roles of the synonymous mutations in the various sodium

channel functions should also be examined in terms of protein secondary structure formation [23] and protein folding [24], as identified in other living systems.

Materials and Methods

Mosquito strains

Three strains of mosquito *Cx. quinquefasciatus* were studied: $MAmCq^{G0}$, the field parental resistant strain collected from Mobile county, Alabama, USA [25]; $MAmCq^{G6}$, the 6th generation offspring of laboratory permethrin-selected $MAmCq^{G0}$; and S-Lab, an insecticide-susceptible strain.

Permethrin treatment

Preliminary concentration ranges for larvae were utilized to generate concentration ranges of LC_{10}, LC_{50}, and LC_{90} for each mosquito strain (Table 6.3) and then used to treat each of the *Culex* strains, $MAmCq^{G0}$ and $MAmCq^{G6}$, generating 8 larval groups with different levels of resistance to the permethrin insecticide. Briefly, ~1500 4th instar larvae of each *Culex* strain were treated with permethrin at their respective LC_{10} concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 1 of each mosquito population (i.e., $MAmCq^{G0}$-$<LC_{10}$, or $MAmCq^{G6}$-$<LC_{10}$). The surviving mosquitoes were then exposed to permethrin LC_{50} concentrations. Eight hours after this treatment, the dead mosquitoes were collected as group 2 of each mosquito population ($MAmCq^{G0}$-LC_{10-50}, or $MAmCq^{G6}$-LC_{10-50}). The surviving mosquitoes from the permethrin LC_{50} concentration treatment were then exposed to permethrin LC_{90} concentrations. Eight hours after treatment, the dead and surviving mosquitoes were separately collected as group 3 ($MAmCq^{G0}$-LC_{50-90}, or $MAmCq^{G6}$-LC_{50-90}) and group 4 ($MAmCq^{G0}$-$>LC_{90}$ or $MAmCq^{G6}$-$>LC_{90}$). Each treatment was repeated 2 times. In this study, the criterion applied was that only individuals that had all 9 mutations could be utilized for the

analyses. Data from a total of 40 individual mosquitoes that met this criterion in each of the 8 groups was collected and analyzed.

Nucleotide polymorphism (SNP) determination for the nucleotide polymorphisms in *Cx. quinquefasciatus*

SNP determinations utilizing an ABI Prism SNaPshot Multiplex Kit were analyzed on an ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer's instructions (A&B Applied Biosystems, [9]). Total RNAs were extracted from a pool of adult mosquitoes for each of the populations. Two replications were performed for each experiment and a total of 40 individual 4th instar larvae were used for each of permethrin treated groups with 20 for each replication. The first strand cDNAs were synthesized from each individual mosquito using the oligo(dT) primer as follows. Three PCR primer pairs, KDR S16 /KDR AS34, PG_KDR S4/KDR AS02, and KDR S03/KDR AS09 (Table 6.6) were designed according to the specific sequences of the full length *Culex* sodium channel cDNAs ([14], accession numbers: JN695777, JN695778, and JN695779) to amplify three sodium channel cDNA fragments from each of the individual mosquitoes with polymorphisms. For each PCR reaction, the cDNA template and 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. PCR products were then used as the templates for the SNP determination. Each PCR reaction was performed 3 times on the cDNA of each of a total of 40 individual 4th instar larvae (20 for each experimental replication) from each of the mosquito groups and for 60 individual 3 day old adults (10 males and 10 females for each experimental replication) from each mosquito population. The PCR products also served as the replication for the SNP determination of each polymorphism. 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, *kdr* cDNA fragments, PCR products of each mosquito sample were sequenced at least once each. The alleles at the polymorphism site of each mutation were analyzed using Genemapper software according to the manufacturer's instructions and as described by Xu et al. [26,27]. The frequency (prevalence) of polymorphic allelic expression for each of the mutations between and among the groups or populations of the mosquitoes was also measured.

Data analysis

The statistically significant difference of the frequency of each of the nucleotide polymorphisms between and among the mosquito samples was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant. Pairwise Goeman's Bayesian scores [15] were tested for significant correlations between resistance levels and SNP combination frequencies of the paired samples using the AssotesteR package in R [16] based on the recommendations for analyzing multiple SNPs in a given gene [17]. Data were stratified by group within each generation, with SNP combinations representing the cases. A total of 500 permutations were conducted for each pairwise comparison.

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Chapter 7 Multiple Sodium Channel Variants in the Mosquito *Culex quinquefasciatus*

Abstract

Voltage-gated sodium channels are the target sites of both DDT and pyrethroid insecticides. The importance of alternative splicing as a key mechanism governing the structural and functional diversity of sodium channels and the resulting development of insecticide and acaricide resistance is widely recognized, as shown by the extensive research on characterizing alternative splicing and variants of sodium channels in medically and agriculturally important insect species. Here we present the first comparative study of multiple variants of the sodium channel gene in the mosquito *Culex quinquefasciatus*. The variants were classified into two categories, CxNa-L and CxNa-S based on their distinguishing sequence sizes of ~6.5 kb and ~4.0 kbs, respectively, and generated via major extensive alternative splicing with minor small deletions/ insertions in susceptible S-Lab, low resistant $HAmCq^{G0}$, and highly resistant $HAmCq^{G8}$ *Culex* strains. Four alternative CxNa-L splice variants were identified, including three full length variants with three optional exons (2, 5, and 21i) and one with in-frame-stop codons. Large, multi-exon-alternative splices were identified in the CxNa-S category. All CxNa-S splicing variants in the S-Lab and $HAmCq^{G0}$ strains contained in-frame stop codons, suggesting that any resulting proteins would be truncated. The ~1000 to ~3000-fold lower expression of these splice variants with stop codons compared with the CxNa-L splicing variants may support the lower importance of these variants in S-Lab and $HAmCq^{G0}$. Interestingly, two alternative splicing variants of CxNa-S in $HAmCq^{G8}$ included entire ORFs but lacked exons 5 to18 and these two variants had much higher expression levels in $HAmCq^{G8}$ than in S-Lab and $HAmCq^{G0}$.

These results provide a functional basis for further characterizing how alternative splicing of a voltage-gated sodium channel contributes to diversity in neuronal signaling in mosquitoes in response to insecticides, and possibly indicates the role of these variants in the development of insecticide resistance.

Key words: Sodium channel, transcript variants, alternative splicing, insecticide resistance, *Culex quinquefasciatus*

Introduction

The targets for insecticides such as DDT and pyrethroids are the insects' voltage-gated sodium channels (Bloomquist 1996, Narahashi 1996, Soderlund 2005), which are responsible for the rising phase of action potentials in the membranes of neurons and most electrically excitable cells (Catterall 2000). Pyrethroids and DDT deliver their toxic, insecticidal effects primarily by binding to the sodium channel, thus altering its gating properties and keeping the sodium channel open for unusually long times, thereby causing a prolonged flow of sodium current that initiates repetitive discharges and prevents the repolarization phase of action potentials (Narahashi 1988, 2000, Catterall 2000). The common feature found in sodium channels is that relatively small changes (such as point mutations or substitutions, short sequence insertions or deletions, or alternative splicing) in the structure of these channels significantly affect their behavior and are sufficient to change neuronal firing, resulting in different phenotypes. Modifications of the insect sodium channel structure can cause insensitivity of the channels to DDT and pyrethroids via a reduction in or an elimination of the binding affinity of the insecticides to proteins (Narahashi 1988, 2000), and hence result in the development of insecticide resistance.

In mammalian systems, molecular characterization of voltage-gated sodium channel genes has revealed the existence of multiple genes (Meisler et al. 2010, Catterall 2000, Goldin

2002): ten paralogous voltage-gated sodium channel genes have been identified in humans (Goldin 2001); 8 in zebra fish (Novak et al. 2006); and 6 in electric fish (Lopreato et al. 2001). Several invertebrate species have also been found to include multiple sodium channel genes in their genome; for example, four sodium channel genes have been characterized in *Hirudo medicinalis* (leech) and two in *Holocynthia roretzi* (ascidia) (Blackshaw et al. 2003, Nagahora et al. 2002, Okamura et al. 2002). Compared to the fairly well defined multiple vertebrate sodium channel genes, it appears that a single sodium channel gene that has been well characterized in many insect species, homologous to *para* (currently $DmNa_V$) of *Drosophila melanogaster*, (Shao et al. 2009) encodes the equivalent of the α-subunit of the mammalian sodium channels. While mammals rely on the selective expression of at least ten different sodium channel genes in various tissues to achieve sodium channel diversity (Goldin et al., 2000), insects may produce a range of diverse sodium channels with different functional and pharmacological properties from a single sodium channel by extensive alternative splicing (Davies et al. 2007, Dong 2007, Olson et al. 2008, Lin et al. 2009).

Because of the importance of alternative splicing as a key mechanism for generating structural and functional diversity in sodium channels (Tan et al. 2002), following the first discovery of the existence of alternative splicing of the *para* sodium channel gene from *Drosophila melanogaster* (Loughney et al. 1989), alternative splicing events were subsequently characterized in many medically or agricultural important insect and arachnid pest species (Loughney et al. 1989, Thackeray and Ganetzky 1995, O'Dowd et al. 1995, Olson et al. 2008, Lee et al. 2002, Tan et al. 2002, Davies et al. 2007, Sonodo et al. 2008, Shao et al. 2009, Wang et al. 2003). As yet, however, there have been no reports of alternative splicing in *Culex quinquefasciatus*, an important mosquito vector of human pathogens such as St. Louis

encephalitis virus (SLEV), West Nile virus (WNV), and the parasitic *Wuchereria bancrofti* nematode in many urban settings throughout the tropical and temperate regions of the world (Sardelis et al. 2001, Jones et al. 2002, Arensburger et al. 2010). Here we present the first comparative study of full length sequences of the para-orthologue sodium channel genes from the *Culex quinquefasciatus* mosquito and examine multiple variants obtained through the mechanism of alternative splicing.

Materials and methods

Mosquito strains

Three strains of mosquito Cx. quinquefasciatus were studied. S-Lab is an insecticide susceptible strain provided by Dr. Laura Harrington (Cornell University). HAmCq^{G0} is a low insecticide resistant strain with a 10-fold level of resistance to permethrin compared with the laboratory susceptible S-Lab strain (Xu et al. 2006). It was originally collected from Huntsville, Alabama, in 2002 and established in our laboratory without further exposure to insecticides (Liu et al. 2004). The HAmCq^{G8} strain is the 8th generation of permethrin-selected HAmCq^{G0} offspring and has a 2,700-fold level of resistance (Xu et al. 2006, Li and Liu 2010). All mosquitoes were reared at 25±2°C under a photoperiod of 12:12 (L:D) h.

Amplification of the full length of sodium channel transcripts in *Cx. quinquefasciatus*

For each of the three mosquito populations, total RNA was extracted from the 4th instar larvae and different tissues (head + thorax, and abdomen) from 2-3 day-old adult females before blood feeding using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott 1997). mRNAs were isolated using Oligotex-dT suspension (QIAGEN). The full length of the *Cx. quinquefasciatus* sodium channel cDNA was subsequently isolated from each of the mosquitoes populations by RT-PCR using the Expand Long Range, dNTPack kit (Roche) with a specific

primer pair, KDR S16 (TGTTGGCCATATAGACAATGACCGA) /KDR AS09 (GCTTCTGAATCTGAATCAGAGGGAG) (Table 7.1), synthesized based on the respective 5’ and 3’ end sequences of the putative sodium channel genes (Xu et al. 2012, accession numbers: JN695777, JN695778, and JN695779). The PCR reaction was conducted following a PCR cycle of 92°C for 2 min, 10 cycles of 92°C for 10 s, 55°C for 15 s, and 68°C for 6 min, and 35 cycles of 92°C for 10 s, 55°C for 15 s, and 68°C for 6 min and 20 s, with a final extension of 68°C for 10 min. All PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced. Cloning and sequence analyses of sodium channel cDNA fragments were repeated at least two times for each mosquito strain with different preparations of RNAs and mRNAs. The inserts of *Culex* sodium channel clones were sequenced and analyzed.

Table 7.1. Oligonucleotide primers used in qRT-PCR reactions for amplifying sodium channel variants

Mosquito population	Varaints	Formard primer	Reverse primer
S-Lab	CxNa-L_v1	5’ AATCAGCTGTAAAAGTGATGGCGC 3’	5’ AGCTCGTAGTACCCTGAATGTTCT 3’
	CxNa-L_v2	5’ CACTGCAAAAGCCCGTAAAGTGAG 3’	5’ ATAGTATACTGGAACGATGATTGCA 3’
	CxNa-L_v3	5’ ACAAGGGCAAGAAGAACAAGCAGC 3’	5’ CTTTATACTGGCAGTGTCATCGTC 3’
	CxNa-S_v1	5’ ATCGATATCTGAGAGAACGTAGTT 3’	5’

			TTCATCCTCGTCCTCATCGTCGTA 3’
HAmCq^{G0}	CxNa-L_v4	5’ GGTCGGAAGAAAAAGAAAAGAGA 3’	5’ TATCCTTTCCTTTACTAACTACTA 3’
	CxNa-L_v5	5’ GCCAAAAAAAGTACTACAACGCAA 3’	5’ TCCCGTCTGCTTGTAGTGAT 3’
	CxNa-L_v6	5’ AGCACAACCATCTCAGTTGGATAT 3’	5’ TCGTCGTCGAGTTCTTCGTCAATT 3’
	CxNa-S_v2	5’ CAAAAGTTCGACATGATCATCATG 3’	5’ TGAAGAACGACATCCCGAAGATG 3’
	CxNa-S_v3	5’ TACTACATGGACAGGATATTCAC 3’	5’ CAGGTTTATGAGCGAGAGCATCA 3’
HAmCq^{G8}	CxNa-L_v7	5’ TCGAGTGTTCAAGCTAGCAAA 3’	5’ AATGCAGAGCACAAACGTCAG 3’
	CxNa-L_v8	5’ TTCCAGTATACTATGCTAATTTAG 3’	5’ TTGGTGTCGACGTAGGACATGTT 3’
	CxNa-S_v4	5’ TCCAAGGTGATAGGCAATTCTATT 3’	5’ TCAATTCCTAGGTCCTCCTTGCT 3’

CxNa-S_v5	5’ ACTACTACGAATGTCTTAATGTTT 3’	5’ TGTACTAAAATATAAATAGCTACG 3’

Quantitative real-time PCR (qRT-PCR)

The total RNA (0.5 µg/sample) from each mosquito sample was reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 µl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR, which was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 µl final volume) contained 1x SYBR Green master mix, 1 µl of cDNA, and a sodium channel transcript specific primer pair designed according to each of the sodium transcript or allele sequences (Table 7.1 shows the accession number for each of the sodium channel transcripts or alleles) at a final concentration of 3-5 µM. All samples, including the A ‘no-template’ negative control, were performed in triplicate. The reaction cycle consisted of a melting step of 50ºC for 2 min then 95°C for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Specificity of the PCR reactions was assessed via a melting curve analysis for each PCR reaction using Dissociation Curves software (Wittwer et al. 1997). Relative expression levels for the sodium channel transcripts were calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software (Livak and Schmittgen 2001). The 18S ribosome RNA gene, an endogenous control, was used to normalize the expression of target genes (Liu et al. 2007, Aerts et al. 2004, Zhu and Liu 2008, Zhu et al. 2008). Preliminary qRT-PCR experiments with a primer pair (Table 7.1) designed according to the sequences of the 18S ribosome RNA gene had revealed that the expression of this gene remained constant among all 3 mosquito strains, so the

18S ribosome RNA gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated three to four times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant. Significant overexpression was determined using a cut-off value of a ≥2-fold change in expression (Strode et al. 2008).

Results

Generation of sodium channel transcripts in *Cx. quinquefasciatus*

To examine the number of transcripts of the para-type sodium channel gene in the genome of *Culex* mosquitoes, RNAs isolated from S-Lab, $HAmCq^{G0}$ and $HAmCq^{G8}$ were subjected to PCR amplification using a primer pair: KDR S16 (TGTTGGCCATATAGACAATGACCGA)/KDR AS09 (GCTTCTGAATCTGAATCAGAGGGAG), synthesized based on the respective 5' and 3' end sequences of the putative sodium channel genes (Table 7.1) (Li et al. 2012). Two distinct molecular sizes of sodium channel cDNAs with ~6.5 and ~4 kb were generated by PCR amplification from each of the three mosquito strains, namely susceptible (S-Lab), intermediate ($HAmCq^{G0}$), and highly resistant ($HAmCq^{G8}$), when only a single primer pair, KDR S16/ KDR AS09 was used (Fig. 7.1). The PCR products of both the ~6.5 and ~4.0 kb fragments from each strain were then cloned and sequenced. Sequence analysis of insertions of clones with ~6.5 kb and ~4.0 kb PCR amplified products from each strain indicated that all the cDNA clones were indeed the sodium channel genes. Interestingly, nucleotide sequence analysis of these sodium channel transcripts revealed the existence of multiple variants in each of ~6.5 and ~4.0 kb PCR

amplification products in each mosquito strains. These variants were then assigned to two categories, CxNa-L and CxNa-S, based on their sizes of ~6.5 and ~4.0 kb, respectively.

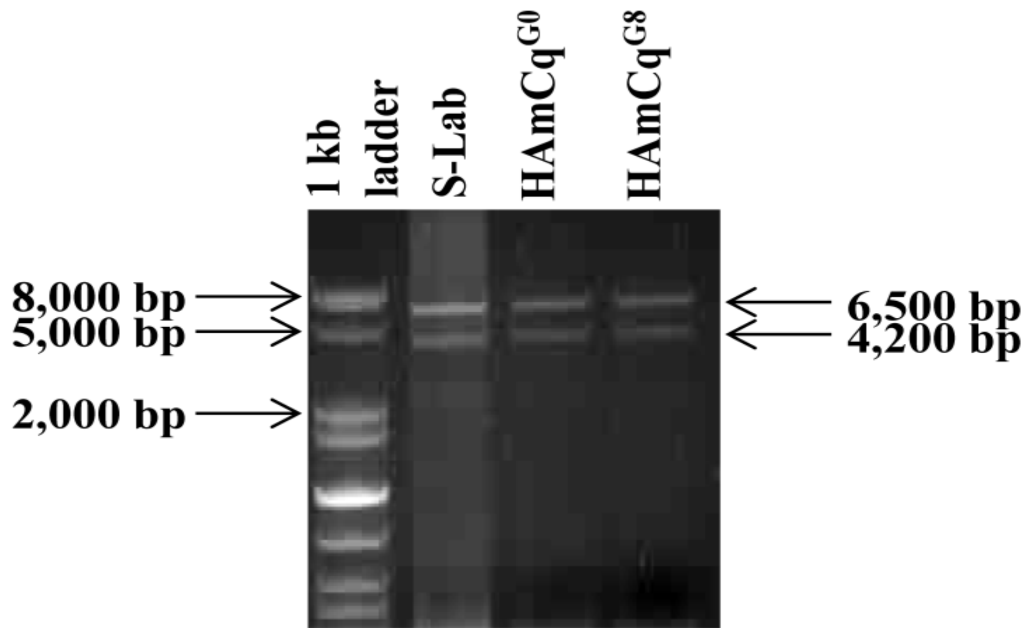

Figure 7.1. Polymerase chain reaction (PCR) amplification of *pa-ra*-type sodium channel transcripts from genomic RNAs of *Culex* mosquitoes. Sodium channel cDNA transcripts amplified from RNAs isolated from S-Lab, HAmCqG0 and HAmCqG8 mosquito strains were subjected to PCR amplification using a primer pair: KDR S16 (TGTTGGCCATATAGACAATGACCGA)/KDR AS09 (GCTTCTGAATCTGAATCAGAGGGAG), synthesized based on the respective 5' and 3' end sequences of the putative sodium channel genes.

The CxNa-L PCR products included three cDNA sequences of the sodium channel, CxNa-L_v1, CxNa-L_v2, and CxNa-L_v3, in the S-Lab strain, with molecular sequence sizes of 6246, 6273, and 6234 bps, respectively (Fig. 7.2); three sodium channel cDNA sequences, CxNa-L_v4, and CxNa-L_v5 and CxNa-L_v6, in the HAmCq^{G0} strain, with 6276, 6285, and 6063 bps, respectively (Fig. 7.3); and two cDNA sequences, CxNa-L_v7 and CxNa-L_v8, in the

HAmCq^{G8} strain, with 6267 and 6273 bps, respectively (Fig. 7.4). In contrast, the CxNa-S PCR products contained only one cDNA sequence of the sodium channel in S-Lab, CxNa-S_v1, with a molecular size of 3891 bps (Fig. 7.2); two cDNA sequences in HAmCq^{G0}, CxNa-S_v2 and CxNa-S_v3, with molecular sizes of 3615 and 3417 bps, respectively (Fig. 7.3); and two sodium channel cDNA sequences in HAmCq^{G8}, CxNa-S_v4 and CxNa-S_v5, with 4068 and 3987 bps, respectively (Fig. 7.4). This discovery provides strong evidence supporting the existence of multiple transcripts of the sodium channel gene in the mosquito *Cx. quinquefasciatus,* as we previously suggested (Xu et al. 2011).

```
                              exon1
CxNa-Lv1   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETGFGRKKKKKE  60
CxNa-Lv2   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETVPGRKKKKKE  60
CxNa-Lv3   MTDDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETGFGRKKKKKE  60
CxNa-Sv1   MTDDLDRYLRE-RSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGE-----------  48

                              exon3
CxNa-Lv1   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYANIKTFVVVSKG 120
CxNa-Lv2   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYANIKTFVVVSKG 120
CxNa-Lv3   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYANIKTFVVVSKG 120
CxNa-Sv1   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYANIKTFVVVSKG 108

                            exon4        IS1
CxNa-Lv1   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 180
CxNa-Lv2   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 180
CxNa-Lv3   KDIFRFSATNASYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 180
CxNa-Sv1   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIIPSTPTVEST 168

                 IS2                  exon5         IS3
CxNa-Lv1   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT 240
CxNa-Lv2   E------------------------------------------YVTMGIDLGNLAALRT 197
CxNa-Lv3   EVIFTGIYTFDQL*SDGARFHITTVYLS*-RCMELVGLRSNSISICNYGYRFG*SRCIEN 239
CxNa-Sv1   E----------------------------------------------------------- 169

           exon6  IS4                                IS5
CxNa-Lv1   FRVLRAPKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC 300
CxNa-Lv2   FRVLRALKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSAFALMGLQIYMGVLTQKC 257
CxNa-Lv3   IQGTTSSQNSGHRSRSQDHRRRCHRVRKESQRCDNFNNVFVVGVCFNGAADLHGRADAKV 299
CxNa-Sv1   ------------------------------------------------------------

                 exon7                              exon8
CxNa-Lv1   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD 360
CxNa-Lv2   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPVCGNSSGAGQCEEGYVCLQGFGD 317
CxNa-Lv3   HQGVPDGRLVGQDPRELGAAPFERFQLVLFRNRGHAPLR-QFVGCWPM*GRICMFTRFWR 359
                       Δ1
CxNa-Sv1   ------------------------------------------------------------

                     exon9          IP                       IS6
CxNa-Lv1   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 420
CxNa-Lv2   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 377
CxNa-Lv3   *SKLRVYKF*YFRMGILICLSSHDPGLLGDLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 419
CxNa-Sv1   ------------------------------------------------------------

                     exon10
CxNa-Lv1   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 480
CxNa-Lv2   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 437
CxNa-Lv3   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 479
CxNa-Sv1   ------------------------------------------------------------ 178

             exon11
CxNa-Lv1   PSDFSCHSCELFVGQEKGNDDNNKEKMSIRSEGLESASLSLPGSPFNLRRGSRGSHQFTI 540
CxNa-Lv2   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESASLSLPGSPFNLRRGSRGSHQFTI 522
                                                 Δ2
CxNa-Lv3   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESASLSLPGSPLNLRRGSRGSHQFTI 539
CxNa-Sv1   ------------------------------------------------------------

                     exon12
CxNa-Lv1   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGSRHSSYTSHQSRI 600
CxNa-Lv2   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGSRHSSYTSHQSRI 594
                                                           Δ3
CxNa-Lv3   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGSRHSSYTSHQSRI 599
CxNa-Sv1   ------------------------------------------------------------

                              exon13
CxNa-Lv1   SYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPANMAASAASVTGAGSGAPNMPYVDTNP 660
CxNa-Lv2   SYTSHGDLLGGMTKESRLRSRTQRNTDHSIVPPANMAASAASVTGAGSGAPNMSYVDTNH 654
CxNa-Lv3   SYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPARTWRPRRRR*RVPARAR-PTCPTSTP 658
CxNa-Sv1   ------------------------------------------------------------

                              exon14                    exon15
CxNa-Lv1   KGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQTVVDMKDVMVLNDIIEQAAGRHSRAS 720
CxNa-Lv2   KGQQRDFDQSQDYTDHAGTIKHNDNPFIEPSQTQTVVDMKDVMVLNDIIEQAAGRHSRAS 714
CxNa-Lv3   TTRASSATLISPKTTQIMLVK*NTTTILSSSPLKPKP**KT*WC*NDIIEQAAGRHSRAS 720
                                                 Δ4
CxNa-Sv1   ------------------------------------------------------------

                                           exon16            IIS1
CxNa-Lv1   DHGEDDDEDGPTFKHKAAEFGMRMIDIFCVWDCCWVWLKFQEWVSFIVFDPFVELFITPC 780
CxNa-Lv2   DHGEDDDEDGPTFKDKAVEFGMRMIDIFCVWDCCWVWLKFQEWVSFIVFDPFVELFITLC 782
              Δ5
CxNa-Lv3   DHGEDDDEDGPTFKDKAVEFGMRMIDIFCVWDCCWVWLKFQEWVSFIVFDPFVELFITLC 780
```

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CxNa-Sv1  ------------------------------------------------------------
                                             IIS2         exon17
CxNa-Lv1  IVVNTLFMALDHHDMNPDMERALKSGNYFFTATFAIEATMKLIAMSPKWYFQEGWNIFDF  840
CxNa-Lv2  IVVNTLFMALDHHDMNPDMERALKSGNYFFTATFAIEATMKLIAMKPQSDVLCQETVREH  842
CxNa-Lv3  IVVNTLFMALDHHDMNPDMERALKSGNYFFTATFAIEATMKLIAMSPKWYFQEGWNIFDF  840
CxNa-Sv1  ------------------------------------------------------------
            IIS3                   IIS4                 exon18   IIS5
CxNa-Lv1  IIVALSLLELGLEGVQGLSVLRSFRLLRVFKLAKSWPTLNLLISIMGRTMGALGNLTFVL  900
CxNa-Lv2  FRFHHRSLELRVWRAFRRSRLRSFRLLRVFKLAKSWPTLNLLISIMGRTMGALGNLTFVL  907
                Δ6          Δ7
CxNa-Lv3  IIVALSLLELGLEGVQGLSVLRSFRLLRVFKLAKSWPTLNLLISIMGRTVGALGNLTFVL  900
CxNa-Sv1  ------------------------------------------------------------
                                          Exon1                IIP
CxNa-Lv1  CIIIFIFAVMGMQLFGKNYIDNVDRFPDKDLPRWNFTDFMHSFMIVFRVLCGEWIESMWD  960
CxNa-Lv2  CIIIFIFAVMGMQLFGKNYIDNVDRFPDKDLPRWNFTDFMHSFMIVFRVLCGEWIESMWD  967
CxNa-Lv3  CIIIFIFAVMGMQLFGKNYTDNVDRFPDKDLPRWNFTDFMHSFMIVFRVLCGEWIESMWD  960
CxNa-Sv1  --------------------QRGPLPGQGPATVELHRLHALIHDRVPGAVRRVDRIHVGL  209
                      IIS6
CxNa-Lv1  CMLVGDVSCIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSAPTADNETNKIAEAFNWI  1020
CxNa-Lv2  CMLVGDVSCIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSAPTADNETNKIAEAFNRI  1027
CxNa-Lv3  CMLVGDVSCIPFFLATVVIGNLVVLNLFLALLCPTVGSSSLSAPTADNETNKIAEAFNRI  1020
CxNa-Sv1  HAGGRRVLHSLSSGHRSDRKFSRS*PFPSLAFVQLWFLEFVGAHSRQRNAQDRRGVQPDI  269
                   exon20               exon21i
CxNa-Lv1  SRFSNWIKANIAAALKFVKNKLTSQIASVQPAGKGVCPCISAEHGENELELTPDDILADG  1080
CxNa-Lv2  SRFSNWIKANIAAALKFVKNKLTSQIASVQPAGKGVCPCISAEHGENELELTPDDILADG  1087
CxNa-Lv3  SRFSNWIKANIAAALKFVKNKLTRQIASVQPAGEQDNHLSWIWKRRAWK*AGINSR*HPG  1081
                                                        Δ8
CxNa-Sv1  ALLQLDQGEHRGRAQVREKQVNKPDCVRAARR--------AAEHGENELELTPDDILADG  321
              exon21ii                             exon22
CxNa-Lv1  LLKKGVKEHNQLEVAIGDGMEFTIHGDLKNKGKKNKQLMNNSKVIGNSISNHQDNKLEHE  1140
CxNa-Lv2  LLKKGVKEHNQLEVAIGDGMEFTIHGDLKNKGKKNKQLMNNSKVIGNSISNHQDNKLEHE  1147
CxNa-Lv3  RRAAEKGRQGAQPAGGGDRRRDGVYDTRRPQEQGQEEQAADEQFQGDRQFYARTES*GHV  1150
                                                             Δ9
CxNa-Sv1  LLKKGVKEHNQLEVAIGDGMEFTIHGDLKHKGKKNKQLMNNSK-----------------  364
                                    exon23
CxNa-Lv1  LNHRGMSLQDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKEDLGIDEELDD  1200
CxNa-Lv2  LNHRGMSLQDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKEDLGIDEELDD  1207
CxNa-Lv3  LTGRKVPMAGR*HCQYKVLWQSQEPPLQGRKPQGQCRNAGGRRKARRQQGGPRN*RRTRR  1215
              Δ10
CxNa-Sv1  ---------DDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKEDLGIDEDLDD  415
                                        exon24
CxNa-Lv1  ECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQGWGNLRLK  1260
CxNa-Lv2  ECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQGRGNLRLK  1267
CxNa-Lv3  RVRG*-GGSSGRGNDHPRGRGRGDRGRAGRLLPGLLQAVPGTGRRRRRAVLAGLGQPAAQ  1275
                                              Δ11
CxNa-Sv1  ECEGEEGPLDGEMIIHAEEDEVIEDAPADSFPDNCYKRFPALAGDDDAPFWQGWGNLRLK  475
                   IIIS1                   exon25        IIIS2
CxNa-Lv1  TFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIFFLEMLIK  1320
CxNa-Lv2  TFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIFFLEMLIK  1327
CxNa-Lv3  DVPADREQVLETAVITMILLSSLALALEDVHLPHRPILQDVPYYMDRIFTVIFFLEMLIK  1336
                    Δ12
CxNa-Sv1  TFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIFFLEMLIK  535
                         IIIS3              exon26        IIIS4
CxNa-Lv1  WLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRPLRAMSRM  1380
CxNa-Lv2  WLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRPLRAMSRM  1387
CxNa-Lv3  WLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRPLRAMSRM  1396
CxNa-Sv1  WLALGFRVYFTDAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRPLRAMSRM  595
                exon27                  IIIS5
CxNa-Lv1  QGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAMMGVQLFAGKYFKCVDTNKTTLSHEIIP  1440
CxNa-Lv2  QGMRVVVNALVQAIPSTFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTTLSHEILP  1447
CxNa-Lv3  QGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTTLSHEIIP  1456
CxNa-Sv1  QGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTTLSHEIIP  655
                     exon28                 IIIP
CxNa-Lv1  DVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFRGWIQIMNDAIDSRDIGKQPIRETN  1500
CxNa-Lv2  DVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDIGKQPIRETN  1507
CxNa-Lv3  DVNACIAENYTWENSPMNFDHVGKAYLCVFQVATFKGWIQIMNDAIDSRDIGKQPIRETN  1516
CxNa-Sv1  DVNACIAENYTWENSPMNFDHVGKAYLCLFQVAPFKGWIQIMNDAIDSRDIGKQPIRETN  715
                 IIIS6                                Exon29
CxNa-Lv1  IYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYYNAMKEMG  1560
CxNa-Lv2  IYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYYNAMKKMG  1567
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CxNa-Lv3   IYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYYNAMKKMG 1576
CxNa-Sv1   IYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYYNAMKKMG 775
                                    IVS1                 exon30
CxNa-Lv1   SKKPLKAIPRPKWRPQAIVFEICTNKKFDMIIMLFMGFNMLTMTLDHYKQTETFSAVLDY 1620
CxNa-Lv2   SKKPLKAIPRPKWRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTETFSAVLDY 1627
CxNa-Lv3   SKKPLKAIPRPKWRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTETFSAVLDY 1636
CxNa-Sv1   SKKPLKAIPRPKWRPQAIVFEICTNKKFDMISMLFIGFNMLTKTLDHYKQTETFSAVLDY 835
                IVS2                          IVS3
CxNa-Lv1   LNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKYFVSPTLL 1680
CxNa-Lv2   LNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKYFVSPTLL 1687
CxNa-Lv3   LNMIFICIFSSECLMKIFALRYHYFIEPGNLFDFVVVILSILGLVLSDLIESTSSRRCSV 1697
                                                                  Δ13
CxNa-Sv1   LNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKYFVSPTLL 895
                IVS4                    exon31                IVS5
CxNa-Lv1   RVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFGMSFFMHV 1740
CxNa-Lv2   RVVRVAKVGRVLRLVQGPRASGTLLFALAMSLPALFNICLLLFLVMFIFAIFGMSFFMHV 1748
                             Δ14
CxNa-Lv3   WCAWPRSVGCCVSSRAPASGRCCLRWPCRCRRCSTSVCCCSW*CSSSPSSECRSSCT*-- 1756
                             Δ14
CxNa-Sv1   RVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFGMSFFMHV 955
                                        IVP                 exon32
CxNa-Lv1   KDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDDDKGYPGNCGSA 1800
CxNa-Lv2   KDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGYPGNCGSA 1808
CxNa-Lv3   RTRAGWTTCTTSRRSARA*SCCFRCQRLRGGTVRWMVSSTRRTACRRITTRVTPGTAGSA 1819
                                                                  Δ15
CxNa-Sv1   KDKSGLGDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGYPGNCGSA 1015
                      IVS6                             exon33
CxNa-Lv1   TIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIWQQFDPDG 1860
CxNa-Lv2   TIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIWQQFDPDG 1868
CxNa-Lv3   SRTCWHIWSSVS*SLSTCTSLSFSRITRRPRRTCRRV*--RTTRSTTCTTRLWQQFDPDG 1877
CxNa-Sv1   TIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYCEIWQQFDPDG 1075

CxNa-Lv1   TQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILDALTKDFFARK 1920
CxNa-Lv2   TQYIRYDQLSDLLERAGTACRFTNPNKYKIISMDIPICRGDMMFCVDILDALAKDFFARK 1929
                                 Δ17
CxNa-Lv3   TQYEYVRPAVGLFRRAGTAADSQTERVQDHLDGHSDLRR---------RHDVLRGHSGRA 1930
                Δ16              Δ18
CxNa-Sv1   TQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILDALTKDFFARK 1135

CxNa-Lv1   GNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGGVGGGGGG 1980
CxNa-Lv2   GNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGGVGGGGGG 1989
CxNa-Lv3   DEGLLRAEGPPDRGQCRDG*--GPAAAGRGRLRAVDVVAPTGFGVLRAVDTARVPEL*GT 1989
                                                      Δ19
CxNa-Sv1   GNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGGVGGGGGG 1195

CxNa-Lv1   GGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPSPKESPDG 2040
CxNa-Lv2   GGGGGGGGGEGAGDDTDADACDNEPGIGGPGAVSGGGGSIAGGGFQANLGAPPPKESPDG 2049
CxNa-Lv3   RRCWWRRRRWRWWRRRWWRRCRR*HRRRCL**RARDRESRRG---QRRWPQHRGRRLP-- 2044
CxNa-Sv1   GGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPSPKESPDG 1255

CxNa-Lv1   NNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 2082
CxNa-Lv2   NNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 2091
CxNa-Lv3   -GSPRAAVTQRIARWQSSRSSNGR----PSRK*WICN*      2078
                           Δ20
CxNa-Sv1   NNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 1297
```

Figure 7.2. Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel transcripts (Cx-Na) in S-Lab *Culex* mosquitoes. Transmembrane segments are indicated on the line over the sequence. Exons are indicated above the sequence with solid triangle symbols to indicate the boundaries between exons. The differences in the aa sequences are indicated by shading. A stop codon is marked by an asterisk (*). – indicates deletions. Δ

indicates insertions with the sequences of Δ1: P; Δ2: VSEITRTTAPTATAAGTAKARKVSA; Δ3: GAIIVPVYYANL; Δ4:*I; Δ5: VSVYYFPT; Δ6: GPFR; Δ7: E; Δ8:*; Δ9: **SSR**VR; Δ10: *HCQY; Δ11:*; Δ12: G; Δ13: R; Δ14: R; Δ15: RRR; Δ16: T; Δ17: R; Δ18: A; Δ19: G; Δ20:**

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                                 exon1
CxNa-Lv4   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETGFGRKKKKRE  60
CxNa-Lv5   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGE-----------  49
CxNa-Lv6   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGE-----------  49
CxNa-Sv2   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGE-----------  49
CxNa-Sv3   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGE-----------  49

                                 exon3
CxNa-Lv4   IRYDDEDEDEGPRADSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG 120
CxNa-Lv5   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG 109
CxNa-Lv6   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG 109
CxNa-Sv2   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG 109
CxNa-Sv3   ------------------------------------------------------------

                     exon4                  IS1
CxNa-Lv4   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 180
CxNa-Lv5   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTIRGNCILMIMPPTPTVEST 169
CxNa-Lv6   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 169
CxNa-Sv2   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST 169
CxNa-Sv3   ------------------------------------------------------------

              IS2            exon5                    IS3
CxNa-Lv4   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT 240
CxNa-Lv5   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT 229
CxNa-Lv6   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT 229
CxNa-Sv2   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT 229
CxNa-Sv3   ------------------------------------------------------------

             IS4 exon6                            IS5
CxNa-Lv4   FRVLRALKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC 300
CxNa-Lv5   FRVLRALKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC 289
CxNa-Lv6   FRVLRALKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC 289
CxNa-Sv2   FRVLRALKTVAIVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC 289
CxNa-Sv3   ------------------------------------------------------------

             exon7                         exon8
CxNa-Lv4   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD 360
CxNa-Lv5   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD 349
CxNa-Lv6   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD 349
CxNa-Sv2   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD 349
CxNa-Sv3   ------------------------------------------------------------

             exon9                      IP                   IS6
CxNa-Lv4   NPDYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 420
CxNa-Lv5   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 409
CxNa-Lv6   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 409
CxNa-Sv2   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFLGSFYLVN 409
CxNa-Sv3   ------------------------------------------------------------

                  exon10                         exon11
CxNa-Lv4   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 480
CxNa-Lv5   LILAIVAMSYDELQKRAEEEEAAEEGALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 469
CxNa-Lv6   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 469
CxNa-Sv2   LILAIVAMSYDELQKKAEEEEAAEEEALREAEEAAAAKQAKLEAHAAAAAAAANPEIAKS 469
CxNa-Sv3   ------------------------------------------------------------

CxNa-Lv4   PSDFSCHSYELFVGQEKGNDDNNSEKMSIRSEGLESASLSLPGSPSNLRRGSRGSHQFTI 540
CxNa-Lv5   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESASLSLPGSPFNLRRGSRGSHQFTI 554
                                                Δ1
CxNa-Lv6   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESASLSLPGSPFNLRRGSRGSHQFTI 554
                                                Δ1
CxNa-Sv2   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESVSEITRTTAPTATAAGTAKARKVS 529
CxNa-Sv3   ------------------------------------------------------------

                     exon12
CxNa-Lv4   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGAIIVPVYYANLGS 600
CxNa-Lv5   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGAIIVPVYYANLGS 614
CxNa-Lv6   RNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGAIIVPVYYANLGS 614
CxNa-Sv2   A----------------------------------------------------------- 530
CxNa-Sv3   ------------------------------------------------------------
                       exon13
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CxNa-Lv4   RHSSYTSHQSRISYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPANMAASAASVTGAGS  660
CxNa-Lv5   RHSSYTSHQSRISYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPANMAASAASVTGAGS  674
CxNa-Lv6   RHSSYTSHQSRISYASHGDLLGGMTKESRLRSRTQRNTNHSIVPPANVAASAASVTGAGS  674
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   ------------------------------------------------------------

                                              exon14
CxNa-Lv4   GAPNMSYVDTNHKGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQTVVDMKDVMVLNDI  720
CxNa-Lv5   GAPNMSYVDTNHKGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQTVVDMKDVMVLNDI  734
CxNa-Lv6   GAPNMSYVDTNHKGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQTVVDMKDVMVLNDI  734
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   ------------------------------------------------------------

                   exon15                                  exon16
CxNa-Lv4   IEQAAGRHSRASDHGVSVYYFPTEDDDEDGPTFKDKAVEFGMRMIDIFCVWDCCWVWLKF  780
CxNa-Lv5   IEQAAGRHSRASDHGVSVYYFPTEDDDEDGPTFKDKAVEFGMRMIGIFCVWDCCWVWLKF  794
CxNa-Lv6   TEPAAGRHSRASDHGVSVYYFPTEDDDEDGPTFKDKAVEFGMRMIDIFCVWDCCWVWLKF  794
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   ------------------------RTTTRTVRRSRTRRSSSGCG*STSSACGTAAGCGSKS  86

                       IIS1                                   IIS2
CxNa-Lv4   QEWGSFIVFDPFVELFITLCIVVNTLFMALDHHDMNPDMERALKSGNYFFTATFAIEATM  840
CxNa-Lv5   QEWVSFIVFDPFVELFITLCIVVNTLFMALDHHDMNPDMERALKSGNYFFTATFAIEATM  854
CxNa-Lv6   QEWVSFIVFDPFVELFITLCIVVNTLLMALDHHDMNPDMERALKSGNYFFTATFAIEATM  854
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   SSRSGCPLSCSTRSSSCSSRSASWSTRCSWRSTTTT*TRTWSGRSRAVTTSSRRRSRGQR  146

                  exon17        IIS3                            IIS4
CxNa-Lv4   KLIAMSPKWYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLLRVFKLAKSWPTLN  900
CxNa-Lv5   KLIAMSPKWYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLLRVFKLAKSWPTLN  914
CxNa-Lv6   KLIAMSPKWYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLLRVFKLAKSWPTLN  914
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   R*S*SR*APSGTSRKVGTFSISSSWPFRCSSSVXRAFRDCQYYVHSVCFECSS*QSRSPR  206

                exon18                IIS5
CxNa-Lv4   LLISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYTDNVDRFPDKDLPRWNFTDFM  960
CxNa-Lv5   LPISIMGRTVGALGNLTSVLCIIIFIFAVMGMQLFGKNYTDNVDRFPDKDLPRWNFTDFM  974
CxNa-Lv6   LLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGGNYIDNVDRFPDKDLPRWNFADFM  974
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   *TYSFPSWAERWAR*VI*RLCSALSSSSLP*WGCSCSARTTSTTWTASRTRTCHGGTLVT  266

                     IIP          Exon1              IIS6
CxNa-Lv4   HSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNFVVLNLFLALLLSNFGSSS  1020
CxNa-Lv5   HSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNFVVLNLFLALPLSIFGSSS  1034
CxNa-Lv6   HSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNVVVLNLFLALLLSNFGSSS  1034
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   SCTHS*SCSGCCAASGSNPCGTACWWATCPAFRSSWPP***EI*SFLTFS*PCFCPTPRV  326
                     exon20
CxNa-Lv4   LSAPTADNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIASVQPAEHGENERE  1080
CxNa-Lv5   LSAPTADNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIASVQPAD-------  1087
CxNa-Lv6   LSAPTADNETNKIAEAFNRDIALLQLDQG-EHRGRAQVREKQVNKPDCVRAARR*-----  1088
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   CRRPQPTTKRTRSPRRSTGYRASPTGSRRTSRPRSSS*KTS*QARLRPCSP---------  377
                exon21ii                                  exon22
CxNa-Lv4   LTPDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGDLKNKGKKNKQLMNNSKVIGNSIS  1140
CxNa-Lv5   ----DILADGLLKKGVKEHNQLEVAIGDGMEFTIHGDLKNKGKKNKQLMNNSKVIGNSIS  1143
CxNa-Lv6   AAQPSQLDMERRQRGMSMYICRAW*K*AGINSR*HPGRREKGRQGAQPAGGGDRRRDGVY  1150
                                                     Δ2
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   --PDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGDPKNKGKKNKQLMNNSK-------  428
                                             exon23
CxNa-Lv4   NHQDNKLEHELNHRGMSLQDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKE  1200
CxNa-Lv5   NHQDNKLEHELNHRGMSLQDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKE  1203
CxNa-Lv6   DTRPQEQGQEEQAADEQFQGR*YHAYKVLWQSQESPLQGRKPQGQCRNAGGRRKARRQQG  1212
              Δ3
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   ------------------DDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKE  469
                                          exon24
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CxNa-Lv4   DLGIDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPF 1260
CxNa-Lv5   DLGIDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPF 1263
CxNa-Lv6   GPRN*RRTRRRVRG*GGSAGRGNDHPRGRGRSDRGRTGRLLPGQLLQAVPGAGRRRRRAV 1273
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   DLGIDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPF 529
                          IIIS1                        exon25  IIIS2
CxNa-Lv4   WQGWGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFT 1320
CxNa-Lv5   WQGWGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFT 1323
                                                [Δ4]
CxNa-Lv6   LAGLGQPAAQDVPADREQVLRDGRHHDDPAEGPGPRGCAPATPTNPAGRPVLHGQD-IHG 1333
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   WQGWGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFT 589
                                IIIS3                exon26    IIIS4
CxNa-Lv4   VIFFLEMLIKWLALGFRVYFTDAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRA 1380
CxNa-Lv5   VIFFLEMLIKWLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRA 1376
CxNa-Lv6   DLFFRDVDQVVGAR-LPGVLYERLVLARFHHCDGVLNQLRGFTLWSGWYSSIQNYANS*G 1392
CxNa-Sv2   ------------------------------------------------------------
CxNa-Sv3   VIFFLEMLIKWLALGFRVYFTNAWCWLDFIIVMLSLINFAIWVGAAD-IPAFRSMRTLRA 649
                                                 [Δ5]
                                  exon27          IIIS5
CxNa-Lv4   LRPLRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTN 1440
CxNa-Lv5   LRPLRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTN 1436
CxNa-Lv6   TASATCHVPYAGYEGCRQCIGTGYTVHLQRVIGVFDLLVDFRHHGRPAVCRKVLQVRRHE 1451
CxNa-Sv2   --------------VVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTN 576
CxNa-Sv3   LRPLRACLSLGGHESCRQCIGTGYTVHLQRVIGVFDLLVDFRHHGRPAVCRKVLQVRRHE 709
                      exon28                                 IIP
CxNa-Lv4   KATLSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRD 1500
CxNa-Lv5   KTTLSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRD 1496
CxNa-Lv6   QDDTVARDHPGRERVHRGELHLGELPDEL*PRGEGLPVFVPG----GHVQGMDPDRERRD 1511
CxNa-Sv2   KTTLSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRD 636
CxNa-Sv3   QDDTVARDHPGRERVHRGELHLGELPDEL*PRGEGLPVFVPGGHVQGMDPDHERRDRLAG 769
                        IIIS6                                 Exon29
CxNa-Lv4   IGKQPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKRAGGSLEMFMTEDQK 1560
CxNa-Lv5   IGKQPIRETNIYMYLYFVFFIIFGSFFTLSLFIGVIIDNFNEQKKKAGGSLEMFMTEGQK 1556
CxNa-Lv6   RLARHWKAAHPRNQHLHVLVLCVLHHLRIVLHAEPLHRCHY*QL*---RTEEEGWGIARD 1567
CxNa-Sv2   IGKQPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQK 696
CxNa-Sv3   HRKAAHPRNQHLHVLVLCVLHHLRIVLHAEPLHRCHY*QL*RTEEEGWGIARDVYDGGLK 829
                                           exon30      IVS1
CxNa-Lv4   KYYNAMKKMGSKKPLKAIPRPKWRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQ 1620
CxNa-Lv5   KYYNAMKKMGSKKPLKAIPRPKRRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQ 1616
CxNa-Lv6   VYDGGPKKVLQRNEEDGLEEATEGHSAAQVATTSNSVRNLHKQKVRHDHHVVHRLQHVDD 1624
CxNa-Sv2   KYYNAMKKMGSKKPLKAIPRPKWRPQAIVFEICTNKSST*SSCCSSASTC*R*RWITTSR 756
CxNa-Sv3   KVLQRNEEDGLEEATEGHSAAQVATTSNSVRNLHKQKVRHDHHVVHRLQHVDDDAGSLQA 889
                          IVS2                                IVS3
CxNa-Lv4   TETFSAVLDYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLI 1680
CxNa-Lv5   TGTFSAVLDYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLRDLI 1676
CxNa-Lv6   G---AGSLQAVGHVQRGAGLPEHDLHLYLQ*---RVSDEDLRAALPLLYRTVEPVRFRRR 1684
CxNa-Sv2   RKRSARCWTT*T*SSSVSSVASV**RSSRCATTTLSNRGTCSISSSSSCPFWAWC*AT*S 816
CxNa-Sv3   DGNVQRGAGLPEHDLHLYLQ*RVSDEDLRAALPLLYRTVEPVRFRRRHPVHFGPGAERPD 949

              exon31         IVS4                                   IVS5
CxNa-Lv4   EKYFVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFA 1740
CxNa-Lv5   EKYFVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFA 1735
CxNa-Lv6   HPVHFGPGAERPDR-----EVLRLADAAPCARGQGRSGAASRQGRQGHPDVAVCAGHVAA 1738
                                       [Δ6]
CxNa-Sv2   KSTSSRRRCSVWCAWPRSVGCCVSSRAPRASGRCCLRWPCRCRRCSTSVCCCSW*CPSSP 876
CxNa-Sv3   RKVLRLADAAPCGARGQGRSGAASRQGRQGHPDVAVCAGHVAAGAVQHLSAAVPGDVHLR 1009
                                                 IVP          exon32
CxNa-Lv4   IFGMSFFMHVKDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDND 1800
CxNa-Lv5   IFGMSFFMHMKDKSGLDGVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDND 1795
CxNa-Lv6   GAVQHLSAAVPGDVHLRHLWDVVLHAR------EGQERAGRRVQLQDVRPEHDPAVSDVN 1794
CxNa-Sv2   SSGCRSSCT*RTRAGWTTCTTSRRSARA*SCCFRCQRLRGGTVCWMVSSTRRTACRRITT 936
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CxNa-Sv3    HLRNVVLHAREGQERAGRRVQLQDVRPEHDPAVSDVNVCGVGRCAGWYHQRGGLPAAGYR 1069
                           IVS6                                exon33
CxNa-Lv4    KGYPGNCGSATIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLMDDDYDMYY 1860
CxNa-Lv5    KGYPGNCGSATIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYY 1855
CxNa-Lv6    VCWVGRCAGWYHQRGGLLAAG*-------------------RQGLPRELRVGDDRHHVPA 1848
CxNa-Sv2    RVTRGTAGRRRSASRTCWRIWSSVS*SLSTCTSLSFSRITRRPRRTCRRV*RTTTTTCTT 996
CxNa-Sv3    QGLPRELRVGDDRHHAPAGISGHQFPDRYQHVHRCHSRELLAGHGGRAGGSDGRRLRHVL 1129

CxNa-Lv4    EIWQQFDPDGTQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILD 1920
CxNa-Lv5    EIWQQFDPDGTQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILD 1915
CxNa-Lv6    GISGHQFPDRYQHVHRCHPRELLAGHGGRAGGSDGRRLRHVLRDLAAVRSGRYAVHPVRP 1889
CxNa-Sv2    RSGSSSIRTVRSTSGTTSCRTFWTCRNRRCRFTNRTSTRSSRWTFRSVAAT*CSAWTFWT 1056
CxNa-Sv3    RDLAAVRSGRYAVHPVRPAVGLFGRAGTAAADSQTEQVQDHLDGHSDLSRRHDVLRGHSG 1189

CxNa-Lv4    ALTKDFFARKGNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKE 1980
CxNa-Lv5    ALTKDFFARKGNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKE 1975
CxNa-Lv6    AVG--LFGRAGTAAADS-QTEQVQDHLD-------------------------GHSDLSR 1921
CxNa-Sv2    R*RRTSSRGRSTRSRTVPRWVRSSSGRTRSVTSRFRRRCGANGRSTARG*YSTRTGTLRN 1116
CxNa-Sv3    RADEGLLRAEGQPDRGQCRDG*GPAAAGRGRLRAGFVDVVAPTGGVLRAVDTARVPEL*G 1249

CxNa-Lv4    RGGVGGGGGGGGGGGGGGGEAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLG 2040
CxNa-Lv5    RGGVGGGGGGGGGGGGGGGEAGDDTDADACDNEPGIGSPGAVSGGDSSIAGGGSQANLG 2035
CxNa-Lv6    RHDVLRGHSGRADEGLLRAEGQ---PDRGQCRDG*-----GPAAAGRGRLRAGFVDVVAP 1973
CxNa-Sv2    EAVLVAAAAVEVVEEEVVAKVPEMTPTPMPVITSPGSGVPARSAAVAAASPAEAPRLT*A 1176
CxNa-Sv3    TRRCWWRRRRWRWWRRRWWRRCRR*HRRRCL**RARDRESRRGQRRWRQHRRRRLPG*PR 1309

CxNa-Lv4    PPSPKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV*        2092
CxNa-Lv5    PPSPKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV*        2095
CxNa-Lv6    TGGVLRAVDTTRVPEL*----GTRRCWWRRRWRRRWRWWRRCRR*HRRRCL**        2021
CxNa-Sv2    RLTQRIVRWQ**SSRSSNGRPSRK*WICN*                               1205
CxNa-Sv3    AAVTQRIARWQ**SSRSSNGRPSRK*WICN*                              1339
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Figure 7.3. Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel reanscripts (Cx-Na) in $HAmCq^{G0}$ *Culex* mosquitoes. Transmembrane segments are indicated on the line over the sequence. Exons are indicated above the sequence with solid triangle symbols to indicate the bounderies between exons. The differences in the aa sequences are indicated by shading. A stop codon is marked by an asterisk (*). – indicates deletions. Δ indicates insertions with the sequences of Δ1: VSEITRTTAPTATAAGTAKARKVSA; Δ2: AA; Δ3: R; Δ4: *F; Δ5: L; Δ6: G.

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                                    exon1                                  exon2
CxNa-Lv7   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETGFGRKKKKKE  60
CxNa-Lv8   MTDDLDSISEEERSLFRPFTRESLLAIEERIANEQAKQRELEKKRAEGETGFGRKKKKKE  60
CxNa-Sv4   MTDDLDSISEEERSLFRPFARESLLVIEERIANEQAKQRELEKKRAEGETGFGRKKKKKE  60
CxNa-Sv5   MTEDLDSISEEERSLFRPFTRESLLVIEERIANEQAKQRELEKKRAEGETGFGRKEKKKE  60
                                     exon3
CxNa-Lv7   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG  120
CxNa-Lv8   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG  120
CxNa-Sv4   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPHEDIDAFYSNIKTFVVVSKG  120
CxNa-Sv5   IRYDDEDEDEGPQPDSTLEQGVPIPVRMQGSFPPELASTPLEDIDAFYSNIKTFVVVSKG  120
                         exon4                      IS1
CxNa-Lv7   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST  180
CxNa-Lv8   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST  180
CxNa-Sv4   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST  180
CxNa-Sv5   KDIFRFSATNALYVLDPFNPIRRVAIYILVHPLFSFFIITTILGNCILMIMPSTPTVEST  180
                IS2             exon5               IS3
CxNa-Lv7   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT  240
CxNa-Lv8   EVIFTGIYTFESAVKVMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRT  240
CxNa-Sv4   E-----------------------------------------------------------  181
CxNa-Sv5   E-----------------------------------------------------------  181
           exon6  IS4                                  IS5
CxNa-Lv7   FRVLRALKTVAIVPGLKTIVGAVTESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC  300
CxNa-Lv8   FRVLRALKTVATVPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKC  300
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                exon7                      exon8
CxNa-Lv7   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD  360
CxNa-Lv8   IKEFPTDGSWGNLTHENWERHHSNDSNWYFSETGDTPLCGNSSGAGQCEEGYVCLQGFGD  360
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                exon9                   IP                        IS6
CxNa-Lv7   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLFFIVIIFSGSFYLVD  420
CxNa-Lv8   NPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGPWHMLVFIVIIFLGSFYLVN  420
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                       exon10                                exon11
CxNa-Lv7   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQARLEAHAAAAAAAANPEIAKS  480
CxNa-Lv8   LILAIVAMSYDELQKRAEEEEAAEEEALREAEEAAAAKQAELEAHAAAAAAAANPEIAKN  480
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------

CxNa-Lv7   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESVSEITRTTAPTATAAGTAKARKVS  540
CxNa-Lv8   PSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESVSEITRTTAPTATAAGTAKARKVS  540
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                              exon12
CxNa-Lv7   AASLSLPGSPFNLRRGSRGSHQFTIRNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADD  600
CxNa-Lv8   AASLSLPGSPFNLRRGSRGSHQFTIRNGRGRFVGVPGSDRKPLVLSTYLDAQEHLPYADD  600
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------

CxNa-Lv7   SNAVTPMSEENGSRHSSYTSHQSRISYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPAN  660
CxNa-Lv8   SNAVTPMSEENGSRHSSYTSHQSRISYTSHGDLLGGMTKESRLRSRTQRNTNHSIVPPAN  672
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                               exon13                     exon14
CxNa-Lv7   MAASAASVTGAGSGAPNMSYVDTNHKGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQT  720
CxNa-Lv8   MAASAASVTGAGSGAPNMSYVDTNHKGQQRDFDQSQDYTDDAGKIKHNDNPFIEPSQTQT  732
CxNa-Sv4   ------------------------------------------------------------
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CxNa-Sv5   ------------------------------------------------------------

                                  exon15                                exon16
CxNa-Lv7   VVDMKDVMVLNDIIEQAAGRHSRASDHGVSVYYFPTEDDDEDGPTLKDKAVEFGMRMIDI  780
CxNa-Lv8   VVDMKDVMVLNDIIEQAAGRHSRASDHGVSVYYFPTEDDDEDGPTFKDKAVEFGMRMIDI  792
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                                       IIS1
CxNa-Lv7   FCVWDCCWVWLKFQEWVSFIVFDPFVELFITLCIVVNTLFMALDHHDMNPDMERALKSGN  840
CxNa-Lv8   FCVWDCCWVWLKFQEWVSFIVFDPFVELFITLCIVVNTLFMALDHHDMNPDMERALKSGN  852
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------

                  IIS2           exon17            IIS3                IIS4
CxNa-Lv7   YFFTATFAIEATMKLIAMSPKWYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLL  900
CxNa-Lv8   YFFTATFAIEATMKLIAMSPKWYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLL  912
CxNa-Sv4   ------------------------------------------------------------
CxNa-Sv5   ------------------------------------------------------------
                                exon18              IIS5
CxNa-Lv7   RVFKLAKSWPTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYIDNVDRFP  960
CxNa-Lv8   RVFKLAKSWPTLNLLISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYTDNVDRFP  972
CxNa-Sv4   -----------------------------------------------------QRGPLPG  188
CxNa-Sv5   -----------------------------------------------------QRGPLPG  188
                    exon19            IIP                      IIS6
CxNa-Lv7   DKDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNFVVLNL  1020
CxNa-Lv8   DKDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNFVVLNL  1032
CxNa-Sv4   QGPATVELHRLHALIHDRVPGAVRRVDRIHVGLHAGGRRVLHSVLLGHRSDRKFCRSYTL  248
CxNa-Sv5   QGPATVVNPRLHALIHDRVPGAVRRV-IESMWDCMLVGDVSCIPFFLATVVIGNFVVLNL  247
                    exon20
CxNa-Lv7   FLALLLSNFGSSSLSAPTADNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIA  1080
CxNa-Lv8   FLALLLSNFGSSSLSASTADNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIA  1092
CxNa-Sv4   FLALLLSNFGSSSLSAPTAGNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIA  308
CxNa-Sv5   FLALLLSNFGSSSLSAPTADNETNKIAEAFNRISRFSNWIKANIAAALKFVKNKLTSQIA  307

                    exon21i                   exon21ii
CxNa-Lv7   SVQPAGKGVCPCISAEHGENELELTPDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGD  1140
CxNa-Lv8   SVQPA----------EHGENELELTPDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGD  1142
CxNa-Sv4   SVQPAGKGVCPCISAEHGENELELTPDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGD  381
                 Δ2
CxNa-Sv5   SVQPAGKGVCPCISAEHGENELELTPDDILADGLLKKGVKEHNQLEVAIGDGMEFTIHGD  380
                 Δ3
                exon22                     exon23
CxNa-Lv7   LKNKGKKNKQLMNNSKDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASREDLG  1200
CxNa-Lv8   LKNKGKKNKQLMNNSKDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKEDLG  1202
CxNa-Sv4   LKNKGKKNKQLMNNSQDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRGASKEDLG  467
                          Δ4
CxNa-Sv5   LKNKGKKNKQLMNNSKDDDTASIKSYGSHKNRPFKDESHKGSAETLEGEEKRDASKEDLG  440

                                   exon24
CxNa-Lv7   IDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQG  1260
CxNa-Lv8   IDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQG  1262
CxNa-Sv4   IDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQG  527
CxNa-Sv5   IDEELDDECEGEEGPLDGEMIIHAEEDEVIEDAPADCFPDNCYKRFPALAGDDDAPFWQG  500
                               IIIS1                          exon25  IIIS2
CxNa-Lv7   WGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIF  1320
CxNa-Lv8   WGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIF  1322
CxNa-Sv4   WGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIF  587
CxNa-Sv5   WGSLRLKTLQLIENKYFETAVITMILLSSLALALEDVHLPHRPILQDVLYYMDRIFTVIF  560
                                    IIIS3                   exon26  IIIS4
CxNa-Lv7   FLEMLIKWLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRP  1380
CxNa-Lv8   FLEMLIKWLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRP  1382
CxNa-Sv4   FLEMLIKWLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRP  647
CxNa-Sv5   FLEMLIKWLALGFRVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRP  620
                            exon27                IIIS5
CxNa-Lv7   LRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTT  1440
CxNa-Lv8   LRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTT  1442
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CxNa-S$_v$4 LRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDTNKTT 707
CxNa-S$_v$5 LRAVSRMQGMRVVVNALVQAIPSIFNVLLVCLISWLIFAIMGVQLFAGKYFKCVDTNKTT 680

exon28 IIP
CxNa-L$_v$7 LSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDIGK 1500
CxNa-L$_v$8 LSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDIGK 1502
CxNa-S$_v$4 LSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDIGK 767
CxNa-S$_v$5 LSHEIIPDVNACIAENYTWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDIGK 740

IIIS6 Exon29
CxNa-L$_v$7 QPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYY 1560
CxNa-L$_v$8 KPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYY 1562
CxNa-S$_v$4 QPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYY 827
CxNa-S$_v$5 QPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYY 800

exon30 IVS1
CxNa-L$_v$7 NAMKKMGSKKPLKAIPRPKRRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTET 1620
CxNa-L$_v$8 NAMKKMGSKKPLKAIPRPKRRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTET 1622
CxNa-S$_v$4 NAMKKMGSKKPLKAIPRPKRRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTET 887
CxNa-S$_v$5 NAMKKMGSKKPLKAIPRPKRRPQAIVFEICTNKKFDMIIMLFIGFNMLTMTLDHYKQTET 860

IVS2 IVS3
CxNa-L$_v$7 FSAVLDYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKY 1680
CxNa-L$_v$8 FSAVLGYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKY 1682
CxNa-S$_v$4 FSAVLDYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVAILSILGLVLSDLIEKY 947
CxNa-S$_v$5 FSAVLDYLNMIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVLSDLIEKY 920

exon31 IVS4 IVS5
CxNa-L$_v$7 FVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFG 1740
CxNa-L$_v$8 FVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFG 1742
CxNa-S$_v$4 FVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFG 1007
CxNa-S$_v$5 FVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFG 980

IVP exon32
CxNa-L$_v$7 MSFFMHVKDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGY 1800
CxNa-L$_v$8 MSFFMHVKDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGY 1802
CxNa-S$_v$4 MSFFMHVKDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGY 1067
CxNa-S$_v$5 MSFFMHVKDKSGLDDVYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGY 1040

IVS6 exon33
CxNa-L$_v$7 PGNCGSATIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIW 1860
CxNa-L$_v$8 PGNCGSATIGITYLLAYLAISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIW 1862
CxNa-S$_v$4 PGNCGSATVGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIW 1127
CxNa-S$_v$5 PGNCGSATIGITYLLAYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIW 1100

CxNa-L$_v$7 QQFDPDGTQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILDALT 1920
CxNa-L$_v$8 QQFDPDGTQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDILDALT 1922
CxNa-S$_v$4 QQFDPDGTQYIRYDQLSDFLDVLEPPLQIHKPNKYKIISMDIPICRGDMMFCVDTLDALT 1187
CxNa-S$_v$5 QQFDPDGTQYIRYDQLSGFLDVLEPPLQIHKPNSNKIISMDIPICRGDMVFCVDILDALT 1160

CxNa-L$_v$7 KDFFARKGNPIEDSAEMGGVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGG 1980
CxNa-L$_v$8 KDFFARKGNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGG 1982
CxNa-S$_v$4 KDFFARKGNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGG 1247
CxNa-S$_v$5 KDFFARKGNPIEDSAEMGEVQQRPDEVGYEPVSSTLWRQREEYCARLIQHAYRNFKERGG 1220

CxNa-L$_v$7 VGGGGGGGGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPS 2040
CxNa-L$_v$8 VGGGGGGGGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPS 2042
CxNa-S$_v$4 VGGGGGGGGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPS 1307
CxNa-S$_v$5 VGGGGGGGGGGGGGGGEGAGDDTDADACDNEPGIGSPGAVSGGGGSIAGGGSQANLGPPS 1280

CxNa-L$_v$7 PKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 2089
CxNa-L$_v$8 PKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 2091
CxNa-S$_v$4 PKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 1356
CxNa-S$_v$5 PKESPDGNNDPQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRSADV* 1329

Figure 7.4. Alignment of deduced amino acid transcript sequences of the *para*-type sodium channel transcripts (Cx-Na) in HAmCq^{G8} *Culex* mosquitoes. Transmembrane segments are indicated on the line over the sequence. Exons are indicated above the sequence with solid triangle symbols to indicate the bounderies between exons. The differences in the aa sequences are indicated by shading. A stop codon is marked by an asterisk (*). – indicates deletions. Δ indicates insertions with the sequences of Δ1: GAIIVPVYYANL Δ2: GEQHSHLSWIWSE; Δ3: GEQHNHLSWIWSE; Δ4: VIGNSISNHQDNKLEHELNHRGMSLQ.

Structural analysis of deduced sodium channel protein sequences within each strain and/or among different strains of *Culex* mosquitoes

The putative amino acid sequences for the CxNa-L and the CxNa-S transcript sequences were compared for each of the three mosquito strains studied. In the S-Lab strain, of the three transcripts identified in the CxNa-L category, two, CxNa-L_v1 and CxNa-L_v2 (JN695777 and JX424546), consisted of full length sodium channel sequences encoding the entire ORFs of the sodium channel proteins eith 2082 and 2091 amino acid residues, respectively. These exons were numbered 1 through 33 (Fig. 7.5), based on the silkworm *Bombyx mori* sodium channel *BmNav* and house fly sodium channel sequences (Shao et al. 2009). However, the *Culex* mosquito sodium channel lacked the exon 12 present in both the *BmNav* and $DmNa_V$ sodium channel sequences. CxNa-L_v1 and CxNa-L_v2 shared very high sequence similarity (96%), except for a missing exon 5 as a result of the alternative splicing (Figs 7.2 and 7.5) and several short insertions identified in the CxNa-L_v2 sequence (Fig. 7.2 and 7.5). The remaining transcript, CxNa-L_v3, incorporated several in-frame premature stop codons, with the first occurring at domain I segment 2 (IS2) (Fig. 7.2 and 7.5). Short deletions and insertions were also identified in CxNa-L_v3 compared with CxNa-L_v1 and CxNa-L_v2.

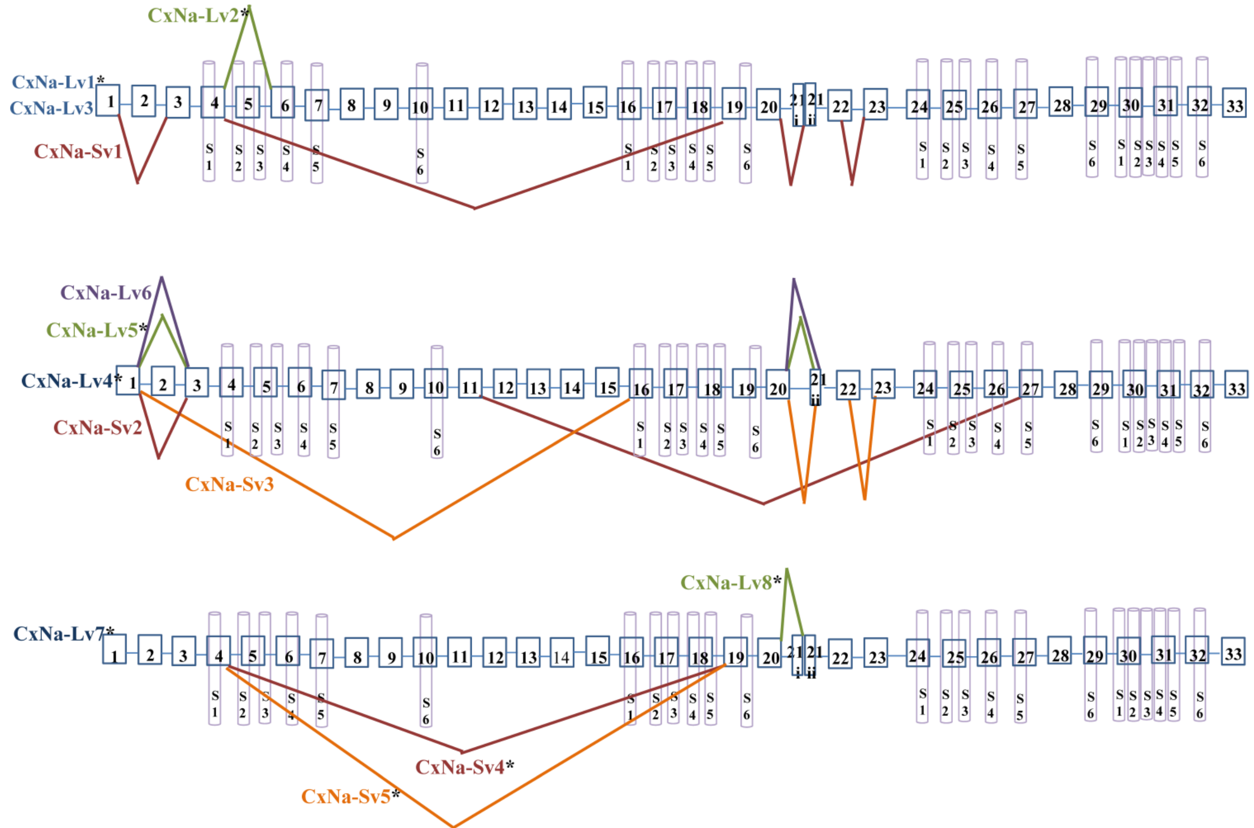

Figure 7.5. Alternative splicing of Cx-Nav from mosquitoes *Culex quinquefasciatus*. Boxes represent exons. The junctions of exons are indicated with straight lines or bridge lines. The schematic of the predicted 6 segments (S1 to S6) in each of the 4 domains (I, II, III, and IV) in the structure of Cx-Nav protein are shown. *The transcript had an entire ORF.

A similar expression pattern was identified in the CxNa-L transcripts of the HAmCq^{G0} mosquitoes (Fig. 7.3 and 7.5), in which two of the three transcripts identified, CxNa-L_v4 and CxNa-L_v5 (Accession numbers: JN695778 and JX424547), were entire sodium channel ORFs, encoding 2092 and 2095 amino acid residues, respectively, and sharing 99% sequence similarity. Comparing the transcript sequences of CxNa-L_v4 and CxNa-L_v5 revealed that an alternative splicing exon 2, a short in-frame insertion in exon 12, and a short in-frame deletion in exon 21ii were present in CxNa-L_v5 (Figs. 7.3 and 7.5). The third CxNa-L transcript, CxNa-L_v6, in the HAmCq^{G0} mosquitoes was again the exception, incorporating several in-frame premature stop

codons, with the first occurred in the linker between IIS3 and IIIS1, in addition to short insertions and deletions (Fig. 7.4 and 7.5). CxNa-L_v6 also exhibited an alternative splicing of exon 2 compared to that identified in CxNa-L_v5. The two CxNa-L transcripts, CxNa-L_v7 and CxNa-L_v8, identified in HAmCq^{G8} were both full length sodium channel transcripts encoding entire ORFs of sodium channels proteins (Fig. 7.4 and 7.5). CxNa-L_v7 and CxNa-L_v8 shared very high sequence similarity (99%), except for an alternative splicing of exon 21i present in CxNa-L_v8 (Figs. 7.4 and 7.5). The above results indicate that multiple ~full length transcripts presented in the mosquitoes, with at least two transcripts in each mosquito strain, had entire ORFs. The other transcripts found in both S-Lab and HAmCq^{G0} incorporated in-frame premature stop codons and, as such, any resulting proteins would be truncated from those regions onward and thus less likely to be functional transcripts.

The sequences of the CxNa-S transcripts with the ~4.0 kb sized sodium channels in each of the mosquito strains were found to be similar to those of the full length CxNa-L sequences; i.e., one or more transcripts were present in each of the mosquito strains. The main difference in the CxNa-S transcripts compared with those of the full length sequences were the internal exons missing through the alternative splicing, along with some minor short deletions or insertions. In the S-Lab strain, only one transcript was observed, CxNa-S_v1, containing a single in-frame stop codon at the IIS6 region in the sequence (Fig. 7.2). However, the CxNa-S_v1 transcript also lacked exons 2, 5 to18, 21i, and 22 as a result of the alternative splicing, and thus had a short sodium channel sequence. Two transcripts, CxNa-S_v2 and CxNa-S_v3, were identified in the HAmCq^{G0} strain (Fig. 7.3). Both of these sequences exhibited alternative splicing of exons, in-frame stop codons, and short deletions and insertions. The CxNa-S_v2 sequences were found to have alternative splicing of exons 2, 12-26, whereas the CxNa-S_v3 sequences lacked exons 2-15,

and parts of exons 21 and 22 (Fig. 7.3) due to the alternative splicing, once again resulting in a short sodium channel sequence. The HAmCq^{G8} strain contained 2 transcripts, CxNa-S_v4 and CxNa-S_v5, with entire ORFs, encoding 1356 and 1329 amino acid residues (Accession numbers: JX424549 and JX424550), respectively, and sharing 98% sequence similarity (Fig. 7.4). Compared with the PRFs of the CxNa-L transcripts, these two CxNa-L transcripts lacked exons 5-18 as a result of the exon alternative splicing (Figs. 7.4 and 7.5). Thus, among all the CxNa-S transcripts identified in the tested mosquitoes, only CxNa-S_v4 and CxNa-S_v5 in the highly resistant HAmCq^{G8} mosquitoes contained the entire ORFs of the sodium channels.

Expression analysis of sodium channel transcripts in *Culex* mosquitoes

The extent of the variation in alternative transcript expression was also addressed by determining the levels of expression of individual sodium channel transcripts in the 4th instar larvae and different tissues from the adult mosquitoes in each strain using qRT-PCR. Characterizing the developmental and regional expression of the sodium channel transcripts in mosquitoes is critical to our understanding of their relative biological importance (Gazina et al. 2010). We therefore determined the relative expression levels of sodium channel RNAs for all the transcripts identified in all three mosquito strains, S-Lab, HAmCq^{G0} and HAmCq^{G8}. Total RNAs were extracted from whole bodies of 4th instar larvae, as well as the head+thorax, and abdomen tissues of 2-3 day old adults. The expression levels were determined using qRT–PCR and the expression ratios for the head + thorax and larval samples were then calculated relative to the quantity of the transcript expression in the corresponding abdomen samples for each strain (Fig. 7.6). The results show that the sodium channel expression in all three strains shared a number of common features. The expression levels were relatively high in the head + thorax tissues compared to the abdomen tissues; the full length sodium channel transcripts of CxNa-L with an ORF of ~6.6 kb

had abundant expression compared with those of CxNa-S ~4.4 kb transcripts, the transcripts with in-frame-stop codons, and CxNa-L_v5 with in-frame-stop codons; and the expression was similar in the head + thorax tissues and larvae with the exception of S-Lab, where the expression of sodium channels was significantly lower ($P \leq 0.05$) in the larvae compared with that in the head + thorax tissues (Fig. 7.6). Comparing the transcripts with the full length ORFs in each of the three strains, even though the transcripts had undergone alternative splicing events the expression levels were similar, suggesting that the variants may have equivalent functional importance in the tissues and the mosquitoes. Indeed, the transcripts with in-frame stop codons were detected in both the S-Lab and $HAmCq^{G0}$ mosquitoes, but at extremely low levels. The difference in the sodium channel expression between the CxNa-L and CxNa-S transcripts was particularly pronounced for S-Lab and $HAmCq^{G0}$, where the CxNa-S transcripts were expressed at levels more than 1000-fold lower than the CxNa-L sodium channel transcripts (Fig. 7.6a, b). In contrast, only about a 10-fold difference in expression between the CxNa-L and CxNa-S transcripts was identified in $HAmCq^{G8}$ (Fig. 7.6c). This feature, plus the markedly higher expression in $HAmCq^{G8}$, might reflect their function in $HAmCq^{G8}$.

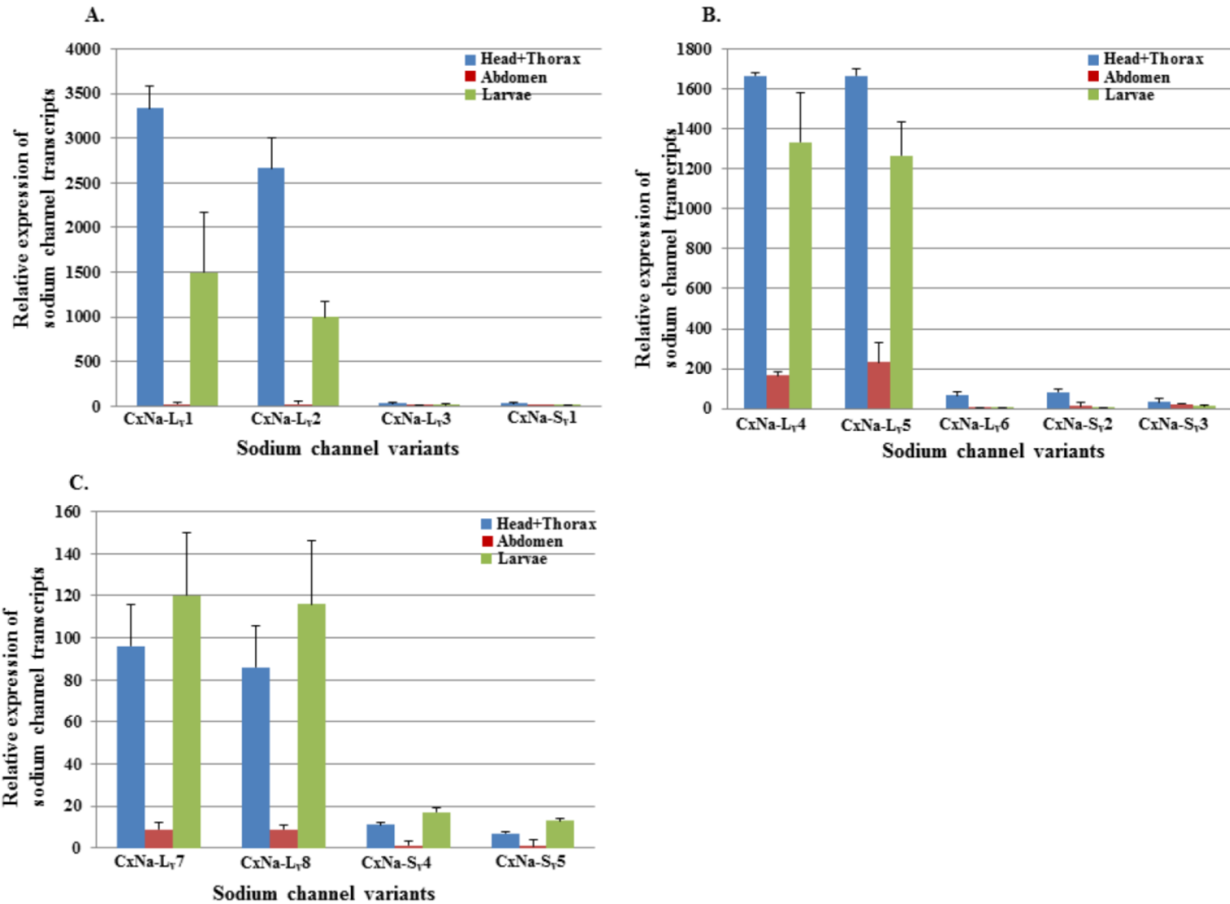

Figure 7.6. Expression of *Cx-Nav* in larvae and head+ thorax and abdomen tissues of 2-3 day-old female adult *Culex* mosquitoes. The relative level of gene expression shown along the Y axis represents the ratio of the gene expression in each tissue from the adults or larvae compared to that measured in the abdomen tissue of the same strain (ratio=1 indicates equal amounts). The experiments were performed three times. The results are shown as the mean ± S.E. No significant difference ($P \leq 0.05$) in the levels of sodium channel transcript ex-pression was found in samples labeled with the same alphabetic letter (i.e., a, b, or c).

Discussion

Voltage-gated sodium channels are essential for the action potential generation of the neuron membrane and play a critical role in membrane excitability (Narahashi 1988, 2000). Over the last few years, a great deal of evidence has accumulated that supports the expression of

diverse distinct sodium channel variants in insects through extensive alternative splicing of a single gene (Davies et al. 2007, Dong 2007, Olson et al. 2008, Tan et al. 2002, Lin et al. 2009). The growing interest in alternative splicing of the sodium channels is propelled by its prominent contribution as a key mechanism generating the structural and functional diversity of sodium channels (Tan et al. 2002, Dong 2007). Following the first reported cloning, sequencing and characterization of multiple variant transcripts from *Drosophila melanogaster* (Loughney et al. 1989), the alternative splicing of sodium channels has now been characterized in many medically or agriculturally important insect and arachnid pest species, including *Drosophila melanogaster* (Thackeray and Ganetzky 1994, O'Dowd et al. 1995, Olson et al. 2008), the house fly *Musca domestica* (Lee et al. 2002), German cockroach *Blattella germanica* (Tan et al. 2002), the mosquito *Anopheles gambiae* (Davies et al., 2007), diamondback moth *Plutella xylostella* (Sonodo et al. 2008), silkworm *Bombyx mori* (Shao et al. 2009) and varroa mite *Varroa destructor* (Wang et al. 2003). The current study represents the first investigation of the transcripts of sodium channels in *Cx. quinquefasciatus* and has revealed multiple variants of sodium channels generated from extensive alternative splicing and small deletions/ insertions, which is consistent with the results of the previous studies of the sodium channels of other insect species.

These multiple variants of *para*-type sodium channel transcripts presented in the mosquito *Cx. quinquefasciatus* can be classified in terms of two categories, CxNa-L and CxNa-S, based on their distinguishing sizes of ~6.5 kb and ~4.0 kb, which were present in all three mosquito strains tested, the susceptible S-Lab strain, the low resistant HAmCq^{G0} strain, and the highly resistant HAmCq^{G8} strain. The main difference in the sequences obtained for these two subcategories is the presence of multiple internal exons obtained through alternative splicing. In

all, nine alternatively splice variants were identified in *Culex* mosquitoes. In the CxNa-L sodium channel category, four splice variants were identified, of which three were full length variants with three optional exons (2, 5, and 21i) and one incorporated in-frame-stop codons. Exon 2 is located in the N-terminus, which is an optional exon corresponding to optional exon 2 of the sodium channel in the silkworm and optional exon j of the *para* in *Drosophila* and which is also conserved in other insect sodium channel genes (Shao et al. 2009). Exon 5 is located between IS2 and IS3. Interestingly, skipping of exon 5 also occurs in the silkworm (Shao et al. 2009), German cockroaches (Song et al. 2004) and the mosquito *Anopheles gambiae* (Davies et al. 2007), suggesting that like exon 2, exon 5 may be a conserved optional exon in insects. Exon 21 is located in the intracellular linker connecting domains II and III of the *Culex* mosquito sodium channels. The 5' portion of exon 21, named 21i, is optional in *Culex* mosquitoes. Exon 21i corresponds to optional exon f in the *para* gene of *Drosophilar* and exon 22i in the silkworm (Shao et al. 2009). These variants with optional exons 2, 5, and/or 21i are all entire ORFs of sodium channels, which may suggest the functional importance of these transcripts in mosquitoes. It has been reported that, when expressed in *Xenopus* oocytes, the alternative splicing variants could exhibit different gating properties and generate sodium channel proteins with differing sensitivities to pyrethroids (Tan et al. 2002, Lin et al. 2009). Whether these variants identified in the *Culex* mosquitoes also have different protein properties and different responses to insecticides remains to be seen.

Investigation of the putative amino acid sequences of alternative splicing variants in the CxNa-S sodium channel category, i.e. the ~4.5 kb transcripts, revealed that in contrast to the findings of CxNa-L, the alternative splicing identified in the sodium channel of *Culex* mosquitoes has resulted in large size or multi-exon-splicing. All the CxNa-S splicing variants in

both the susceptible S-Lab and low resistance parental HAmCq^{G0} strains had in-frame stop codons, suggesting that these splicing variants and any resulting proteins would be truncated from those regions onward. As it has been reported that a truncated channel does not produce any sodium current when expressed in *Xenopus* oocytes (Tan et al. 2002), the transcripts identified in our study that contain in-frame stop codons may not be functional transcripts. Furthermore, the ~1000 to ~3000-fold lower expression of the splice variants with stop codons compared to the CxNa-L splicing variances may further support the conclusion that these variances in mosquitoes are relatively unimportant. Nevertheless, two alternative splicing variants of CxNa-S splicing in HAmCq^{G8} had no in-frame stop codons but still had ORFs encoding sodium channel transcripts lacking exons 5 to18. In addition, these two variants in HAmCq^{G8} had relatively high expression levels, with only ~10-fold lower expression levels compared with the CxNa-L variants. Nevertheless, these variants both lacked IS4 and IIS4 as a result of the alternative splicing. Since the S4 segments act as voltage sensors that initiate voltage-dependent activation (Davies et al. 2007, Shao et al. 2009), the issue of whether these two alternative splicing variants identified in the highly resistant HAmCq^{G8} strain perform some function in the sodium channels of mosquitoes requires further investigation.

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Chapter 8 A Rhodopsin-Like G-Protein-Coupled Receptor Involved the Regulation Pathway of Cytochrome P450 Gene Expression through Activation of Protein Kinase A in the Permethrin-Resistant Mosquito, *Culex quinquefasciatus*

Abstract

Insecticide resistance has been an immense practical problem associated with the chemical control of mosquito vectors. Understanding genes and their regulatory function involved mosquito resistance will be fundamental for designing novel strategies to control mosquitoes, especially the resistant ones. Our previous studies have identified a rhodopsin-like G protein-coupled receptor (GPCR) gene overexpressed in pyrethoid-resistant mosquitoes of *Culex quinquefasciatus*. Rhodopsin-like GPCRs are involved in the G-protein-coupled signal transduction system and regulate many essential physiology processes and functions in insects. The current study investigated that the expression pattern of the rhodopsin-like GPCR was significantly up-regulated in resistant mosquitoes and the up-regulation was increased in mosquitoes following permethrin selection. Furthermore, the expression of rhodopsin-like GPCR gene was up-regulated by permethrin insecticide challenge in highly resistant mosquitoes. Functional studies using double-stranded RNA-mediated gene interference (RNAi) technique revealed that knockdown of the rhodopsin-like GPCR gene in resistant mosquitoes caused a reduction of mosquitoes' tolerance to permethrin, simultaneously a decrease in the expression of a protein kinase A gene (PKA), and 4 cytochrome P450 genes that have been suggested to be involved in the detoxification of insecticides and development of resistance in mosquitoes.

Moreover, the involvement of a PKA gene in the permethrin resistance through the rhodopsin-like GPCR regulatory pathway by regulation of P450 gene expression has been identified. We further confirmed the rhodopsin-like GPCR function using transgenic lines of *Drosophila melanogaster*, in which the tolerance to permethrin and the expression of P450 genes were increased. Taken together, these functional studies strongly revealed the role of the rhodopsin-like GPCR-mediated pathway in the regulation of cytochrome P450 genes that, in turn, involved in detoxification of insecticides and evolution of resistance in *Culex* mosquitoes.

Key Words: Insecticide resistance, G-protein-coupled receptors, Cytochrome P450, Regulation, Protein kinase A, *Culex quinquefasciatus*

Introduction

G-protein-coupled receptors (GPCRs) are largest super-family of cell-surface molecules, which have diverse number involved in cell signal transduction events to regulate numerous physiological pathways in insects (Caers, et al. 2012), such as neuronal excitability, behavior (Mitri, et al. 2009), reproduction (Simonet, et al. 2004), development (Hauser, F. et al. 2006; Bai, et al., 2011), metabolism (Spit, et al. 2012), and by coupling with extracellular ligands and triggering the activation of the heterotrimeric G-protein, which active or inhibit effectors including adenylyl cyclase, levels of cAMP, protein kinases, phospholipase and ionic channel to elicit a cellular response (Dorsam, et al. 2007). The Class A, rhodopsin-like receptors, is largest family of GPCRs, which could be a transducer transfer a striking and extraordinary range of ligands including light, peptides, lipids, nucleotides, etc. in both vertebrates and invertebrates (Müller, et al. 2012). Interestingly, previous studies showed that G protein-coupled receptor (GPCR) gene, for instance a rhodopsin GPCR gene (Liu, et al. 2007) and one opsin gene (Hu, et

al. 2007), which were up-regulated in resistant mosquito strains of *Culex* mosquitoes (compared with the susceptible ones). One arrestin, which was overexpressed in deltamethrin resistance strain of *Culex pipiens*, involved in the regulation of opsin gene and one P450 gene expression in cell line (Sun, et al. 2012). Furthermore, many GPCRs in insects have sequence similarity to known drug target, which reveal new opportunities for the development of novel insecticides (Nene, et al. 2007; Bai, et al. 2011). However, there are no more evidences for GPCR signal transduction pathway involved in insecticide resistance.

Mosquito vector control is the critical part of the current global strategy against the mosquito-borne diseases. Pyrethroids are particularly suitable for veterinary and public health purposes to control pests. However, obtained resistance to insecticide in mosquito vector becomes an urgent and widespread problem for mosquito-borne disease control (Ranson, et al. 2011; Asidi, et al. 2012; Wondji, et al. 2012). The mosquito *Culex quinquefasciatus* is a primary vector of West Nile encephalitis, eastern equine encephalitis, Saint Louis encephalitis, and lymphatic filariasis pathogens inhabiting tropical and subtropical regions worldwide (Sardelis, et al. 2001; Jones, et al. 2002; Reisen, et al. 2005). This species is extendedly treated by pyrethroid (Tungu, P. et al. 2010). However, pyrethriod resistance has broadly developed in *Culex* mosquito species (Liu, et al. 2004; Bisset, et al. 2008; Ranson, et al. 2011). The mainly molecular mechanisms of insecticide resistance in mosquitoes are: 1) increased detoxification of pesticide (alterations in the levels or activities of detoxification proteins), such as cytochrome P450s, GSTs, or esterases, and 2) target-site insensitivity, such as mutations in sodium channels, GABA receptors, or acetycholinesterase (Hemingway, et al. 2004; Liu, et al. 2007). Cytochrome P450s play pivotal role in insecticide detoxification in mosquitoes via increasing their expression level in resistant mosquitoes (Scott, 1999; Pridgeon, et al. 2003; Liu, et al. 2011; Yang, et al.

2011; David, et al. 2013). However, the regulatory pathway of P450 expression-involved insecticide resistance is still unclear. Understanding the mechanisms of insecticide resistance and their regulatory pathways will be fundamental for designing novel strategies to control mosquitoes, especially developing new substances. Liu and Scott (Liu, 1997) have characterized a regulatory factor for P450 gene up-regulation in house fly. Other studies showed that the AMP-actived protein kinase (AMPK) involved in the induction of P450 gene expression in human (Rencurel, et al. 2005) and chick cells (Blättler, et al. 2007), and activation or inhibition of an AMP-activated protein kinase could increase or decrease P450 4F2 expression, respectively, in human hepatocyte cell line (Hsu, et al. 2011). The protein kinase A (PKA) regulated-signaling pathway played crucial role in P450 1B1 expression in rat and mouse tumor cells (Deb, et al. 2011) and also PKA may play a role on P450c17 phosphorylation (Wang, et al. 2010). However, the involvement of protein kinase signal pathway in the regulation of P450 gene expression in insects is unclear.

Taken together, in this study, we, at the first time, characterized the function of a rhodopsin-like GPCR gene in insecticide resistance by regulating the permethrin resistance-related P450 gene expression (Liu, et al. 2011; Yang, et al. 2011) through PKA activity, and also this result has been conducted in *Drosophila* transgenic line to further confirm the rhodopsin-like GPCR gene function in insecticide resistance and regulatory function of P450 gene expression.

Materials and Methods

Culex quinquefasciatus Mosquito Strains

Five mosquito strains were used in this study. They were S-Lab strain, an universal insecticide susceptible strain obtained from Dr. Laura Harrington (Cornell University, Ithaca, NY); HAmCq^{G0}, a field resistant mosquito strain collected from Madison county, AL collected

from Huntsville, Alabama; HAmCq^{G8}, the 8th generation of permethrin-selected HAmCq^{G0} offspring with elevated levels of resistance (~2700-fold resistance compared to S-Lab); MAmCq^{G0}, a field resistant mosquito strain collected from Mobile county, AL; MAmCq^{G6}, the 6th generation of permethrin-selected MAmCq^{G0} offspring with elevated levels of resistance (~570-fold resistance compared to S-Lab) (Liu, et al. 2004; Xu, et al. 2006; Li, et al. 2010). All mosquitoes were reared 25±2^{o}C under a photoperiod of 12:12 (L: D) h.

RNA Extraction and mRNA Preparation

Total RNAs were extracted from 4th instar larvae and 3-day-old adults from HAmCq^{G0}, HAmCq^{G8}, MAmCq^{G0}, MAmCq^{G6} and S-Lab mosquitoes using the acidic guanidine thiocyanate (GIT)-phenol-chloroform method (Liu, et al. 1997). mRNA was isolated with oligo-dT suspension(QIAGEN) as described by the manuscript.

Quantitative Real-time PCR (qRT-PCR) and Gene Expression Analysis

qRT-PCR was performed by ABI 7300 Real-time PCR system(ABI Biosystems). The quantity of total RNA was measured using a spectrophotometer prior to DNA-free. Ten microgram of RNAs was completed DNA-free with TURBO DNA-*free*™ DNase (Ambion) in 50uL reaction following kit introduction. Total RNA samples (0.2ug/sample) were reverse-transcribed to cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche) in a total volume of 20uL. cDNA concentration was measured using a spectrophotometer prior to qRT-PCR. Each qRT-PCR reaction was run in 3 replicates, in a total reaction volume of 25 µL, containing a 2x SYBR Green master mix, 3-5 pmol of a primer pair of target gene, and a 1µg cDNA template from each mosquito sample. A ‘no-template’ negative control was performed. The reaction cycle consisted of a melting step of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 20sec. Relative expression level of

rhodopsin-like GPCR gene was calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software. The 18S ribosome RNA gene was used as endogenous control with primer pair of 18S rRNA F1 and 18S rRNA R1 (Table 8.1), because of the 18S rRNA gene remained constant expression in different tissues and in all strains. Each experiment was repeated 3 times with 3 independently isolated RNA samples from each mosquito strain. The statistical significance of the gene expression was calculated using a Student's t-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons, a value of $P \leq 0.05$ was considered statistically significant.

Table 8.1. Oligonucleotide primers used in PCR and qRT-PCR reaction

Primer description	Primer Name	Primer Sequence
18S Ribosomal RNA	18S rRNA F1	5'CGCGGTAATTCCAGCTCCACTA 3'
	18S rRNA R1	5' GCATCAAGCGCCACCATATAGG 3'
Rhodopsin-like gene amplification	Cx#27f3	5' GAGCTCTAACAAGTCCATCCAAG 3'
	C_2	5' TAATACGACTCACTATAGGGAGA 3'
Drosophila construction	RhoF	5'CCGGAATTCCAAAATGGCATCTTACGCAGCATGGAC3'
	RhoR	5'CTAGTCTAGAGGCCTTCTCGTCCGAAGCG3'
Rho.-like GPCR dsRNA synthesis	dsRNA RhoF	5'TAATACGACTCACTATAGGGGCCATCTTCTTCCTGTGC3'
	dsRNA RhoR	5'TAATACGACTCACTATAGGCGGGGCGAAGTACACGAA3'
Rho.-like GPCR Northern blot analysis	Northern RhoF	5'GGGCCATCTTCTTCCTGTGC3'
	Northern RhoR	5'GCGGGGCGAAGTACACGAAC3'
Rho.-like GPCR Real-time PCR	qPCR iRho F	5'ACTACCTCACCGACACCTTCTC3'
	qPCR iRho R	5'GCCTTGATGATGAAGATG3'
PKA	dsRNA PKA F	5' TAATACGACTCACTATAGTGAAGCAGATCGAGCACGTCAAGA3'
	dsRNA PKA R	5' TAATACGACTCACTATAGAGATGCCGAACGGATTATCGTCGT3'
PKA	qRT PKA F	5'TTGATTGGTGGGCATTAGGCGTTC3'
	qRT PKA R	5'AGCAGCTTCTTGACCAGGTCCTTT3'
GFP gene dsRNA synthesis	dsRNA GFPF	5'TAATACGACTCACTATAGGGAGAAGAACTTTTCACTGG3'
	dsRNA GFPR	5'TAATACGACTCACTATAGCTTCTACCTAGGCAAGTTGA3'
P450CYP9M10 (mosquito)	qRTP450-1CxF	5' ATGCAGACCAAGTGCTTCCTGTAC 3'
	qRTP450-1CxR	5' AACCCACTCAACGTATCCAGCGAA 3'
P450CYP9J40 (mosquito)	qRTP450-23CxF	5' ACCCGAATCCGGGCAAGTTTGAT 3'
	qRTP450-23CxR	5' AACTCCAAACGGTAAATACGCCGC 3'
P450CYP6AA7 (mosquito)	P4505959F	5' ATGACGCTGATTCCCGAGACTGTT 3'
	P4505959R	5' TTCATGGTCAAGGTCTCACCCGAA 3'

P450CYP9J34	P45010546F	5' ATCCGATGTCGGTAAAGTGCAGGT 3'
(mosquito)	P45010546R	5' TGTACCTCTGGGTTGATGGCAAGT 3'
P450CYP6a2	CYP6a2(D)F	5' TGGACGGAAAGAAGTGGAAGGAC3'
(drosophila)	CYP6a2(D)R	5' AGTTCATGTTCCCGACGGTGATCA 3'
P450CYP12d1	Cyp12d1(D)F	5' GCTCGGCTCAAATGTGCTGATGAA3'
(drosophila)	Cyp12d1(D)R	5'TGACCTGCATCTTCTTTCCGGTCT3'
P450CYP6a8	Cyp6a8(D)F	5'ACGAGTGCACCAAGGATCTGAAG3'
(drosophila)	Cyp6a8(D)R	5' ATTGACCAGCCTCGATGACGAAGT3'
P450CYP6g1	Cyp6g1(D)F	5'CGGCTGAAGGACGAGGCTGT3'
(drosophila)	Cyp6g1(D)R	5' GCTATGCTGTCCGTGGAGAACTGA3'
P450CYP6a19	Cyp6a19 (D) F	5' AATCGACAGTGTGCTGGAAA3'
(drosophila)	Cyp6a19 (D) R	5' TGCCGGCCTCTATGAAATAC3'

Nrothern Blotting Analysis

Northern blot analyses were performed according to Sambrook et al. (1989). Five micrograms of mRNA from 4th instar larvae of 5 strains were fractionated on 1% formaldehyde denaturing agarose gel containing ethidium bromide and transferred to Nytran membranes. The rhodopsin-like GPCR gene (~500bp) was amplified using Northern RhoF and Northern RhoR primer pair (Table 8.1). The PCR product of rhodopsin-like GPCR gene was labeled with [α-^{32}P]dCTP using a Prime-It II Random Primer Labeling kit (Agilent Technologies, Stratagene) following the manufacturer's instructions, and hybridized with RNA blots using QuickHyb solution (Agilent Technologies, Stratagene). The quantity of RNA loaded in each lane was standardized by comparing the density of 18S ribosomal RNA (rRNA) band on the agarose gel under UV light before transfer (Spiess, et al. 1998). All Northern blot analyses were repeated three times with different preparations of RNA samples.

Permethrin Challenge Experiment

Later third instar larvae of resistant strains, HAmCq^{G0} and HAmCq^{G8}, and S-Lab, were treated with permethrin, following mosquito larva bioassay method (Li, et al. 2010). Preliminary concentration range, time course, and rhodopsin-like GPCR gene induction assays for larvae

were performed with corresponding concentration ranges of LC_{10}, LC_{50}, and LC_{70} for each mosquito strain (Table 8.2). Briefly, ~400 later 3rd instar larvae of each *Culex* strain were treated with permethrin at their different LC_{10}, LC_{50} and LC_{70} concentrations, respectively. Twelve, 24, 48 and 72 hours after treatments, the surviving mosquitoes ~ 30 larvae were collected from each mosquito population of LC_{10}, LC_{50}, and LC_{70} treatment, respectively. The surviving mosquito larvae collected for RNA extraction after exposure to permethrin. The control mosquitoes, which had not been exposed to permethrin but 1% acetone treated, were collected as same time point as the permethrin treated mosquitoes. Each treatment experiment was repeated 3 times.

Table 8.2. Permethrin treatment with dose range in the mosquito strains

	LC_{10}	LC_{50}	LC_{70}
S-Lab	0.003ppm	0.005ppm	0.015ppm
$HAmCq^{G0}$	0.02ppm	0.05ppm	0.15ppm
$HAmCq^{G8}$	5ppm	15ppm	20ppm

Double-Stranded RNA Preparation and RNA Interference

Adult injection with dsRNA of rhodopsin-like GPCR gene

To initially investigate the function of rhodopsin-like GPCR gene in *Culex* mosquitoes, we used the double-stranded RNA interference (RNAi) technique, which is a powerful tool to silence the gene expression post-transcriptionally (Bellés, 2010). A ~500-bp PCR product of the rhodopsin gene and a green fluorescent protein (GFP-pMW1650) gene were generated complementary to rhodopsin-like GPCR cDNA sequences and a pMW1650 plasmid, respectively. There is T7 promoter sequences (5’-TAATACGACTCACTATAGGG-3’) appended the 5’ end of each PCR primer (Table 8.1). Double-stranded RNA (dsRNA) was synthesized from the PCR template with the opposing T7 promoter sequences by *in vitro* transcription, using the MEGAscrip T7 High Yield Transcription kit. For dsRNA purification, phenol/chloroform extraction followed by ethanol precipitation method was applied. We injected

the nuclease free water-dissolved dsRNAs (~500 ng) of the rhodopsin-like GPCR gene into the mesothorax of 12-hr to 24hr-old female adult HAmCq^{G8} and MAmCq^{G6} mosquitoes using Nanoject II (Drummond Scientific Company). To avoid gene injection affects for target gene expression, we injected with dsRNA of the GFP served as the control, and the HAmCq^{G8} mosquitoes that received no injection served as the calibrator. Total RNAs were extracted from 3-4 day post-injection and non-injected HAmCq^{G8} adults, and the relative expression of rhodopsin-like GPCR gene, P450 genes, *CYP9M10*, *CYP6AA7*, *CYP9J40*, and *CYP9J34* (Liu, et al. 2011), and one cAMP-dependent protein kinase gene, PKA (XM_001842369.1) were investigated by using qRT-PCR with primer pair, qPCR iRho F and qPCR iRho R, qRTP450-1CxF and qRTP450-1CxR, qRTP450-23CxF and qRTP450-23CxR, P4505959F and P4505959R, P45010546F and P45010546R, qRT PKA F and qRT PKA R, respectively (Table 8.1). Each experiment was repeated 6 times with independently isolated RNA samples from mosquitoes.

Embryo injection with dsRNA of rhodopsin-like GPCR and protein kinase A gene

According to the literature about *Drosophila* embryo injection with dsRNA (Ivanov, et al. 2004) and mosquito transgenic method (Adelman, et al. 2008), the function of the rhodopsin-like GPCR gene in larvae of resistant mosquito strains, HAmCq^{G8} and MAmCq^{G6}, was tested by injection of the rhodopsin-like GPCR dsRNA to mosquito embryo, detecting the gene expression corresponding to the permethrin resistance in 2nd -3rd instar larvae. One thousand embryos were used for dsRNA injection in each time. Grey embryos were collected from HAmCq^{G8} and MAmCq^{G6} mosquito cages, in which the mosquito had been feed with blood three days early and reared under insectory conditions. Around 120 freshly laid eggs were collected and arranged on a piece of paper filter. Embryos were allowed to dry 2-3 min and transferred to a clear microscope

slide cover using double sided tape, and embryos were then covered with Halocarbon 700 oil. Capillaries for microinjection were prepared by using a Flaming/Brown micropipette puller, and tip was opened by using a K. T. Brown Type micro-pipette beveler. We injected 0.2-0.5nl of dsRNA (3.5ug/ul) into embryo posterior, corresponding to approximately 1-5% of the embryo volume by using Picospritzer III injector system. Injected embryos were clear one by one and transferred to water solution and keep the water container under insectory condition for 3-4 days. Each time ~500 embryos were injected with dsRNA of rhodopsin-like GPCR gene and dsRNA of GFP gene, respectively. Hatched 2^{nd} -3^{rd} instar larvae were separated into two groups, one was tested for bioassay with 5 dose range of permethrin, and another was prepared for gene expression identification of rhodopsin-like GPCR and permethrin resistance-related P450 genes, *CYP9M10*, *CYP9J34*, *CYP9J40*, *CYP6AA7* (Liu, et al. 2011; Yang, et al. 2011), and PKA by using qRT-PCR. Each experiment was repeated 6 times with independently isolated RNA samples. Both gene expression and permethrin sensitivity were detected for identification of the rhodospin-like GPCR gene function.

To identify the function of the PKA gene in insecticide resistance through the regulation pathway of the rhodopsin-like GPCR, we used RNAi method to knockdown the gene expression of PKA gene and also tested four P450 gene expression in dsRNA injected mosquitoes compared with GFP-injection and non-injection. The dsRNA synthesis primers of PKA were dsRNA PKA F and dsRNA PKA R (Table 8.1). The gene knockdown efficient was tested by using real-time PCR with primer pairs of qRT PKA F and qRT PKA R (Table 8.1).

Mosquito Bioassay

Adult bioassay To test the resistant function of rhodopsin-like GPCR in *Culex* mosquitoes, we used the topical application for the adult bioassays; a 0.5-μl drop of insecticide

in acetone was delivered to the thoracic notum of each mosquito adult using a 25- μl Hamilton gastight syringe (Fisher Scientific). Three to 4 days post-injection and non-injected female adult $HAmCq^{G8}$ and $MAmCq^{G6}$ mosquitoes were used in this study. Each bioassay consisted of 20 mosquitoes per dose and four or five doses that yielded >0 and <100% mortality (Li, et al. 2010). Control groups received acetone alone. Treated mosquitoes were reared in 6-oz Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with 10% sugar water on cotton. Mortality was assessed at 24 h post-treatment. The criterion for death was the mosquitoes' inability to move. All tests were run at 25^{o}C and replicated 6 times.

Larva bioassay The stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) were prepared in acetone. Each bioassay consisted of 20 2^{nd}-3^{rd} instar mosquito larvae with rhodopsin-like or PKA injection, GFP-injection and non-injection in 10 ml glass vial with regular tap water and 1% insecticide solution in acetone at the required concentration, with four or five concentrations that resulted in >0 and 100%< mortality (Li, et al. 2010). Control groups received only 1% acetone. Mortality was assessed after 24 h. all tests were run at 25^{o}C and replicated 6 times.

Cloning and Sequencing of Rhodopsin-Like GPCR Gene from *Cx. quinquefasciatus*

The full length of rhodopsin-like GPCR gene was amplified from cDNA of *Cx. quinquefasciatus* using platinum Taq DNA polymerase High Fidelity (invitrogen) with specific primer pair, Cx#27f3 and C_2 (Table 8.1). PCR product of full length was purified using QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were ligated into pCR 2.1 vector using the Original TA Cloning kit (invitrogen) as described by the manufacturer. The full length of rhodopsin-like gene was cloned in One Shot TOPO 10F' cell using One Shot TOP10F' Chemically Competent *E. coli* kit (invitrogen) and grown in LB plate (Kan.$^{+}$). The single clone

was picked up and grown in TB solution (Kan.$^+$). The plasmid was extracted by using EndoFree plasmid Maxi Kit (Qiagen) following the manuscript and sequenced. Cloning and sequence analyses of the rhodopsin-like GPCR cDNAs were repeated at least three times.

Construction of Transgenic Fly Strains

The rhodopsin-like GPCR gene full length was amplified from the above TA clone plasmid using primer pair of Rho F and Rho R with protecting bases CCG in RhoF and CTA in RhoR, and restriction enzyme cutting site EcoRI (GAATTC) in Rho F and Xbal (TCTAGA) in Rho R (Table 8.1) based on the 5' and 3' end sequence of the putative rhodopsin-like GPCR gene. Rhodopsin-like GPCR gene as above described and pUASTattB vector were digested with EcoRI-HF and Xbal-HF restriction enzyme (NEB). The digested product was purified by using QIAquick Gel Extraction Kit (Qiagen). The rhodopsin-like GPCR and digested pUASTattB vector were ligated by using T4 DNA ligase (invitrogen). The plasmid of pUASTattB-rhodopsin-like GPCR was cloned in Max Efficiency DH5α cell (invitrogen) and grown in TY solid media with $CaCl_2$ (Amp$^+$). Single colon was picked up and grown in TY liquid media with $CaCl_2$ (Amp$^+$). The plasmid of pUASTattB-rhodopsin-like was extracted by using EndoFree plasmid Maxi Kit (Qiagen) following the manuscript and transformed into the germline of *D. melanogaster* 58A strain using standard P-element-mediated transformation techniques (Rainbow Transgenic Flies, Inc. CA). The inserted DNA construct in a transformed line was mapped on chromosome 2. We chose five P450 genes *(CYP6a2*, *Cyp12d1*, *Cyp6a8*, *Cyp6g1*, and *Cyp6a19*) (Daborn, et al. 2007) in drosophila to detect the relative expression of P450 genes in transgenic and non-transgenic drosophila lines using qRT-PCR with primer pairs, CYP6a2(D)F and CYP6a2(D)R, Cyp12d1(D)F and Cyp12d1(D)R, Cyp6a8(D)F and Cyp6a8(D)R,

Cyp6g1(D)F and Cyp6g1(D)R, Cyp6a19 (D) F and Cyp6a19 (D) R (Table 8.1). Each experiment was repeated 3 times with 3 independently isolated RNA samples from each mosquito strain.

Drosophila Bioassay

We used 2-3 days post-closion female drosophila for bioassay. Permethrin was evenly distributed on the inside of 15mL class vials by applying 200µL acetone-dissolved permethrin with 0.05µg/µL in series concentration and rolling the vial until all of acetone had evaporated. Fifteen female flies were knockdown by CO_2 and transferred to each prepared vial, which were plugged with cotton balls socked with 5% sucrose. Control groups received acetone alone. The mortality was scored after 24hr exposure to insecticide. For each bioassay, at least 6 replicates were performed. All drosophila were reared 25±2^{o}C under a photoperiod of 12:12 (L: D) h.

Results

Rhodopsin-like GPCR Gene Expression in *Culex* Mosquitoes

To characterize the expression pattern of the rhodopsin-like GPCR gene implicated in insecticide resistance and in the development of mosquitoes, we examined the expression level of rhodopsin-like GPCR gene for 4th instar larvae and 3-days old adults using RT-qPCR and Northern blot analysis. We compared the expression of rhodopsin-like GPCR in S-Lab, HAmCq^{G0}, HAmCq^{G8}, MAmCq^{G0}, and MAmCq^{G6} mosquito strains. We found 2.6-fold expression of rhodopsin-like GPCR gene in HAmCq^{G8} strain higher than both S-Lab and its parental strain HAmCq^{G0}, and ~2-fold expression in MAmCq^{G6} higher than in MAmCq^{G0}. However, there was no significant difference expression of rhodopsin-like GPCR gene between MAmCq^{G6} and S-Lab. Northern blot analysis has consistent expression profile with qRT-PCR in the larvae of 5 strains (Fig. 8.1). The expression of rhodopsin-like GPCR gene is ~200 times overexpressed in larvae more than in adults (Fig. 8.1).

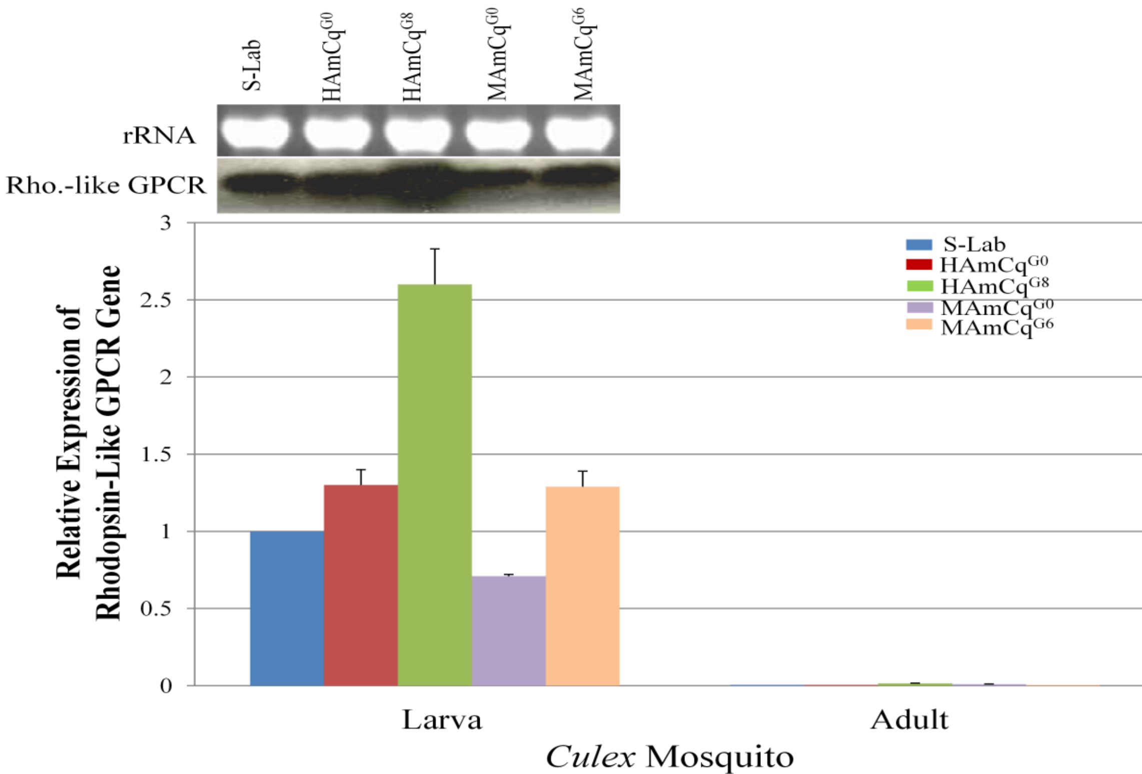

Fig. 8.1. Expression of rhodopsin-like GPCR gene in *Culex* mosquito strains. Relative expression of rhodopsin-like GPCR gene in larva and adult of S-Lab, HAmCq^{G0}, HAmCq^{G8}, MAmCq^{G0}, and MAmCq^{G6} have been identified by using northern blot analysis and real-time PCR. Relative expression levels were normalized by the expression of 18S ribosomal RNA. The data shown are mean ± SEM (n=8).

Response of rhodopsin-like GPCR gene to permethrin challenge in resistant and susceptible mosquitoes

In order to further characterize the rhodopsin-like GPCR gene expression in response to permethrin challenge in *Culex* mosquitoes, we used qRT-PCR to test the expression of rhodopsin-like GPCR gene in 4th instar mosquito larvae with permethrin in acetone and acetone treatment. To minimize the possibility that the rhodopsin-like GPCR gene has nothing to do with resistance but arose solely because of a strain-strain difference, the study used susceptible strain,

S-Lab, and two resistant strains, $HAmCq^{G0}$ and $HAmCq^{G8}$. To examine the effect of permethrin on induction of the rhodopsin-like GPCR gene, we measured the expression of the rhodopsin-like GPCR gene in mosquitoes challenge with permethrin at a corresponding concentration range (LC_{10}, LC_{50}, and LC_{70}) for various durations and different time points of 0-hour, 12-, 24-, 48-, and 72- for time course. Although no induction was detected in either the susceptible strain S-Lab or field resistant strain $HAmCq^{G0}$ for the dose range and time intervals tested, our results showed that permethrin induced the rhodopsin-like GPCR gene up-regulation in $HAmCq^{G8}$ with varying levels in a time point (48h) and concentration (LC_{50})-dependent manner (Fig. 8.2A, B). The significant induction of the rhodopsin-like GPCR gene only in $HAmCq^{G8}$ mosquito at 48 hour and LC_{50} is ~2.5-fold higher than acetone treated. Moreover, the rhodopsin-like GPCR gene was also induced at time points of 12 hour (~2-fold) and 24 hour (~2-fold) compared with acetone treated mosquitoes. However, the expression level was decreased at 72 hour after treatment (Fig. 8.2A).

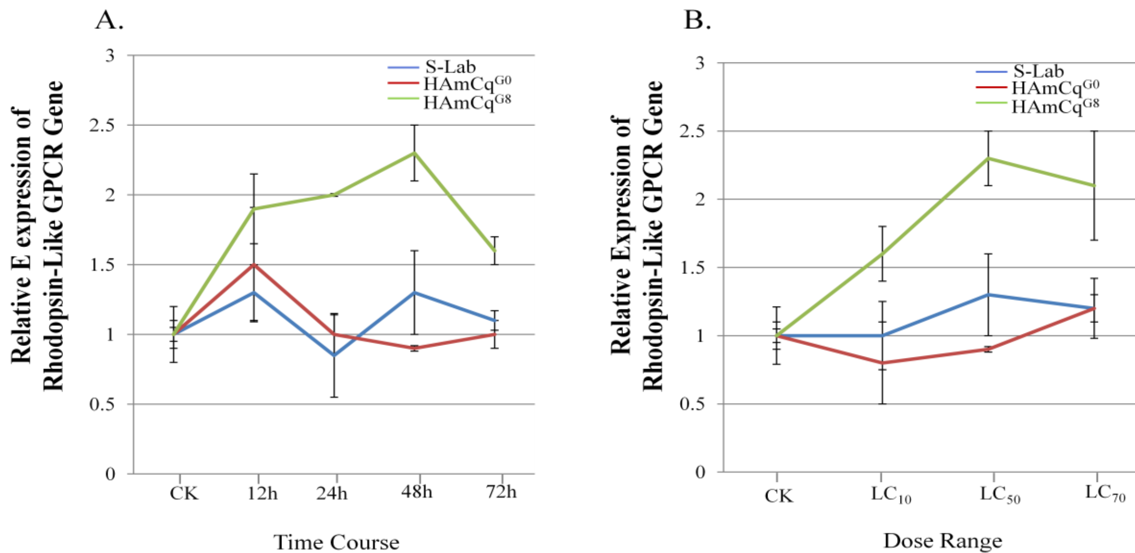

Fig. 8.2. Time and dose-dependent induction of the relative expression of the Rhodopsin-like GPCR gene. A. rhodopsin-like GPCR gene expression was tested at LC_{50}-treatment at different

time points, 0-hour, 12-, 24-, 48-, and 72- in S-Lab, $HAmCq^{G0}$, and $HAmCq^{G8}$. B. rhodopsin-like GPCR gene expression was tested at 48 hour post-treatment at different dose range, LC_{10}, LC_{50}, LC_{70}, and acetone treated CK in early 4^{th} instar larvae of S-Lab, $HAmCq^{G0}$, and $HAmCq^{G8}$. Y-axis is relative expression value of rhodopsin-like GPCR gene.

Initiate the Functional Study of the Rhodopsin-like GPCR in *Culex* Mosquitoes

To initially characterize the rhodopsin-like GPCR function in *Culex* mosquitoes, we used the RNAi technique to inhibit the expression of rhodpsin-like GPCR gene in larvae and adults of resistant strains, $HAmCq^{G8}$ and $MAmCq^{G6}$, respectively. We found a ~2-fold lower in expression of rhodopsin-like GPCR in dsRNA-injected adults of $HAmCq^{G8}$ and $MAmCq^{G6}$ adults than the control (injected with GFP) and calibrator (no injection) mosquitoes (Fig. 8.3A, 8.3C), and also a 3-fold and 1.5-fold lower in expression in dsRNA injected larvae of $HAmCq^{G8}$ and $MAmCq^{G6}$, respectively, than GFP-injected and no injection (Fig. 8.3B, 8.3D). Moreover, to determine whether the knockdown expression of the rhodopsin-like GPCR gene causes suppression of permethrin resistance in $HAmCq^{G8}$ and $MAmCq^{G6}$, we performed adult and larvae bioassay to compare the permethrin resistance levels among injected (with rhodopsin-like GPCR and GFP) and no injected mosquitoes. Bioassay result showed that the decreased resistant level of 3-fold to permethrin in adults and larvae of the rhodopsin-like GPCR injected $HAmCq^{G8}$ compared with GFP injected and no injected mosquitoes (Fig. 8.4A, 8.4B). However, $MAmCq^{G6}$ had no decreased resistance to permethrin in adults and ~1.8-fold lower in larvae of the rhodopsin-like GPCR injected than the GFP-injection and no injection (Fig. 8.4C. 8.4D).

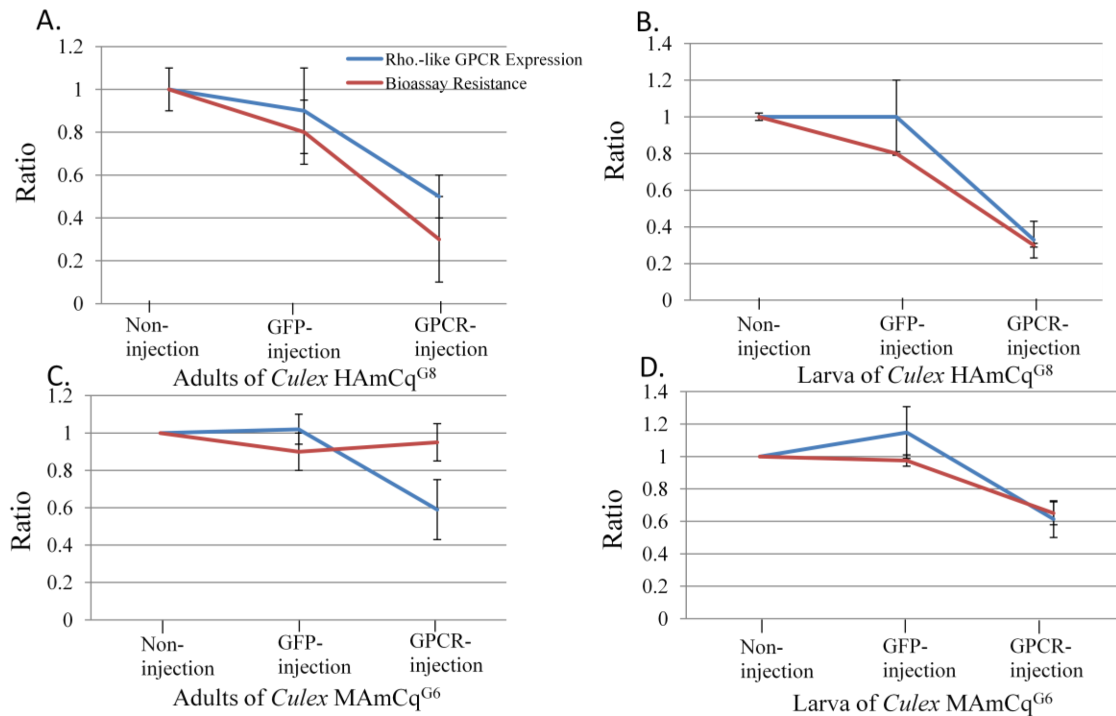

Fig. 8.3. Rhodopsin-like GPCR gene expression and bioassay resistance ratio in the mosquitoes with or without rhodopsin-like-dsRNA injection. Figure A and C. The gene expression of rhodopsin-like GPCR and tolerance to permethrin were decreased in injected adult mosquito of $HAmCq^{G8}$ and $MAmCq^{G6}$ with ds-RNA of rhodopsin-like GPCR gene in comparison with non-injected and ds-GFP-injected adult mosquitoes. B and D. ds-RNA of rhodopsin-like GPCR gene was injected into $HAmCq^{G8}$ and $MAmCq^{G6}$ mosquito embryo. The gene expression level was conducted at 2^{nd} instar larva using qRT-PCR. In rhodopsin-like GPCR gene injected 2^{nd} instar larva, the rhodopsin-like GPCR gene expression and tolerance to permethrin were decreased in comparison with non-injected and ds-RNA of GFP 2^{nd} instar larvae. The result shown is mean ± SEM (n=5).

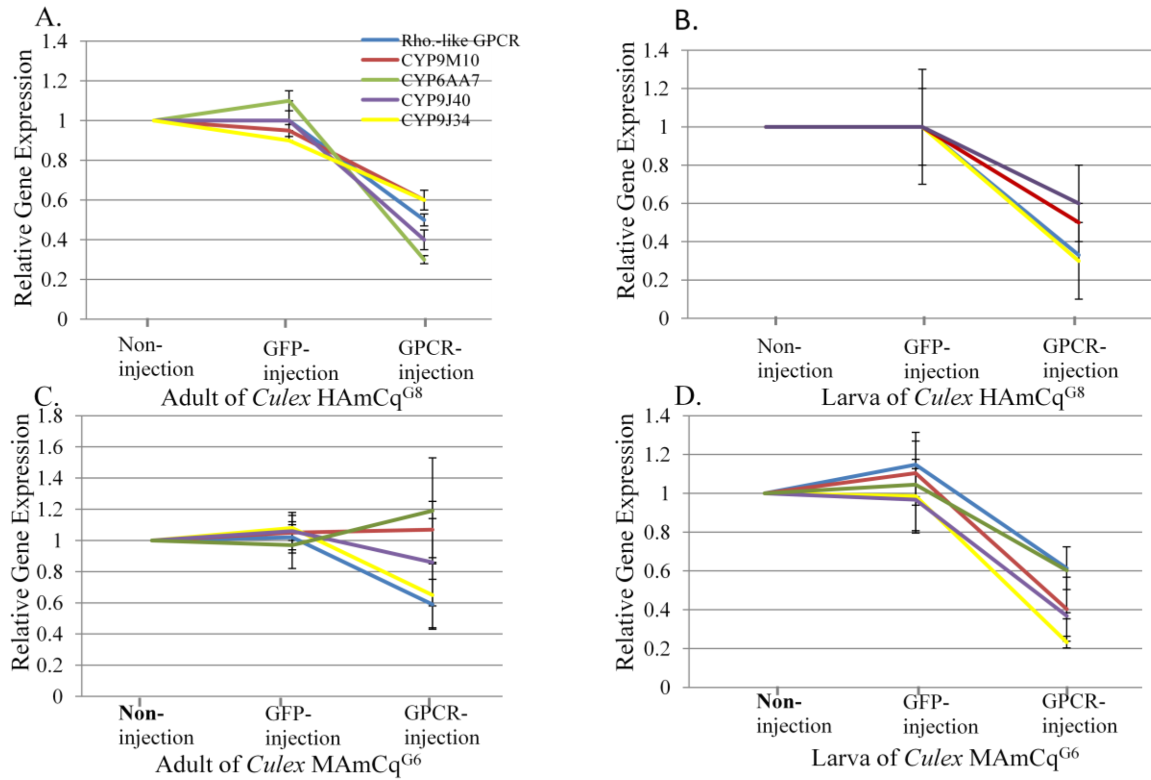

Fig. 8.4. Four P450 Gene Expression in the Mosquitoes with or without dsRNA-rhodpsin-like Injection. A. and C Four P450 gene expression was decreased in rhodopsin-like-ds-RNA injected-HAmCq^{G8} and MAmCq^{G6} adult mosquitoes by comparison with ds-RNA-GFP injection and non-injection mosquitoes. B and D. Four P450 gene expression was decreased in 2nd instar larva of HAmCq^{G8} and MAmCq^{G6} with rhodopsin-like-ds-RNA embryo injection in comparison with ds-RNA GFP injection and non-injection mosquito ones. The result shown is mean ± SEM (n=5).

To verify how the rhodopsin-like GPCR was involved in permethrin resistance in mosquitoes, we identify the function of the rhodopsin-like GPCR gene in the regulation of the expression of 4 permethrin resistance-related cytochrome P450 genes in injected and no injected larvae and adults of HAmCq^{G8} and MAmCq^{G6} mosquitoes. The result indicated that these P450 genes were significantly decreased expression in rhodopsin-like-dsRNA-injected mosquitoes

following rhodopsin-like GPCR gene interference silence in adults and larvae of HAmCq^{G8} and in larvae of MAmCq^{G6} (Fig. 8.4A, 8.4B, 8.4D), however, there no evidence showed decreased expression of the P450 genes in adult of MAmCq^{G6} injected mosquitoes, which was consistent with the no decreased resistance to permethrin in bioassay (Fig. 8.4C).

Initially Functional Study of cAMP/PKA in Resistant Mosquito Strain

To further characterize the rhodopsin-like GPCR-regulated pathway for P450 gene expression, we found a cAMP dependent protein kinase A (PKA) gene was down-regulated in the rhodopsin-like GPCR injected mosquito strains compared with GFP-injection and no injection ones (Fig. 8.5), suggesting the involvement of the PKA in the regulation pathway of rhodopsin-like GPCR. The regulation function of the PKA in P450 gene expression was initially tested by using dsRNA of PKA injection into fresh eggs of HAmCq^{G8} and MAmCq^{G6} mosquitoes. The results showed a strong correlation between 2-fold knockdown of the PKA gene expression and decreased resistance to permethrin (~2.5-fold) in PKA injected HAmCq^{G8} and ~1.5 fold in MAmCq^{G6} compared with GFP-injection and no injection ones in adult (Figs. 8.6A, 8.6C). In the PKA knockdown mosquitoes, decreased expression of the four P450 genes (>2-fold) was investigated in PKA dsRNA-injection of HAmCq^{G8} mosquitoes (Fig. 8.5B), but only two P450 genes, CYP9J34 and CYP9J40, were decreased expression in PKA knockdown of MAmCq^{G6} mosquitoes (Fig. 8.6D).

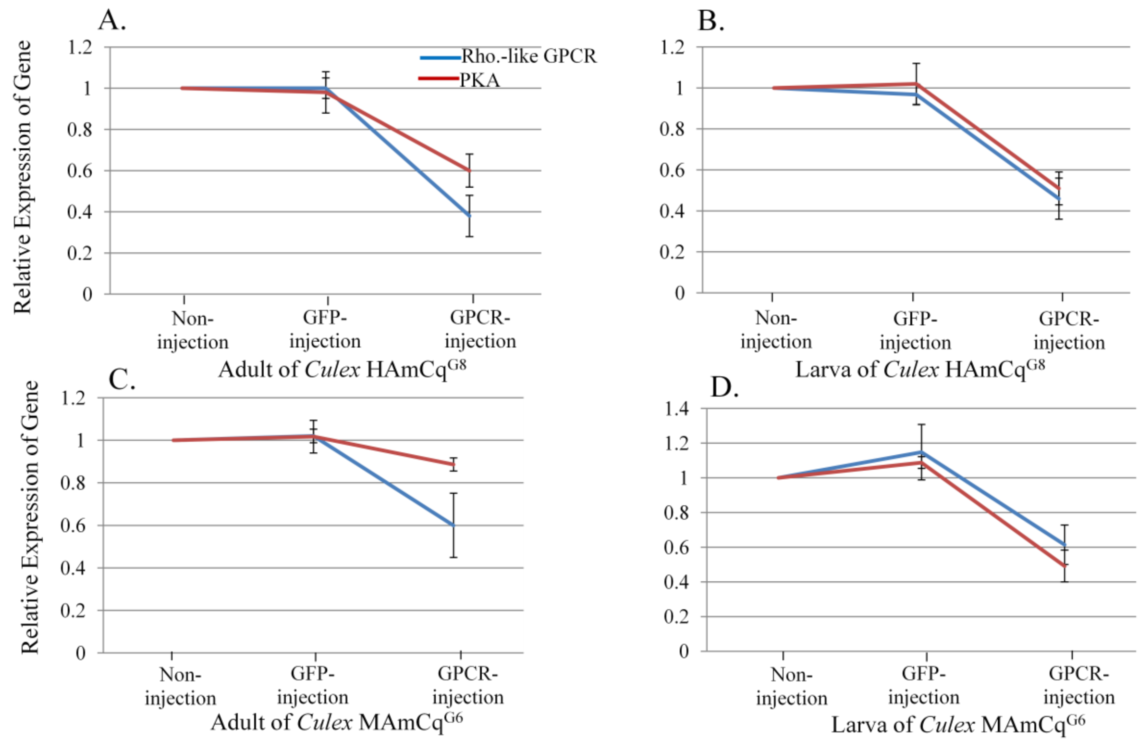

Fig. 8.5. cAMP/PKA gene expression in mosquito with or without rhodopsin-like GPCR gene injection. A. and C PKA gene expression was tested in rhodopsin-like-ds-RNA injected-HAmCq^{G8} and MAmCq^{G6} adult mosquitoes by comparison with ds-RNA-GFP injection and non-injection mosquitoes. B and D. PKA gene expression was decreased in 2nd instar larva of HAmCq^{G8} and MAmCq^{G6} with rhodopsin-like-ds-RNA embryo injection in comparison with ds-GFP injection and non-injection mosquito ones. The result shown is mean ± SEM (n=5).

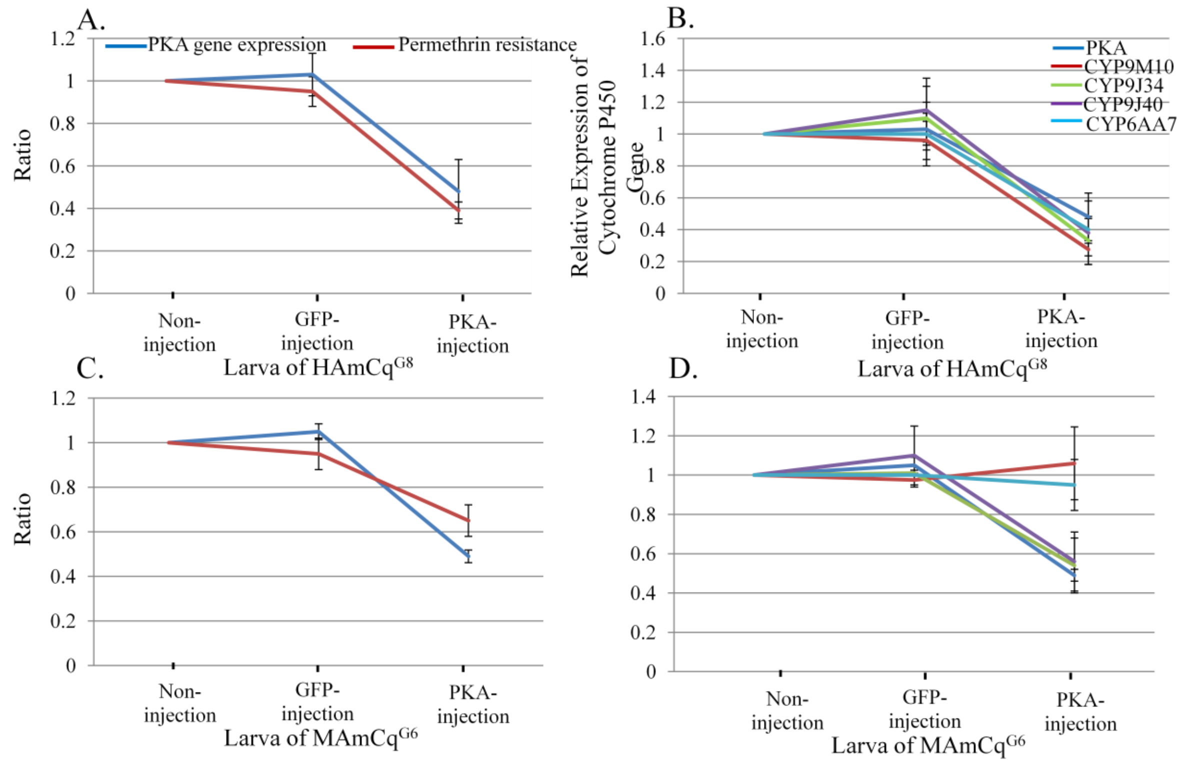

Fig. 8.6. Mosquito injection with or without PKA gene. A and C. The gene expression of PKA and tolerance to permethrin were decreased in embryo injected larva of HAmCq^{G8} and MAmCq^{G6} with ds-RNA of PKA gene in comparison with non-injected and ds-GFP-injected ones. B and D. Four P450 gene expression was tested in 2nd instar larva of HAmCq^{G8} and MAmCq^{G6} with PKA-ds-RNA embryo injection in comparison with ds-GFP injection and non-injection ones. The result shown is mean ± SEM (n=5).

Transgenic Expression of Rhodopsin-like GPCR in *D. melanogaster*

To further identify the expression of rhodopsin-like GPCR gene caused permethrin resistance, the rhodopsin-like GPCR gene was constructed into pUASTattB plasmid forming pUASTattB-rhdopsin-like construction, which was transformed into drosophila 58A strain by P-element-mediated transgenic technique. The virgin female of transgenic line (with orange eye marker) crossed mating with the male of the balancer drosophila (with curly wing marker) to

produce the F1 progeny. The virgin male and female from the F1 progeny with orange eye and curly wing crossed mating and produced the F2 progeny, which was homozygous for the rhodopsin-like gene with orange eye and straight wing. The virgin female of homozygous transgenic drosophila crossed mating with male of Gal 4 strain to produce the progeny, which expressed rhodopsin-like gene in the transgenic drosophila. To avoid the transgenic process influence the drosophila biology changes, the empty vector of pUASTattB transgenic line served as control. Total RNAs were extracted from 3-day-old adults of transgenic with the rhodopsin-like gene expression and vector-transgenic *Drosophila* lines. cDNA was synthesized using total RNA as template following the previous method description. The rhodopsin-like GPCR gene was amplified using cDNA and with primer pairs, showing us that the rhodopsin-like GPCR gene represent in transgenic drosophila line (1122bp) but absent in vector-transgenic lines (Fig. 8.7A). The 2-3-day post-closion drosophila adults from rhodopsin-like transgenic line and vector-transgenic control line were treated by permethrin following previous bioassay description. The bioassay result showed that the transgenic line obtained higher tolerance to permethrin at a diagnostic dose of10ug permethrin per vial (70% survival) than control line (10% survival) (Fig. 8.7B). Furthermore, we also detected expression of 5 drosophila cytochrome P450 genes, which were up-regulated in pyrethriod resistance in drosophila according to previous studies, in which 3 of them were increased expression in the pUASTattB-rhdopsin-like construction transgenic line (Fig. 8.7C). Especially, the up-regulation of CYP12d1 and CYP6a8 has ~5-fold and ~4-fold in transgenic line higher than it in control line, respectively (Fig. 8.7C).

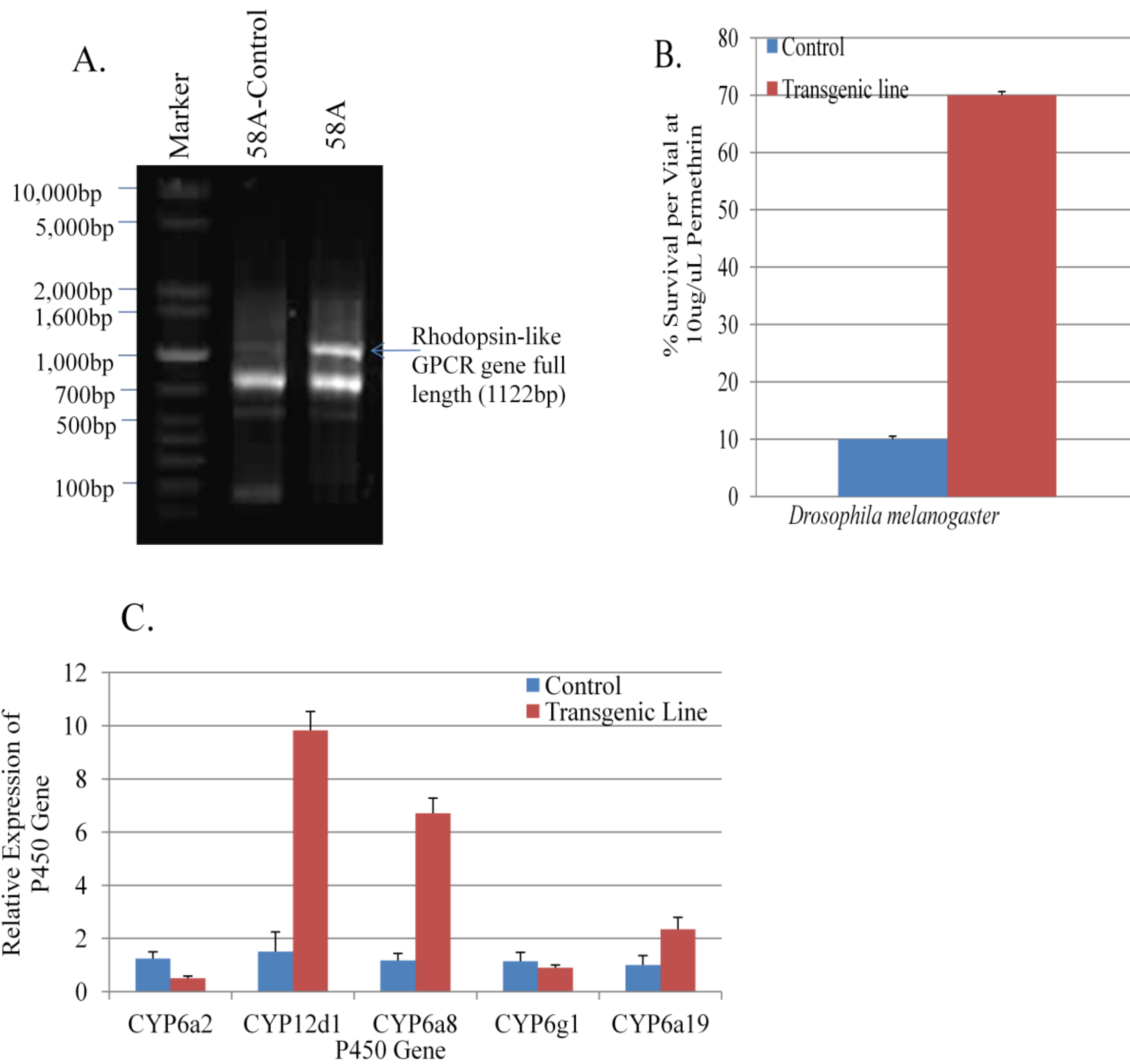

Fig. 8.7. Functional study of rhodopsin-kike GPCR in *Drosophila* transgenic and non-transgenic lines. A. The rhodopsin-like GPCR gene full length (~1122bp) was amplified from transgenic drosophila but no amplification in control lines. B. The percent survival of control strain and transgenic line exposed to 10µg permethrin/ vial. C. The relative expression of five P450 genes

was identified in *Drosophila* control line and rhodopsin-like GPCR transgenic line. The result shown is mean ± SEM (n=5).

Discussion

The evolution of insecticide resistance is conferred through mechanisms, typically requiring the interactions of multiple genes. We hypothesize that the gene(s) involved in insecticide resistance exhibit increased expression following insecticide selection. One of the major mechanisms of resistance to insecticide in mosquitoes is enhanced detoxification ability (Hemingway, et al. 2002, 2004, Scott, 1999, Ranson, et al. 2002; Nardini, et al. 2012), such as cytochrome P450 genes (Kasai, et al. 2000, Nikou and Ranson, 2003, David, et al. 2005, Casimiro, et al. 2006, Liu, et al. 2007, 2011, Muller, et al. 2008, Amenya DA, et al. 2008, Chiu, et al. 2008). G-protein coupled receptors are widely expressed in the body and play a fundamental role in physiology and pathophysiology and have been used as potential targets for therapeutic intervention in many human diseases (Wise et al. 2002; Lappano, et al. 2011; Insel, et al. 2012). Insect GPCRs are cell membrane receptors for neurohormones, neuropeptides, odorant, light (Hauser, et al, 2006, Benton, 2006). The total of 276 GPCR genes has been identified from *Anopheles gambiae* genome, in which 79 candidate odorant receptors as well as 76 putative gustatory receptors may play crucial roles in sensory pathways and disease transmission in *A. gambiae* (Hill, et al. 2002). In *Aedes aegypti*, 135 GPCRs have been separated into four classes. In comparison among genomes of *Ae. aegypti*, *An. gambiae*, and *D. melanogaster* suggest conservation of GPCR-mediated neurological processes across the Diptera (Nene, 2007). According to the whole genome sequence of *Cx. quinquefasciatus* (Arensburger, et al. 2010), 63 GPCRs have been identified by comparison with the genome of *Ae. aegypti*, *An.*

gambiae, and *D. melanogaster*. Forty eight GPCRs could pertain to rhodopsin-like class A. Thus, we hypothesize that the rhodopsin-like class has crucial functions in *Cx. quinquefasciatus*.

Cytochrome P450 genes are expressed in brain, gut and fat body in insects. Zhu, et al (2010) reported that a brain-specific CYP6BQ9 gene was overexpressed in the deltamethrin resistance strain of *Tribolium castaneum*, as well as involved in detalmethrin resistance. Furthermore, GPCR signal pathway may involve in insecticide resistance of mosquitoes (Liu, et al. 2007; Hu, et al. 2007; Sun, et al. 2012), and also an insect orphan GPCR DmXR was able to detect the plant insecticide L-canavanine to avoid the toxic substances in *Drosophila* (Mitri, et al. 2009). Since overexpressed P450 genes and mutation in sodium channel gene provide the increased detoxification and target sit insensitivity, respectively, in response to insecticides, the up-regulation of the GPCR gene may involve in resistance via signaling transduction system that could regulate other insecticide resistance gene expression. However, the gene(s) regulation pathway is still unclear. Consequently, the characterization of gene interactions and regulation in resistance is fundamental for achieving an understanding of the complex processes responsible for resistance.

In current study, we investigated that one rhodopsin-like GPCR gene was significantly overexpressed in resistant mosquito, *Cx. quinquefaciatus*, and the up-regulation was gradually increased in mosquito following permethrin selection. Moreover, the up-regulation of the rhodopsin-like GPCR gene ~200 fold in larva higher than adult was associated with the difference of permethrin resistance ratio between larva and adult in this species (Li, et al. 2010). These results indicated that up-regulated GPCR gene may involved in the development of insecticide resistance in *Culex* mosquitoes. Transcriptional up-regulation of genes in resistant insects appears to be a common determining event in the development of resistance revolution in

insects (Liu, et al. 2007). Furthermore, the rhodopsin-like GPCR gene was up-regulated in HAmCq^{G8} mosquito through the induction mechanisms. Taken together, both the constitutive and induction up-regulation of rhodopsin-like GPCR gene indicated the functional importance of this gene that may play a key role in mosquito resistance to insecticides. However, the regulatory pathways of GPCRs on regulation of P450 gene expression in insects are unknown. We propose that the enzyme systems, such as adenylyl cyclases, protein kinases, and phosphorylases, which are involved in the GPCR signaling pathways in other organisms (Steinberg, et al. 2001), may play similar roles in the regulatory pathways of the rhodopsin-like GPCR on the regulation of P450 gene expression in mosquitoes. AMP-active protein kinase regulated-signal transduction pathway has the regulation function on P450 gene expression in human (Hsu, et al. 2011), and also cAMP-active protein kinase has crucial role on P450 expression in mouse and rat cell (Deb, et al. 2011). Our functional study focused on the role of up-regulated rhodopsin-like GPCR gene in the regulation of resistance related-P450 gene expression through protein kinase involved-signal transduction pathway in resistant mosquitoes.

The initially functional study revealed that a decreased in expression of the rhodopsin-like GPCR in rhodopsin-like-ds-RNA injected larvae and adults of HAmCq^{G8} and larvae of MAmCq^{G6}, which caused decreased tolerance to permethrin. The surprising evidence that four P450 genes, *CYP9M10*, *CYP6AA7*, *CYP9J40*, *CYP9J34*, which were overexpressed and induction in resistant mosquito strains, and one PKA gene were decreased expression in rhodopsin-like-dsRNA-knockdown mosquitoes, indicating the pivotal role of regulatory pathway of the rhodopsin-like GPCR gene in insecticide resistance of *Culex* mosquitoes. However, knockdown of the rhodopsin-like GPCR gene mosquitoes has not caused the decreased permethrin resistance, the PKA and P450 gene expression in adults of MAmCq^{G6} suggesting the probably no function of

this rhodopsin-like gene in insecticide resistance of MAmCq^{G6} adults. Furthermore, knockdown of the PKA gene has affected the decreased expression of 4 P450 genes and resistant tolerance to permethrin in HAmCq^{G8} and 2 P450 genes in MAmCq^{G6}, indicating that the PKA gene was not only involved in the rhodopsin-like GPCR regulatory pathway but also in permethrin resistance by regulating P450 gene expression. In the knockdown of PKA in MAmCq^{G6} mosquitoes, 2 P450 genes, CYP9M10 and 6AA7 haven't shown the decreased expression, suggesting that the function of this PKA gene in MAmCq^{G6} may be different to HAmCq^{G8}. To further confirm our functional study of the rhodopsin-like GPCR gene, we used the transgenic expression of the rhodopsin-like GPCR gene in *D. melanogaster*, which successfully exploited the function of rhodopsin-like GPCR gene in insecticide resistance. The transgenic fruit fly with rhodopsin-like GPCR expression showed high tolerance to permethin. We examined the expression level of five fruit fly P450 genes, which were involved in insecticide resistance in fruit fly. The striking test result showed that three P450 genes, *CYP12d1*, *CYP6a8*, *CYP6a9*, were increased expression level in the rhodopsin-like GPCR transgenic lines, suggesting that the rhodopsin-like GPCR gene was involved in insecticide resistance via regulating the P450 gene expression, which increased the detoxification ability to insecticide, in turn, the up-regulation of rhodopsin-like GPCR play a crucial role in metabolisms on insecticide in insect. The primary goal of this study was to investigate whether insecticide resistant insects may be uniquely resistant to insecticide due to their ability to mount an adequate cellular response when challenged with insecticide by regulating the up-regulation of other insecticide resistance genes, which, in turn, may significantly diminish toxicological effects of the insecticides to these insects. It also appears that the induction of gene expression may reflect a good compromise between energy saving and adjustment to a rapidly changing environment.

Cytochrome P450 expressed in insect play very important role for insect development by action of hydroxylation ecdysone at carbon 20, which is the primary molting hormone (Feyereisen, 1999, Petryk, et al. 2003). Furthermore, a mosquito sterol carrier protein-2 (AeSCP-2) has been shown to aid in the uptake of cholesterol, which is the source of ecdysone synthesis, in mosquito cells (Blitzer, et al. 2005). AeSCP-2 inhibitors (SCPIs) are known to compete with cholesterol for binging to AeSCP-2 (Kim et al. 2005). SCPIs are not only exhibit larvicidal activity against mosquito species such as *Aedes aegypti* (L.), *Culex pipiens pipiens* L., *Anopheles gambiae* s.s., *Culex restuans* Theobald, and *Aedes vexans* (Meigen) (Kim et al. 2005, Larson et al. 2008), but also have valuable synergistic effects tested on permethrin insecticide toxicity in *Cx. quinquefasciatus* (Li et al, 2009). It is therefore possible that cholesterol metabolism may be linked in some way to the pathways that influence to some extent the ability of mosquitoes to develop resistance. However, the relationship with these pathways remains unclear. Cholesterol can modulate membrane receptor function such as the nicotinic acetylcholine receptor and GPCRs by either altering protein conformation or changing the biophysical properties of the membrane lipid bilayer, and more evidence showed that the association of cholesterol and rhodopsin-like (Class A) affected the downstream in cell (Oates, et al. 2011). Thus, we hypothesize that SCPIs inhibit the cholesterol uptake by cell, in turns, the rhodopsin-like GPCR could obtain high level activity, which play a crucial function in insecticide resistance in *Culex* mosquitoes. In our future study, we will focus on all the overexpressed GPCR gene in the regulation of resistance related-gene and investigate the GPCR and GPCR signal pathway in insecticide resistance, which will likely shed new light on understanding molecular mechanisms of resistance development regulation and help us designing new strategies, such as novel insecticide, to control mosquito and its borne diseases.

Acknowledgment

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Chapter 9 Characterization of G-protein-Coupled Receptor Related Genes in Insecticide Resistance of Mosquitoes, *Culex quinquefasciatus*

Abstract

G-protein-coupled receptors and G-protein regulated signal transduction pathways play diverse and pivotal roles in physiological pathways in insects including development and reproduction. To characterize the functions of GPCRs in insecticide resistance of mosquitoes, we tested 120 GPCR-related genes in resistant and susceptible mosquitoes, *Culex quinquefasciatus*. Four up-regulated GPCR-related genes and 13 down-regulated GPCR-related genes were determined in larva and adult of HAmCq^{G8} and MAmCq^{G6}, respectively. To further demonstrate the function of the up-regulated GPCR-related genes in insecticide resistance of mosquitoes, we knockdown an up-regulated GPCR gene in HAmCq^{G8} and 3 in the MAmCq^{G6} strain by using the RNAi, showing that knockdown of these 4 GPCR-related genes caused the decreased resistance to permethrin. Since GPCR signal pathway regulates protein kinase activity, which was involved in P450 gene expression. We also tested the expression of cAMP-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK), and 4 P450 genes in the GPCR-related gene knockdown mosquitoes, indicating the involvement of 3 PKA and AMPK genes in the regulation of P450 gene expression through the GPCR signaling pathway. However, a GPCR-related gene, which was up-regulated in adult of MAmCq^{G6}, has no correlation with these 4 P450 gene expression, indicating there may be either some other pathways involved in insecticide resistance or its regulatory function in other resistance-related P450 gene expression. Our studies not only

determine the potential role of the GPCR-related genes in insecticide resistance, but also illuminate new strategies to control the insects, especially the resistant ones.

Key words: Insecticide resistance, G-protein-coupled receptor, Protein kinase, Cytochrome P450s, *Culex quinquefasciatus*

Introduction

G-protein-coupled receptors (GPCRs) are largest super family of cell-surface molecules, which are integral membrane proteins with the presence of seven α-helical hydrophobic domains and divided into several subfamilies based on the sequence and function similarity (Stevens, R. C. et al. 2013), which carries out specific physiological function (Tadevosyan, et al. 2012). The function or dysfunction of GPCR may cause various cellular response changes. Thus, GPCRs as major drug targets for wide range of disease affect many drugs on the market and play critical role in medicine development in human (Insel, et al. 2012; Shoichet, et al. 2012). GPCRs are activated by conformation changes of GPCRs resulting from different ligand binding (Choe, H., et al. 2011; Zocher, M., et al. 2012). In insects, several GPCRs can regulate physiological pathways (Caers, et al. 2012) and affect insect behavior (Mitri, et al. 2009), reproduction (Simonet, et al. 2004), development (Hauser, F. et al. 2006; Bai, et al., 2011), and metabolism (Spit, et al. 2012). Because of the striking role of GPCRs and whole genome sequence determined in diversity species, 948 GPCRs in human that encode >2% of the total genes of human genome (Takeda, et al. 2002) and ~1000 olfactory receptors in mice (Zhang, et al. 2002) have been reported. Moreover, GPCRs also have been characterized in various insect species, revealing 276 GPCRs in *Anopheles gambiae* (Hill, et al. 2002), 135 non-sensory and opsin GPCRs in *Aedes aegypti* (Nene, et al. 2007). In the red flour beetle, ~70 neurohormone GPCRs were found (Hauser, et al. 2008; Tribolium genome sequencing consortium, 2008). There are 56

neurohormone receptor genes in honey bee *Apis mellifera* and 69 in *Drosophila melanogaster* (Hauser, et al. 2006). The human body louse, *Pediculus humanus humanus*, has 107 GPCRs in whole genome (Kirkness, et al. 2010). GPCRs are subdivided into 6 families based on their sequence similarity and structure features (Guo. et al. 2006; Xiao, et al. 2011). The *Drosophila* GPCRs have been subdivided into four main families including rhodopsin-like, secretin-like, metaboropic glutamate-like and atypical 7TM proteins (Brody, et al. 2000). The Class A, rhodopsin-like receptors, is largest family of GPCRs, which could be a transducer transfer a striking and extraordinary range of ligands including light, peptides, lipids, nucleotides, etc. in both vertebrates and invertebrates (Müller, et al. 2012). Structural study of GPCR is crucial to characterize the function of GPCRs and GPCR-pathways (Rosenbaum, et al. 2009). However, the low efficient transport and insertion of GPCR 7TM structure into the plasma membrane and the toxic effect on host cell causes the low success rate in functionally structural studies (Lundstrom, 2005a). The gene expression profile of GPCR in human and mouse indicated that over 90% of GPCRs were overexpressed in the brain, and most GPCRs were unique (Vassilatis, et al. 2001). The transcriptionally up-regulated gene expression is primary for insect to adapt the changes of environment, such as developed resistance to insecticides. Furthermore, multiple gene interaction and regulation play pivotal role in insecticide resistance (Feyereisen, 1999; Scott, 1999; Hemingway, et al. 2004; Liu, et al. 2007). GPCR and G-protein signal pathway could regulate the protein kinase activity (Dorsam, et al. 2007), which has been determined in regulation of P450 gene expression in human cell (Rencurel, et al. 2005; Hsu, et al. 2011) and in rate tumor cell (Deb, et al. 2011). Interestingly, some up-regulated GPCRs have been determined in the resistant *Culex* mosquitoes (Liu, et al. 2007; Hu, et al. 2007), which is a primary vector of West Nile virus, eastern equine encephalitis, Saint Louis encephalitis, and lymphatic filariasis

pathogens inhabiting tropical and subtropical regions worldwide (Sardelis, et al. 2001; Jones, et al. 2002; Reisen, et al. 2005). Thus the amazing and wide function of GPCRs in cell biology and the blank of GPCRs in insecticide resistance are attracted our interesting. To address the potential function of GPCR and GPCR-related genes in insecticide resistance of the mosquito, *Cx. quinquefasciatus*, we characterized the expression level of GPCR and GPCR-related genes thruogh susceptible strain to highly resistant *Culex* mosquitoes. Furthermore, we used double-stranded RNA interference (RNAi) technique to silence the up-regulated GPCR and GPCR-related genes in resistant mosquito strain to investigate the function of these GPCR and GPCR-related genes in insecticide resistance by regulating the resistant P450 gene expression. Our studies showed the interfering of GPCR and GPCR related genes might provide a new opportunity for resistance prevention.

Materials and Methods

Mosquito strains

Five mosquito strains of *Cx. quiquefasciatus* were used in this study. Two field strains, namely, HAmCq ^{G0} and MAmCq^{G0}, collected from Madison County and Mobile County, AL, respectively, spanning >600 km among the two counties; HAmCq^{G8}, the eight generation of permethrin-selection HAmCq^{G0} offspring; MAmCq^{G6}, the sixth generation of permethrin-selected MAmCq^{G0} offspring; and S-Lab, an insecticide susceptible strain from Dr. Laura Harrington (Cornell University, Ithaca, NY). All the mosquitoes were reared at 25 ± 2°C under a photoperiod of 12: 12 (L: D) h and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University, Auburn, AL).

RNA extraction and cDNA preparation

Total RNAs were extracted from 4th instar larvae and 2-3 day-old adults (without blood feeding) of each mosquito population for each experiment by using the acidic guanidine thiocyanate-phenol-chloroform method (Liu, et al. 1997). The DNA was free from total RNA (5ug) of each mosquito sample using DNase (TURBO DNA-free, Ambion). The DNA-free total RNA was reverse-transcribed to synthesize cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche) and a random hexamer primer following manufacturer's instructions. The quantity of cDNA was measured by a spectrophotometer prior to qRT-PCR. Each experiment repeated six times with RNA preparation and cDNA synthesis.

Quantitative real-time PCR (qRT-PCR)

Each qRT-PCR reaction was run in 3 replicates, in a total reaction volume of 25 μL, containing a 2x SYBR Green master mix, each GPCR-related gene specific primer pair designed according to each of the GPCR-related gene sequences (HYPERLINK "http://cquinquefasciatus.vectorbase.org/", Table 9.1 with accession number for each GPCR-related gene) at a final concentration of 3-5 μM, and a 1μg cDNA template from each mosquito sample. A 'no-template' negative control was performed. The reaction cycle consisted of a melting step of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. Specificity of the GPCR-related gene PCR reactions was assessed by a melting curve analysis using Dissociation Curves software (Wittwer, et al. 1997). Relative expression level of GPCR-related gene was calculated by the $2^{-\Delta\Delta CT}$ method using SDS RQ software (Livak, et al. 2001). The 18S ribosome RNA (rRNA) gene was used as endogenous control, because the preliminary assay shown that the 18S rRNA gene remained constant in different tissues and in all strains (Aerts, et al. 2004; Liu, et al. 2007). Each experiment was repeated 3 times with 3 independently isolated RNA samples from each mosquito strain. The statistical significance of

the gene expression was calculated using a Student's t-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons, a value of $P \leq 0.05$ was considered statistically significant. Significant up-regulation or down-regulation was determined using a cut-off value of a "2-fold change in expression" (Strode, et al. 2008)

Table 9.1. Oligonucleotide primers used in qRT-PCR reactions for amplifying the GPCR genes

Transcript ID[a]	Accession No.	Gene[b]	Forward Primer (5′ to 3′)[c]	Reverse Primer (5′ to 3′)[c]
CPIJ013660	XM_001864055	octopamine receptor oamb (rhodopsin-like receptor activity)	CGAACCCACATGGGCAAGAACTTT	TATACAGAACCCGGACAGCAGGTT
CPIJ015294	XM_001865540	dopamine receptor, invertebrate (rhodopsin-like receptor activity)	TCTACAAGGTGAACAAGGCGTCCA	ATGATGTTGCACGTGAAGAAGGGC
CPIJ019015	XM_001869366	octopamine receptor oamb (rhodopsin-like receptor activity)	TCAGTATGGAAATCACACGCCGGT	TCGTCGAAACCGGGAAAGGAAGAT
CPIJ018698	XM_001869052	ribosomal protein S6 (rhodopsin-like receptor activity)	ACCACATTCCGGATTGCTGATTGC	CAGGATGCACAGTTTGCGGATGTT
CPIJ001044	XM_001842661	muscarinic acetylcholine receptor gar-3 (rhodopsin-like receptor activity)	CGCTGTTGTGTGATTTGTGGCTCT	TTGGTTCGCCAGCTGCGATATTTG
CPIJ008330	XM_001849662	conserved hypothetical protein (rhodopsin-like receptor activity)	TGGTCAGCATGATCCCGGACATTT	AATCGTGTAAACCGAGTCCTGGCT
CPIJ	XM	conserved	AGCTGCAGAACGTCACCAACTAC	TGAACCCAAACGGCCAGTAA

011883	_001863239	hypothetical protein (rhodopsin-like receptor activity)	T	CCTA
CPIJ003188	XM_001844724	g-protein coupled receptor (rhodopsin-like receptor activity)	ATACCAACTTCCGGGATGCCTTCA	ACGGGACGATTTCTGTTGTGGAGA
CPIJ016039	XM_001866016	conserved hypothetical protein (rhodopsin-like receptor activity)	CAAGAACTTCAAGCACAGGCCGTT	TGAAACGACCGTAATTTCCGCTGC
CPIJ015747	XM_001866028	5-hydroxytryptamine receptor 1 (rhodopsin-like receptor activity)	GCAACGCGACATTCCCAAGTTGTA	ACCACACTGAGTGCGTCTGAAGAT
CPIJ014487	XM_001864681	beta adrenergic receptor (rhodopsin-like receptor activity)	CATCTTTGGCAACCTGCTGGTCAT	TGACAGCGTACGGCTTGTTCACTA
CPIJ013218	XM_001863307	tyramine/octopamine receptor (rhodopsin-like receptor activity)	TAAGACTAATCCAACCAGCGCCGT	GCGGAACGATTGTTTCATGCGACT
CPIJ003421	XM_001845139	conserved hypothetical protein (rhodopsin-like receptor activity)	ATTGCCAAGAACCTGATTGCGAGC	AGCAAACAGGTGAAGAATGCGAGC
CPIJ014065	XM_001864128	neuromedin-U receptor 1 (rhodopsin-like receptor activity)	TCGAGCTGTCGACGTTTGTGTTCT	CGAGCGCAACTTCAACCCAATCAA
CPIJ0055	XM_001	sulfakinin receptor (rhodopsin-like	TACACATACGTCAGCAGCAACGGA	AGCAACAGCACAGACAGAGGAAGA

74	847763	receptor activity)		
CPIJ016281	XM_001866703	sulfakinin receptor (rhodopsin-like receptor activity)	GCGCACCATCACCAACCTATTTCT	TGCCACTGAAGAGGCTTGCAGATA
CPIJ013069	XM_001863157	neuropeptide Y receptor type 2 (rhodopsin-like receptor activity)	ACGTGGTCTTCCGGAATAAAGCCA	CGTGTGACAAATGAGCTTGCCGAA
CPIJ007187	XM_001849241	FMRFamide receptor (rhodopsin-like receptor activity)	TGTTCATCTACCTGCTGCCGTTCA	GCAGAAACACAATGACCACGCAGA
CPIJ006984	XM_001848696	g-protein coupled receptor (rhodopsin-like receptor activity)	CGAAGCTGAACTTGACGTTGGACA	TTTGCGGTTGTTGCTGTACTGCTC
CPIJ018265	XM_001869016	g-protein coupled receptor (rhodopsin-like receptor activity)	GCCTAGAGGCGATACAAATTGTTG	CACCATGTGGCGCTGTTGTTGATT
CPIJ014670	XM_001865310	endothelin B receptor (rhodopsin-like receptor activity)	AGAATACCAGTGTAGCGGTGGCAA	CCAAACCAGCAACACCACGATCAA
CPIJ012071	XM_001862415	myokinin receptor (rhodopsin-like receptor activity)	TGTGTGCGGACGGAGAGGATAAAT	ATCCATATCACCAGCGAGTTGCCT
CPIJ018504	XM_001868775	neuropeptide Y receptor (rhodopsin-like receptor activity)	ACAGCTGCTACAACCCGATCATCT	GCTGGTGCTAACATCACTTCGCTT

CPIJ 010469	XM_001854800	somatostatin receptor type 5 (rhodopsin-like receptor activity)	ACTCGGTGTACGAGATTTGCCCAT	TATCAGAAAGCACTGGTCCGCGAT
CPIJ 011191	XM_001859633	somatostatin receptor (rhodopsin-like receptor activity)	TATGCCGACCACGTACACACTGTT	TGTGCATCGTGGCAATCAAGAACG
CPIJ 003158	XM_001844694	G protein-coupled receptor (rhodopsin-like receptor activity)	ACCGCGAAACCTCCTCACAGTAAT	CTGATTGCCGTCAGCGGCATATTT
CPIJ 011716	XM_001862544	g-protein coupled receptor (rhodopsin-like receptor activity)	TTGCCCTGGGTATCAATCTTCCGT	TGGCGAACAGAAAGTACCAGCTCT
CPIJ 016695	XM_001867185	g-protein coupled receptor (rhodopsin-like receptor activity)	TTGCCCTGGGTATCAATCTTCCGT	TGGCGAACAGAAAGTACCAGCTCT
CPIJ 006269	XM_001847618	cardioacceleratory peptide receptor (rhodopsin-like receptor activity)	GCATCATTCCAAAGGCGAAGGTGA	TTGGTCTGCGTTTCCGGAATTTGG
CPIJ 007712	XM_001849183	leucine-rich transmembrane protein (protein-hormone receptor activity)	GTTTCGGTTGGTTGCCCATTCTGT	ATTGTTTCGTGGCCACTTCGGATG
CPIJ 011619	XM_001861641	leucine-rich transmembrane protein (protein-hormone receptor activity)	AGCCGGAAATAGCATAAGCTCCCT	TGAAGGCGTCCTGGTGGATAAAGT
CPIJ 0011	XM_001	gonadotropin-releasing hormone	TCTGTCCGTGGTGATTACGCTGTT	GCGGAATCATGATGAATGCCACCA

99	8428 16	receptor (rhodopsin-like receptor activity)		
CPIJ 0050 00	XM _001 8465 89	opsin-2 (rhodopsin-like receptor activity)	TGCTGGCGTTGATCTACTTTGTGC	AAGGCATTCACCAGAAACAT CGGC
CPIJ 0092 46	XM _001 8511 05	ultraviolet-sensitive opsin (rhodopsin-like receptor activity)	AATCGTAAGCCACGTCGTGAACC A	CCACGAAGCCACAAACAGGA AACA
CPIJ 0134 08	XM _001 8635 11	opsin-2 (rhodopsin-like receptor activity)	CAAGCATCGCTGTTTGTGCTGCTA	AACACCATTGGAATCGCGTA AGCC
CPIJ 0040 67	XM _001 8456 45	opsin-1 (rhodopsin-like receptor activity)	TCGCACTCGTTACGATCTCGCTTT	ACTTGGGATGACTGATGCCG TACA
CPIJ 0120 52	XM _001 8621 30	opsin-1 (rhodopsin-like receptor activity)	TCAAGATCCTGATGGTGTGGGCTT	ACACGAAGATGGCGTAGACG ATGA
CPIJ 0115 71	XM _001 8621 63	opsin-1 (rhodopsin-like receptor activity)	TCAACAGACCAGCGCTGAGATCA A	TGATTCCGTACACGATCGGG TTGT
CPIJ 0115 73	XM _001 8621 65	opsin-1 (rhodopsin-like receptor activity)	ATGTGCCAGGTTTACGCCATGTTG	GAGCAGCGCATTGTTGAAGG TCAT
CPIJ 0115 74	XM _001 8621 66	rhodopsin (rhodopsin-like receptor activity)	TCTACTTTGCGCCTCTGCTGATGA	CAGCTTCATTTCGGTGCTGGT GTT

CPIJ 011983	XM_001862303	conserved hypothetical protein (rhodopsin-like receptor activity)	GAGCCTGCCCAACTTTAAGCCAAA	ATGATGCAGAACGGCAACCAGAAC
CPIJ 013056	XM_001863451	rhodopsin (rhodopsin-like receptor activity)	CGATGGTGCAGATCTTTGGCGTTT	CAGTGGCGCAAAGTACACGAACAT
CPIJ 020021	XM_001870251	rhodopsin (rhodopsin-like receptor activity)	ACCTGTTAGTGGTCAATCTGGCCT	TACAGCCGAACAGTGATCCACACA
CPIJ 011419	XM_001861603	ultraviolet-sensitive opsin (rhodopsin-like receptor activity)	TGTTAACTCCGCTGGGTTCGATGA	ACTGCGAGTGGTTCATCTCCTGTT
CPIJ 014334	XM_001864516	opsin (rhodopsin-like receptor activity)	TCATCCTGCTGAACCTGGTGTGTT	TATGCCCAGCAGGGACATGAAGAA
CPIJ 015116	XM_001865344	wd-repeat protein (rhodopsin-like receptor activity)	CCAGCTGGTTCTGCTGCATTTGAT	TGTCCACACCCGTGAACAGAATGT
CPIJ 000143	XM_001841761	g-protein coupled receptor (rhodopsin-like receptor activity)	GTGCTGACAAGACGTGAAATGCGT	ACACTGCGTAGGGCACATAATCCA
CPIJ 006507	XM_001848139	g-protein coupled receptor (rhodopsin-like receptor activity)	ACTTCACCCAGATACTGCACACCA	TCGTGAAGACAAAGTACGTCGGCA
CPIJ	XM	conserved	AGCTCCAGTTGGGAATGAACGAG	ACGCTGATGATCTTGCTGTTT

011547	_0018618 35	hypothetical protein (G-protein coupled receptor activity)	A	GCG
CPIJ 011549	XM _0018618 37	conserved hypothetical protein (G-protein coupled receptor activity)	AGTGTTCCCACATGACGAGCTTCT	TCCAAACAAGGACAACAAGCAGCC
CPIJ 017258	XM _0018675 08	neural-cadherin (calcium ion binding, homophilic cell adhesion)	ATCATGCCACAGATTCCTGGACCT	GACATCCCGCAATTGTTCCGTTGT
CPIJ 011559	XM _0018621 51	calcitonin receptor (Secretin-like)	TCTGCTGCACTATCTGATGCTGGT	AGTTGTTCCGGAAGGTTGCGTAGA
CPIJ 014419	XM _0018648 61	calcitonin receptor (Secretin-like)	GCGAAACTGAACGAATCCGTCGAA	TGGTGTCGAATCCGAAGACGAACT
CPIJ 009749	XM _0018510 12	calcitonin receptor (Secretin-like)	ACAGCACTGGAATTTGCCTTGTGG	ACCGCGAATGGAGAGGTAAACACT
CPIJ 008822	XM _0018506 59	conserved hypothetical protein (diuretic hormone receptor activity)	TAGAATGCTCCTGGATGCGGGAAT	AGCTTCGTGATCAGGACCCACATT
CPIJ 013929	XM _0018643 08	conserved hypothetical protein (G-protein coupled receptor activity)	TGAGGCCGCATCGGTTACTATGAA	TACACGGAATGAAGTCCCAACCCA
CPIJ 0139	XM _001	conserved hypothetical	TAACGGTTATACCATGCTCGGCGT	TGTTCAGCGTCACACAGATGGTCA

32	864311	protein (G-protein coupled receptor activity)		
CPIJ008641	XM_001850157	gamma-aminobutyric acid type B receptor (GABA-B receptor activity)	TGCAGGAGAACGAGTACTGCCAAT	ACATACCGACGTGTTTGGAGTCGT
CPIJ002466	XM_001844023	gamma-aminobutyric acid type B receptor (GABA-B receptor activity)	GTTGCTTCTGTTGGACGCTTGTGT	GCGTACAACGTTCCAAGCCAACTT
CPIJ008111	XM_001849791	conserved hypothetical protein (GABA-B-like receptor activity)	AGAAGCAGTGTTGCTGGGTTTGTG	TCGATCGGGATTTGGAGGCATTCT
CPIJ001702	XM_001843190	conserved hypothetical protein (rhodopsin-like receptor activity)	GCGCGGTTTCAACGACTTCAATCT	GAACGTCCGAGAACTGGAAGTCAA
CPIJ016970	XM_001867057	uridine cytidine kinase i (rhodopsin-like receptor activity)	GAACAGTTTCGTCATTGCGGTGGT	GCGATGGCGGTTATCAAGCGATTT
CPIJ007717	XM_001849188	conserved hypothetical protein (rhodopsin-like receptor activity)	TTCGTTGTATGGCTGGTGGCACTT	TCATCGGAATGAAGAAGGAGCCCA
CPIJ014671	XM_001865311	substance P receptor (rhodopsin-like receptor activity)	TGACGGCCATCGTATTCCCAAAGA	ACGGTCGTTCCTTGTAGTTTCGGT
CPIJ000410	XM_001842028	smoothened (G protein-coupled receptor activity)	AGCGATTGGGAAGATCGGAAAGGA	ATCGTAATCGTCAACACCAGCGGA

CPIJ 0079 73	XM _001 8498 75	conserved hypothetical protein (rhodopsin-like receptor activity)	AGATCGAATGGAACGGGCAGCTT A	TCGCATCGCTTTGAACAGCA GAAC
CPIJ 0117 57	XM _001 8619 22	5HT-dro2A receptor (rhodopsin-like receptor activity)	ACTCTCCACCATTTCTAACGGCCA	TCCTCGGCGAAGCTTGTAAC TTGA
CPIJ 0200 07	XM _001 8703 41	conserved hypothetical protein (G-protein coupled receptor activity)	TACTGTTCCTGAGAAATCGCCGCT	CACGCCCAACACAAACGAGA AGTT
CPIJ 0111 05	XM _001 8614 25	pyrokinin receptor (rhodopsin-like receptor activity)	TATCGAGTGCAAGCGATCCTCCA A	GTGCTCCATTGTGAGGCAAC CAAA
CPIJ 0111 06	XM _001 8614 26	pyrokinin receptor (rhodopsin-like receptor activity)	GTTCAATCAGGTGGCCTGCATCAT	TTCGAACTTGCCGAAATGGG TGAC
CPIJ 0094 59	XM _001 8513 55	conserved hypothetical protein (rhodopsin-like receptor activity)	TCGCCTTGTCGTACCTGTTTGGAT	GGTCGTTTGTGAGCAGGCAT TTCA
CPIJ 0191 11	XM _001 8695 07	conserved hypothetical protein (G-protein coupled receptor activity)	ACGATCGAGGCAGAACAATCACG A	TGGAGAAGTCGAACAGCGGT TTCT
CPIJ 0000 02	XM _001 8416 20	conserved hypothetical protein (GABA-B-like receptor activity)	TGATGACCCTAGCTTTCCTGCCAA	TTCCGGACTGGCTTCATTAA GGGT
CPIJ	XM	Cap2b receptor	CGGCGACTCATGATGCTTCTTGTT	TTGATCAGCTCCATCAGGTT

018293	_0018683 97	(rhodopsin-like receptor activity)		GGCT
CPIJ015979	XM_00186612 0	conserved hypothetical protein (rhodopsin-like receptor activity)	ACCAAACGACGACGACGATGAAGA	GGATGTGATGGTTGCCAATGTGGT
CPIJ019916	XM_00187015 6	conserved hypothetical protein (rhodopsin-like receptor activity)	TGCTAGCCATCTTCACAGTGGTCA	TTGTTGAAGTGGGTGTTGCTGCTG
CPIJ014753	XM_00186511 8	conserved hypothetical protein (rhodopsin-like receptor activity)	CCAGAGCAAAGAGGAGCAAGTCAA	TCGAGATGAACGTCAACGTGAGCA
CPIJ002213	XM_00184383 0	Odorant receptor 94b (olfactory receptor activity)	ACCTGCTTATTTCACGTTGGCAGC	TCAAACGCCTGCAGTGTGTGAAAG
CPIJ003436	XM_00184515 4	conserved hypothetical protein (G-protein coupled receptor activity)	ATTGTGTACTTTGTGCTGCCCACG	TGCTGAACTCGGTGAAGATACGCA
CPIJ003422	XM_00184514 0	conserved hypothetical protein (rhodopsin-like receptor activity)	TGGTGCCCATTGTCATCCTCAAGA	ACCCATACTTCGGGTGGTGAGTTT
CPIJ003420	XM_00184513 8	conserved hypothetical protein (rhodopsin-like receptor activity)	ACGGCATCAACAAATCTGAGCAGC	GTTGTGGCGTTGATGAAGCAACCT
CPIJ013535	XM_001	GABA-B receptor (GABA-B receptor activity)	TGTGGGATAGTGGTGGCATTAGCA	TGGCTTGGCATACCTTGGGATAGT

	863623			
CPIJ013536	XM_001863624	GABA receptor (GABA-B receptor activity)	TTATGTTACGGCGCATCTAGCCCT	ATGAACACTTCTTCGGCTTGCTGG
CPIJ016679	XM_001866877	conserved hypothetical protein (rhodopsin-like receptor activity)	TGGCGGTGTACTCACAAGAGGTTT	ATGTTCCCGAGCAGCGATACGATT
CPIJ011717	XM_001862545	g-protein coupled receptor (rhodopsin-like receptor activity)	ATGACTTTGATACCGCTGTGGACG	ATCAGCGTACGCCAGATTCACGAT
CPIJ016293	XM_001866977	conserved hypothetical protein (G-protein coupled receptor activity)	TTCCGGTCCAACACGAACCTCAA	TTTCACGTCCCGCTCCTTGTACTT
CPIJ007715	XM_001849186	G protein-coupled receptor (rhodopsin-like receptor activity)	ATCTTCCCACAATCCTCTGCTCGT	TCAGTGAAGCTGCCACGTTTTGTA
CPIJ007716	XM_001849187	G protein-coupled receptor (rhodopsin-like receptor activity)	TGGCTTGGGTTATCTGGGACGAAA	TCGAGCCGAACTCAGAAACTCGTA
CPIJ018158	XM_001868532	frizzled-3 (G-protein coupled receptor activity)	TCCGGAAGCGTCGAGAAGAACTTT	AGTTGTGGTGATGGTGTTGTTGCG
CPIJ007681	XM_001849511	frizzled (G-protein coupled receptor activity)	TGATCGATTCGTCTCGGTTCAGGT	CCTGCGTAATCGTCGATAGCATCT

CPIJ 0076 76	XM _001 8495 06	frizzled (G-protein coupled receptor activity)	TCTTCATGGCCCTGTACTTCTGCT	AATCCTTCAACTTTGCCCAGT GCC
CPIJ 0076 77	XM _001 8495 07	frizzled (G-protein coupled receptor activity)	CTTCTTCAGCGGCCTGTTCATTCT	AGAAGATCTTGCACATCTGG CGGT
CPIJ 0076 82	XM _001 8495 12	frizzled (G-protein coupled receptor activity)	CTTCTTCAGCGGTCTGTTCATCCT	CACCATCTGGGCGTTGTATTT GGT
CPIJ 0088 20	XM _001 8506 57	diuretic hormone receptor (diuretic hormone receptor activity)	CACGCAGAAACCGGAAGAAGGTT T	TCGACGGATCCAAAGCCGTG TAAA
CPIJ 0006 51	XM _001 8423 62	dopamine receptor (rhodopsin-like receptor activity)	GACGTTCGCCGGTGTCAATGATTT	TGAGCGGATCCTTGATGTGG ATGT
CPIJ 0166 78	XM _001 8668 76	corazonin receptor (rhodopsin-like receptor activity)	TTCTTCGGGATGTCCAACAGCCTA	TGTTGCTTGTACTGATTGTGC CGC
CPIJ 0195 66	XM _001 8697 64	G-protein coupled receptor (rhodopsin-like receptor activity)	TGGTGATAGCGTTCTTCCTGTGCT	TGTACGTCAGGATTTCGAAC GCGA
CPIJ 0118 81	XM _001 8632 37	conserved hypothetical protein (rhodopsin-like receptor activity)	AACCTTCAATTCCCGGAGTGACCT	TGCAGAACAGCACATCCAGA CAGA
CPIJ	XM	CCK-like GPCR	TCATCAACACGATGGCCCTGTTCT	AAGCACCGAAACAGGTTCAG

016282	_0018667 04	(rhodopsin-like receptor activity)		GAAG
CPIJ 006268	XM _001847617	cardioacceleratory peptide receptor (rhodopsin-like receptor activity)	TCTCGACGCACTTTGTCAGGACTT	ATTGAATCGCTGCTGTGCTGGTTC
CPIJ 003873	XM _001845489	beta adrenergic receptor (rhodopsin-like receptor activity)	GCATCGAACGGTTGGCTTCATGTT	ATGATTGCGTACGCCTTGTTGACC
CPIJ 014488	XM _001864682	beta adrenergic receptor (rhodopsin-like receptor activity)	TCAACTCGACGCTCAATCCGCTTA	TTTGGAGAAGCAGGGCAGTAGCGAT
CPIJ 014409	XM _001864626	alpha-2 adrenergic receptor (rhodopsin-like receptor activity)	GCGGTGGTGAAACCGTTGAAGTTT	GCCGCGTTATTGTTCTTGTTCCGA
CPIJ 011118	XM _001861441	allatostatin receptor (rhodopsin-like receptor activity)	CTGCCTTCCACATTGCGTTCTTCT	TTGCTCTTCTTCGACTCTGCGGAA
CPIJ 016163	XM _001866351	allatostatin receptor (rhodopsin-like receptor activity)	TCAGCTGAATCCAGACGAGGCAAA	ATGACCTGAATAGGGCACCAGCAA
CPIJ 013095	XM _001863362	allatostatin receptor (rhodopsin-like receptor activity)	TTACAACACCACAGCCATCCCAAC	AAGATGGGCACAATCCGTGAAACG
CPIJ 017622	XM _0018677	conserved hypothetical protein	TCCCGATGACCCTCATCACCATTT	CAGCCACGACAGCACAAACAGAAT

	14	(rhodopsin-like receptor activity)		
CPIJ000934	XM_001842552	conserved hypothetical protein (rhodopsin-like receptor activity)	ATCGGCCTATTCATCACGTGGCTA	TCAGCGGGTACAACACCACGATAA
CPIJ006942	XM_001848937	conserved hypothetical protein (rhodopsin-like receptor activity)	TGTGACCCAACGGGATCCAAACTA	TCTTGAAGAACACCAGCACCGAGA
CPIJ017421	XM_001867456	conserved hypothetical protein (rhodopsin-like receptor activity)	TCTGGTGATTAGCGCGAACCTGAT	TTTCACCGTAAGGCCAGCAGTGTA
CPIJ011756	XM_001861921	conserved hypothetical protein (rhodopsin-like receptor activity)	CAGCAGCAGTTGCAGCTTAAACCA	TGGTTGAGGGTTTCCTGGTCAGAT
CPIJ003683	XM_001845458	5-hydroxytryptamine receptor 2B (rhodopsin-like receptor activity)	AACAACAGCAACCATCAACCACCG	GATTTGGCGTGCTGGCAATGTTCA
CPIJ011755	XM_001861920	conserved hypothetical protein (rhodopsin-like receptor activity)	AGCCATCGATCGCCAAGAAGAAGA	ATCGCGATGATGAAGAACGGCAAC
CPIJ019013	XM_001869365	octopamine receptor oamb (rhodopsin-like receptor activity)	GCCAAAGTTCCAGAGTTCCAAAGT	CTGGAACTCAGAAGTCCTAACTCT
CPIJ019017	XM_001869368	conserved hypothetical protein (rhodopsin-like receptor activity)	TCAGCAGAAGGAGTACTTGACGGT	AAGGGCAACCAACACAGGATGAAC

CPIJ016091	XM_001866585	conserved hypothetical protein (rhodopsin-like receptor activity)	TCCCATGTAATCATCAGCCTGTGC	GCGTGATGATGGTGGCAAAGTACA
CPIJ016092	XM_001866586	adenosine A2 receptor (rhodopsin-like receptor activity)	TCATGATCTGCTGGATTCCGCTGT	ATGCTCATGATCAGATTGCGCAGC
CPIJ019039	XM_001869192	conserved hypothetical protein (GABA-B-like receptor activity)	TACGTACGGTCTGGAAATGGCAGT	TGAAGCAGCTCCAACAACTCCTGA
CPIJ000647	XM_001842358	conserved hypothetical protein (rhodopsin-like receptor activity)	TCATGGGCGTCTTCCTAGTTTGCT	AGATGATCGGATTGAACGCGGAGT
CPIJ000649	XM_001842360	dopamine receptor (rhodopsin-like receptor activity)	AATGGCGTCAAGTACGAAACGTGC	GCAGCAGTTGGACTGCAGTAGATT

Functional study of up-regulated GPCRs

Double-strained RNA (dsRNA) synthesis

Around 500-bp PCR product of the GPCR-related gene and a green fluorescent protein gene (pMW1650 and this gene is not normally expressed in *Cx. quinquefasciatus*) were generated complementary to cDNA sequence of target gene and a pMW1650 plasmid, respectively. There is T7 promoter sequence (5’-TAATACGACTCACTATAGGG-3’) appended the 5’ end of each PCR product (Table 9.2). dsRNA was synthesized from the PCR template with the opposing T7 promoter sequences by *in vitro* transcription, using the MEGAscrip T7 High Yield Transcription

kit. For dsRNA purification, phenol/chloroform extraction followed by ethanol precipitation method was applied.

Table 9.2. Oligonucleotide primers used in PCR and qRT-PCR reactions

Transcript ID[a]	Gene[b]	Primer name	Primer sequence
CPIJ019111	conserved hypothetical protein (G-protein coupled receptor activity)	dsRNA CPIJ019111 F	5’TAATACGACTCACTATAGCGGAGGGCAAGTGCATATAA3’
		dsRNA CPIJ019111 R	5’TAATACGACTCACTATAGTCAAAGACGTGTGGCTACTG3’
CPIJ007717	conserved hypothetical protein (rhodopsin-like receptor activity)	dsRNA CPIJ007717 F	5’TAATACGACTCACTATAGGTATGGCTGGTGGCACTT3’
		dsRNA CPIJ007717 R	5’TAATACGACTCACTATAGCCGTGAAATCCGGTGCT3’
CPIJ014419	calcitonin receptor (Secretin-like)	dsRNA CPIJ014419 F	5’TAATACGACTCACTATAGCCTCAAATGTGCCCGGATAA3’
		dsRNA CPIJ014419 R	5’TAATACGACTCACTATAGACGAAGAGGCCCTGAAATG3’
CPIJ014334	opsin (rhodopsin-like receptor activity)	dsRNA CPIJ014334 F	5’TAATACGACTCACTATAGGCCACCACCTACATCATCTATC3’
		dsRNA CPIJ014334 R	5’TAATACGACTCACTATAGCATCTTCAGCCGGTTCTTCT3’
CPIJ019111	conserved hypothetical protein (G-protein coupled receptor activity)	RT-RNAi CPIJ019111 F	5’CCATAAACTCCGCCCTTCTT3’
		RT-RNAi CPIJ019111 R	5’GCGTATTCATCTCGTTGGATTTC3’
CPIJ007717	conserved hypothetical protein (rhodopsin-like receptor activity)	RT-RNAi CPIJ007717 F	5’TTCGTTGTATGGCTGGTGGCACTT3’
		RT-RNAi CPIJ007717 R	5’TCATCGGAATGAAGAAGGAGCCCA3’
CPIJ014419	calcitonin receptor	RT-RNAi CPIJ014419 F	5’GAGCGGGTTTACGAAACGATA3’

	(Secretin-like)	RT-RNAi CPIJ014419 R	5’CTTGACTTGTGCGATCACCT3’
CPIJ014334	opsin (rhodopsin-like receptor activity)	RT-RNAi CPIJ014334 F	5’GGAAACGCAAACCCTGAAC3’
		RT-RNAi CPIJ014334 R	5’GACAATGATGTTGGTGTAGCTG3’
CPIJ018257	cAMP-dependent protein kinase catalytic subunit	Cx. APK18257F	5’ATACCGTGACTTGAAGCCGGAGAA3’
		Cx. APK18257R	5’AGAACACCTAATGCCCACCAGTCA3’
CPIJ019613	5'-AMP-activated protein kinase catalytic subunit alpha-2	Cx. APK19613F	5’TGGTGTGCTTCTGTGGGAGATCAT3’
		Cx. APK19613R	5’ATCATCCGTACACTGAGCTGGCTT3’
CPIJ000798	c-AMP-dependent protein kinase Catalytic subunit	Cx. APKC00798F	5’TTGATTGGTGGGCATTAGGCGTTC3’
		Cx. APKC00798R	5’AGCAGCTTCTTGACCAGGTCCTTT3’
CPIJ014218	P450CYP9M10	qRTP450-1CxF	5’ATGCAGACCAAGTGCTTCCTGTAC3’
		qRTP450-1CxR	5’AACCCACTCAACGTATCCAGCGAA3’
CPIJ010543	P450CYP9J40	qRTP450-23CxF	5’ACCCGAATCCGGGCAAGTTTGAT3’
		qRTP450-23CxR	5’AACTCCAAACGGTAAATACGCCGC3’
CPIJ010546	P450CYP9J34	P45010546F	5’ATCCGATGTCGGTAAAGTGCAGGT3’
		P45010546R	5’TGTACCTCTGGGTTGATGGCAAGT3’
CPIJ005959	P450CYP6AA7	P4505959F	5’ATGACGCTGATTCCCGAGACTGTT3’
		P4505959R	5’TTCATGGTCAAGGTCTCACCCGAA3’

Adult injection with dsRNA of GPCR genes

To investigate the role of GPCR-related gene in insecticide resistance, we used dsRNA interference (RNAi) technique to knockdown the up-regulated GPCR-related gene (CPIJ014334), in the resistant strain, MAmCq^{G6}. We injected the dsRNAs (~500 ng) of the CPIJ014334 gene in nuclease free water into the mesothorax of 12-24 hour old female adult of

MAmCq^{G6} mosquitoes by using Nanoject II (Drummond Scientific Company). To avoid gene injection affects for target gene expression, we injected with dsRNA of the GFP served as the control, and the MAmCq^{G6} mosquito that received no injection served as the calibrator. Total RNA was extracted from 3-4 day post-injection and non-injected MAmCq^{G6} adult mosquitoes. The relative expression of CPIJ014334, AMPK19613, and 4 P450 genes (CYP9M10, -9J34, -9J40, and 6AA7) were investigated using qRT-PCR with primer pair of these genes (Table 9.2). Each experiment was repeated 3 times with 3 independently isolated RNA samples from mosquitoes.

Mosquito Adult Bioassay

To test the resistant function of CPIJ014334 in *Culex* mosquitoes, we used the topical application for the adult bioassays; a 0.5-μl drop of insecticide in acetone was delivered to the thoracic notum of each mosquito adult with a 25- μl Hamilton gastight syringe (Fisher Scientific). Three to 4 days post-injection and non injected female adult HAMq^{G8} mosquitoes were used in this study. Each bioassay consisted of 20 mosquitoes per dose and four or five doses that yielded >0 and <100% mortality (Li and Liu, 2010). Control groups received acetone alone. Treated mosquitoes were reared in 6-oz Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with 10% sugar water on cotton. Mortality was assessed at 24 h post-treatment. The criterion for death was the mosquitoes' inability to move. All tests were run at 25^{o}C and replicated at least three times.

Embryo injection with dsRNA of up-regulated GPCR-related genes

According to the literature about *Drosophila* embryo injection with dsRNA (Ivanov, et al. 2004) and mosquito transgenic method (Adelman, et al. 2008), the function of the up-regulated GPCR genes (CPIJ019111, -7717, -14419) in larvae of resistant mosquito strains, HAmCq^{G8} and

MAmCq^{G6}, was investigated by injection of the target gene dsRNA to mosquito embryo, detecting the gene expression corresponding to the permethrin resistance in 2nd -3rd instar larvae. One thousand embryos were used for dsRNA injection in each time. Grey embryos were collected from HAmCq^{G8} and MAmCq^{G6} mosquito cages, in which the mosquito had been feed with blood three days early and reared under insectory conditions. Around 120 freshly laid eggs were collected and arranged on a piece of paper filter. Embryos were allowed to dry 2-3 min and transferred to a clear microscope slide cover using double sided tape, and embryos were then covered with Halocarbon 700 oil. Capillaries for microinjection were prepared by using a Flaming/Brown micropipette puller, and tip was opened by using a K. T. Brown Type micro-pipette beveler. We injected 0.2-0.5nl of dsRNA (3.5ug/ul) into embryo posterior, corresponding to approximately 1-5% of the embryo volume by using Picospritzer III injector system. Injected embryos were clear one by one and transferred to water solution and keep the water container under insectory condition for 3-4 days. Each time ~500 embryos were injected with dsRNA of target gene and dsRNA of GFP gene, respectively. Hatched 2nd -3rd instar larvae were separated into two groups, one was tested for bioassay with 5 dose range of permethrin, and another was prepared for gene expression identification of GPCR-related gene and permethrin resistant P450 genes, *CYP9M10*, *CYP9J34*, *CYP9J40*, *CYP6AA7* (Liu, et al. 2011; Yang, et al. 2011), PKA (CPIJ018257, -00798), and AMPK (CPIJ19613) by using qRT-PCR (Table 9.2). Each experiment was repeated 6 times with independently isolated RNA samples. Both gene expression and permethrin sensitivity were detected for identification of the GPCR-related gene function.

Larva bioassay

The stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) were prepared in acetone. Each bioassay consisted of 20 2^{nd}-3^{rd} instar mosquito larvae with rhodopsin-like or PKA injection, GFP-injection and non-injection in 10 ml glass vial with regular tap water and 1% insecticide solution in acetone at the required concentration, with four or five concentrations that resulted in >0 and 100%< mortality (Li, et al. 2010). Control groups received only 1% acetone. Mortality was assessed after 24 h. all tests were run at $25^{o}C$ and replicated 6 times.

Results

GPCR genes in *Cx. quinquefasciatus*

Sixty GPCR gene and 60 GPCR-related genes were identified from *Cx. quinquefasciatus* whole genome sequence. In *Culex* mosquito, the GPCRs mainly fall into Class A (rhodopsin-like), Class B (Secretin-like receptor), and Class C (GABAB receptor) (Table 9.3). Based on the GPCRDB annotation in *Culex* mosquitoes, class A rhodopsin-like have 48 GPCR genes and consists of 12 biogenic amine receptors, a leukotriene B4 receptor, 16 peptide receptor, 2 hormone protein receptor, a gonadotropin releasing hormone receptor, 13 rhodopsin receptor, as well as 3orphan GPCRs. Nine GPCRs belong to secretin-like genes, including 7 secretin-like receptors and 2 methuselah-like receptors. Class C has 3 GPCRs in total with 2 GABAB and a metabotropic glutamate receptor. Also, 60 GPCR-related GPCRs are identified based on the GPCR biologic process relationship (Table 9.3).

Table 9.3. Summary of GPCRs in *Culex quinquefasciatus*

Class A	Amine	Dopamine	2
		Adrenoreceptors	2
		Muscarinic acetylcholine	2
		Serotonin	4
		Octopamine	2
	Leukotriene B4 receptor		1
	Peptide	Neuromedin U like	1
		Cholecystokinin CCK	2
		Melanocortin	1
		Neuropeptide Y	4
		Tachykinin	2
		Somatostatin- and angiogenin-like peptide	3
		Galanin like	2
		Vasopressin like	1
	Hormone protein	Thyrotropin	1
		Gonadotropin type I	1
	Gonadotropin releasing hormone		1
	Rhodopsin	Rhodopsin Arthropod	13
	Orphan		3
Class B	Secretin-like		7
	Methuselah-like proteins		2
Class C	GABAB		2
	Metabotropic glutamate	Metabotropic glutamate type 1	1
Other GPCR-related genes (by Biological Process relationship)			60
Total			120

Relative expression of GPCR-related genes in the *Cx. quinquefasciatus* strains

Understanding of the changes of GPCR-related gene expression in different mosquito strains could elucidate the potential roles of these genes in insecticide resistance of mosquitoes. In this study, we characterized the 120 GPCR-related genes expression level in both larva and adult of mosquito strains, which bear different resistance profiles in response to permethrin, ranging from susceptible strain, S-Lab, through intermediate resistant strain (HAmCq^{G0} and MAmCq^{G0}, filed parental strains) to highly resistant strains (offspring of HAmCq^{G0}, HAmCq^{G8}, and offspring of MAmCq^{G0}, MAmCq^{G6}) (Li, et al. 2010). The GPCR-related gene expression profiles revealed that 4 genes were up-regulated and13 genes were down-regulated in highly

resistant mosquitoes compared with the intermediate resistant strains and susceptible strain. Moreover, 103 genes were equally expressed in all strains.

GPCR genes involved in up-regulation in both larvae and adults of resistant strains, HAmCq^{G8} and MAmCq^{G6}

To identify the correlation between the up-regulated GPCR genes and insecticide resistance of the mosquito, *Cx. quinquefasciatus*, we compared the expression levels of 120 GPCR-related genes in both larvae and adults of susceptible and permethrin resistant strains. We found that a GPCR-related gene (CPIJ019111) was up-regulated ~2.3-fold) in the 4th instar larva of HAmCq^{G8} compared with S-lab and HAmCq^{G0} (Fig. 9.1A). A secretin-like (CPIJ011419) and a GPCR-related gene (CPIJ007717) were up-regulated with ≥2-fold in larva of MAmCq^{G6} compared with S-Lab and MAmCq^{G0} (Fig. 9.1B). An opsin gene (CPIJ014334) expression was 2.4 times higher in adult of MAmCq^{G6} than it in MAmCq^{G0} and S-lab. However, all these up-regulation GPCR-related genes in HAmCq^{G8} and MAmCq^{G6} have the similar expression level between S-Lab and HAmCq^{G0} (Fig. 9.1).

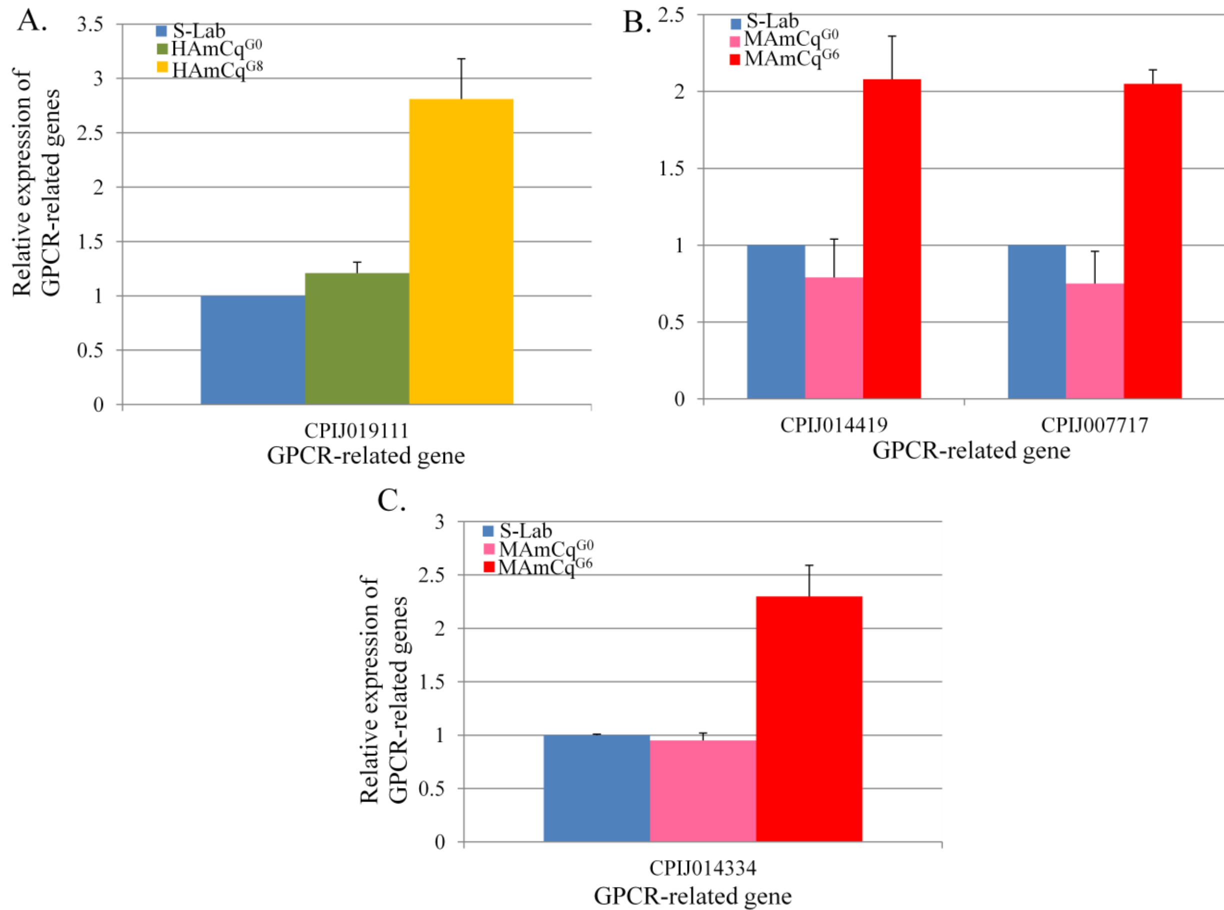

Fig. 9.1. Up-regulated GPCR-related gene expression in mosquito strains of *Cx. quinquefasciatus*. A. Two up-regulated GPCR-related gene expression were determined in 4th instar larva of different mosquito strains through S-Lab, HAmCq^{G0} and HAmCq^{G8}. B. Two up-regulated GPCR-related gene expression were determined in 4th instar larva of different mosquito strains through S-Lab, MAmCq^{G0} and MAmCq^{G6}. C. An up-regulated GPCR-related gene expression was determined in 3-day-old adult of different mosquito strains through S-Lab, MAmCq^{G0} and MAmCq^{G6}.

GPCR genes involved in down-regulation in both larvae and adults of resistant strains, HAmCq^{G8} and MAmCq^{G6}

Besides GPCR-related genes were up-regulated in resistant *Culex* mosquitoes, we found several GPCR-related genes were down-regulated (≤-2-fold) in HAmCq^{G8} and HAmCq^{G6} following the permethrin selection. Seven genes were down-regulated in larva of HAmCq^{G8} compared with HAmCq^{G0}, including a peptide receptor and 6 GPCR-related genes (Fig. 9.2A). The expression of 7 genes has ~2-fold in larva of MAmCq^{G6} less than MAmCq^{G0}. These genes were distributed into a peptide receptor, 2 secretin-like receptor, and 4 GPCR-related genes (Fig. 9.2B). Five genes were down-regulated ~2 times in adult of MAmCq^{G6} than MAmCq^{G0} with a peptide receptor, a secretin-like receptor, and 3 GPCR-related genes (Fig. 9.2C). In summary, CPIJ003158, -003420, -007676, and -003873 genes were down-regulated in the larva of both HAmCq^{G8} and MAmCq^{G6}. The down-regulation of CPIJ011549 in both larva and adult of MAmCq^{G6} was identified. The CPIJ017421 was down-regulated in larva of HAmCq^{G8} and in adult of MAmCq^{G6}.

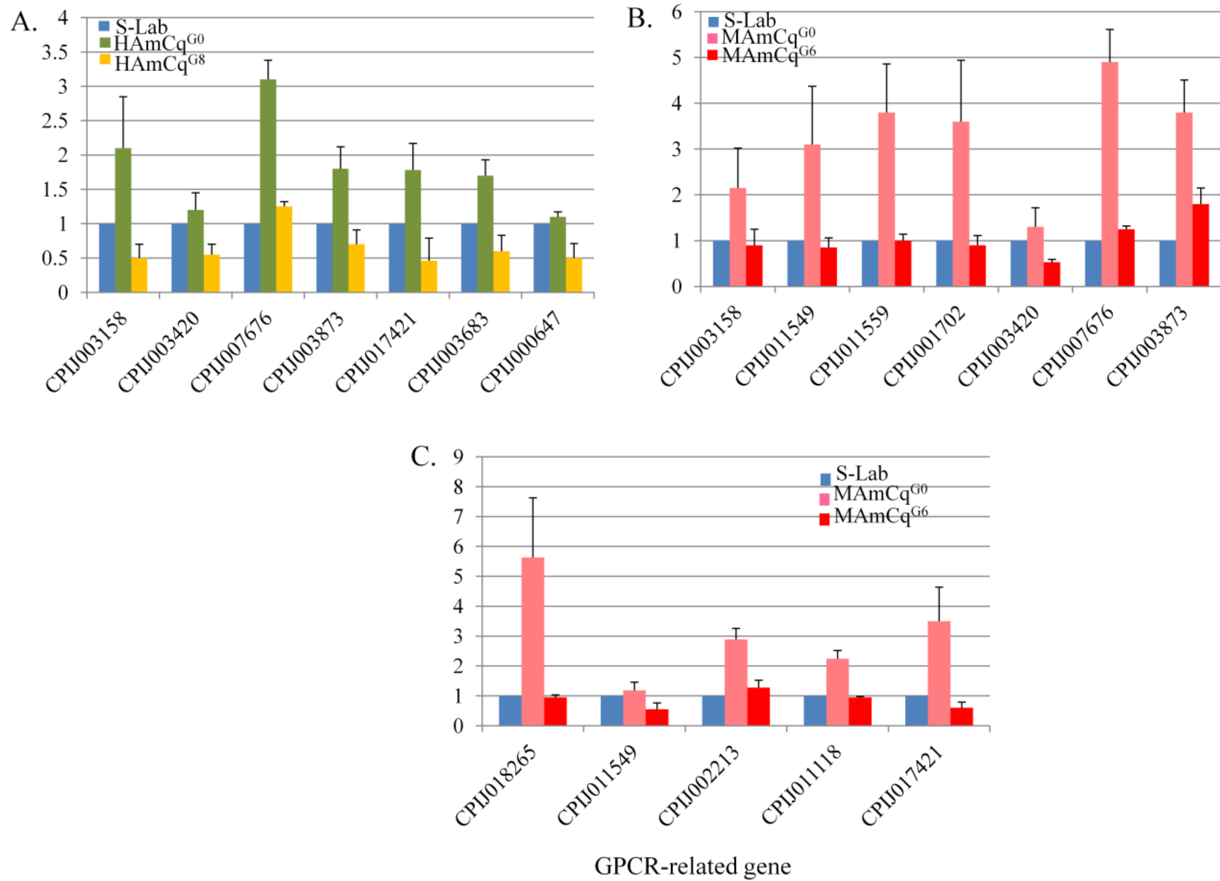

Fig. 9.2. Down-regulated GPCR-related gene expression in mosquito strains of *Cx. quinquefasciatus*. A. seven down-regulated GPCR-related gene expression were determined in 4^{th} instar larva of S-Lab, $HAmCq^{G0}$ and $HAmCq^{G8}$. B. seven down-regulated GPCR-related gene expression were determined in 4^{th} instar larva of S-Lab, $MAmCq^{G0}$ and $MAmCq^{G6}$. C. five down-regulated GPCR-related gene expression were determined in 3-day-old adult of S-Lab, $MAmCq^{G0}$ and $MAmCq^{G6}$.

Microinjection with dsRNA of CPIJ019111 in larva of $HAmCq^{G8}$

To investigate the function of up-regulated GPCR-related genes in *Culex* mosquitoes, we used the powerful technique RNAi to initially knockdown the target CPIJ019111 gene in larva of $HAmCq^{G8}$. The results showed that ~35% of the gene expression of CPIJ019111was decreased, which related to decreased resistance ratio (~50%) to permethrin compared with non-injection

and GFP-injection mosquitoes (Fig. 9.3A). To investigate the involvement of PKA and AMPK in GPCR signaling pathway, we tested the PKA and AMPK gene expression in CPIJ019111 knockdown mosquitoes. The result showed decreased expression of PKA18257 (~40%) and AMPK19613 (~70%) following the knockdown of CPIJ019111 (Fig. 9.3B). Furthermore, knockdown of target gene also caused decreased expression of 4 P450 genes (CYP9M10, -9J34, -9J40, -6AA7) (Fig. 9.3C).

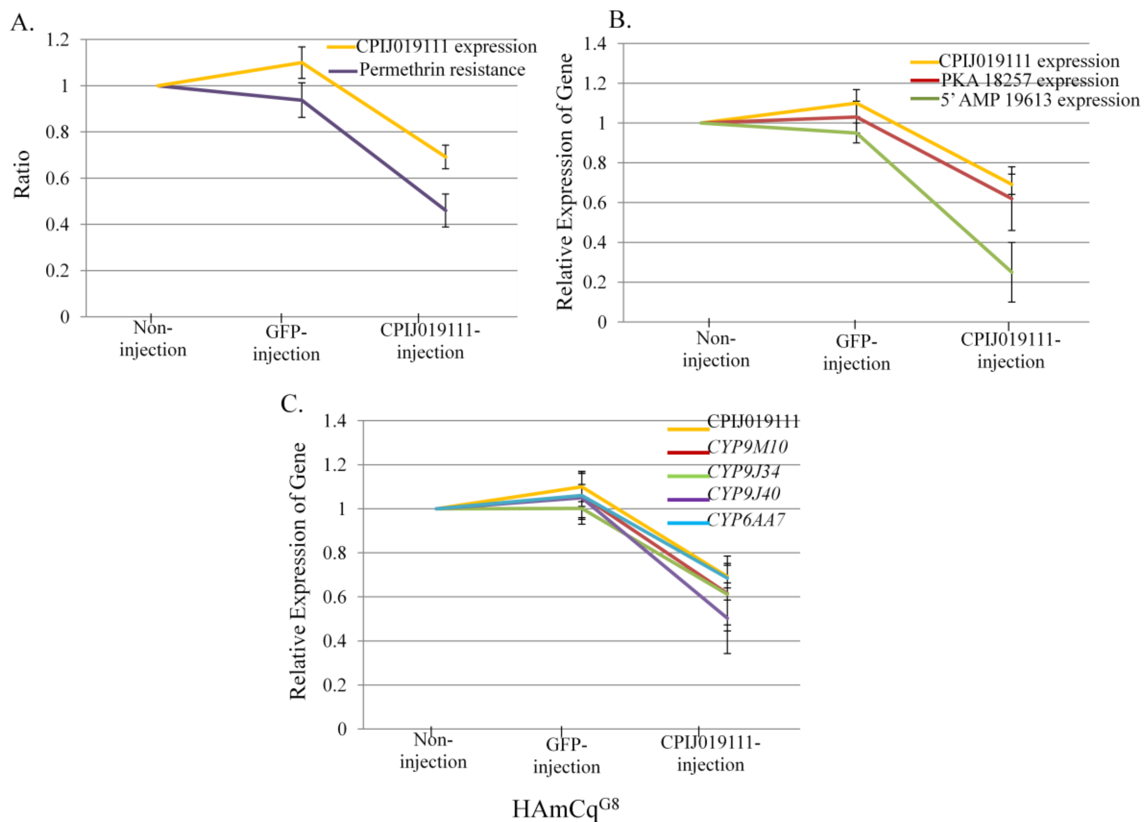

Fig. 9.3. Functional study of up-regulated CPIJ019111gene in permethrin resistance of HAmCq^{G8} strain. A. knockdown of CPIJ019111 gene associated with decreased resistance to permethrin in dsRNA of CPIJ019111-injected HAmCq^{G8} mosquitoes compared with non-injection and GFP-injection ones. B. knockdown expression of CPIJ019111gene caused decreased expression of PKA and 5'AMP genes. C. four cytochromeP450 genes were down-regulated in CPIJ019111knockdown mosquitoes.

Microinjection with dsRNA of CPIJ007717 and -014419 in larva of MAmCq^{G6}

dsRNA of CPIJ007717 and -014419 were injected into fresh embryo of MAmCq^{G6} to investigate the function of these genes in insecticide resistance. Around 40% knockdown expression of CPIJ007717 and -014419 genes caused decreased resistance to permethrin (~50% mortality) in target gene injected mosquitoes compared with non-injection and GFP-injection (Fig. 9.4A, 9.5A). Moreover, we found decrease expression of PKA18257 (~65%) following the knockdown of CPIJ007717 (Fig. 9.4B), and decreased expression of PKA00798 (~55%) in CPIJ014419 knockdown mosquitoes (Fig. 9.5B). All 4 P450 genes were down-regulated in CPIJ007717 injected mosquitoes (Fig. 9.4C), however, only 2 P450 genes, CYP9M10 and –9J34, had significant decreased expression following the knockdown of CPIJ014419 (Fig. 9.5C).

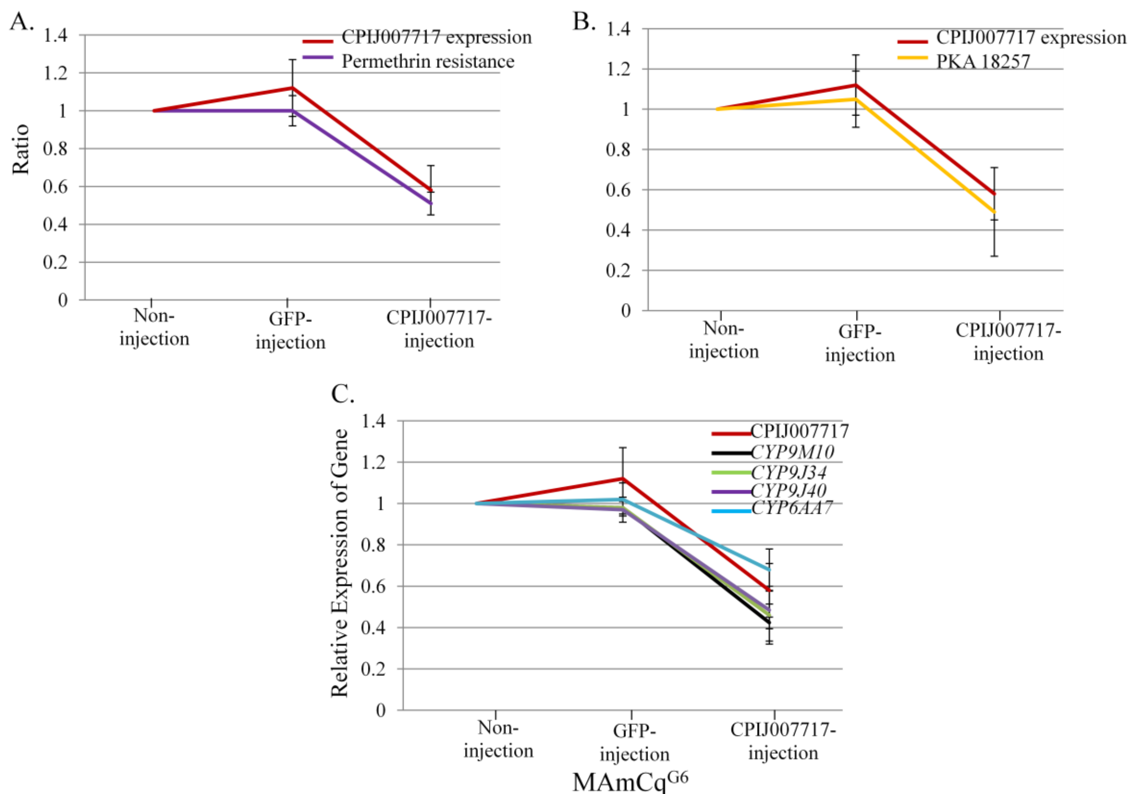

Fig. 9.4. Functional study of up-regulated CPIJ007717gene in permethrin resistance of MAmCq^{G6} strain. A. knockdown of CPIJ007717 gene associated with decreased resistance to

permethrin in dsRNA of CPIJ007717-injected MAmCq^{G6} mosquitoes compared with non-injection and GFP-injection ones. B. knockdown expression of CPIJ007717 gene caused decreased expression of PKA gene. C. four cytochrome P450 genes were down-regulated in CPIJ007717knockdown mosquitoes.

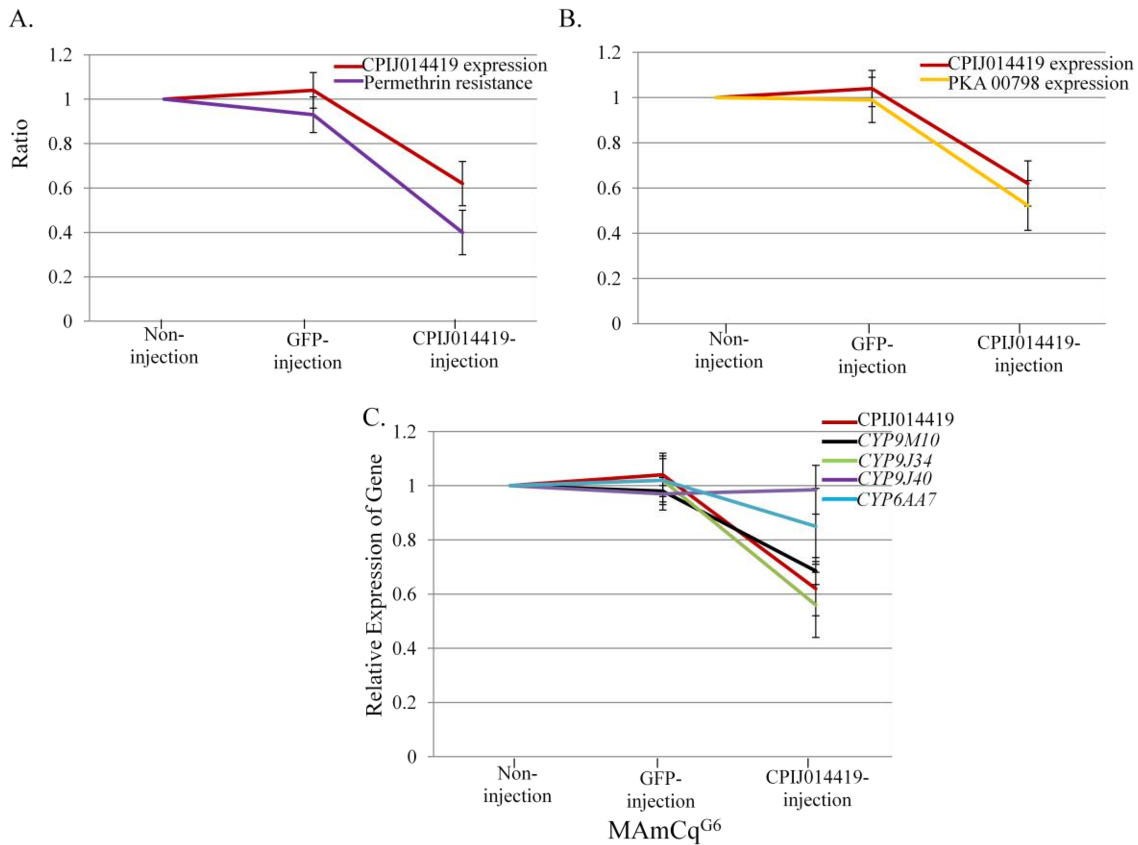

Fig. 9.5. Functional study of up-regulated CPIJ014419 gene in permethrin resistance of MAmCq^{G6} strain. A. knockdown of CPIJ014419 gene associated with decreased resistance to permethrin in dsRNA of CPIJ014419-injected MAmCq^{G6} mosquitoes compared with non-injection and GFP-injection ones. B. knockdown expression of CPIJ014419 gene caused decreased expression of PKA gene. C. four cytochromeP450 genes were down-regulated in CPIJ014419 knockdown mosquitoes.

Microinjection with dsRNA of CPIJ014334 in adult of MAmCq^{G6}

In the adult injection of CPIJ014334, we found ~50% decreased expression of CPIJ014334 correlated with the decreased resistance to permethrin (~40% mortality) in 3-4 day post-injection female mosquitoes compared with the non-injection and GFP-injection (Fig. 9.6A), and also a AMPK 019613 with ~50% decreased expression in CPIJ014334 knockdown adults (Fig. 9.6B). However, there was no change of the P450 gene expression in CPIJ014334 injected mosquitoes (Fig. 9.6C).

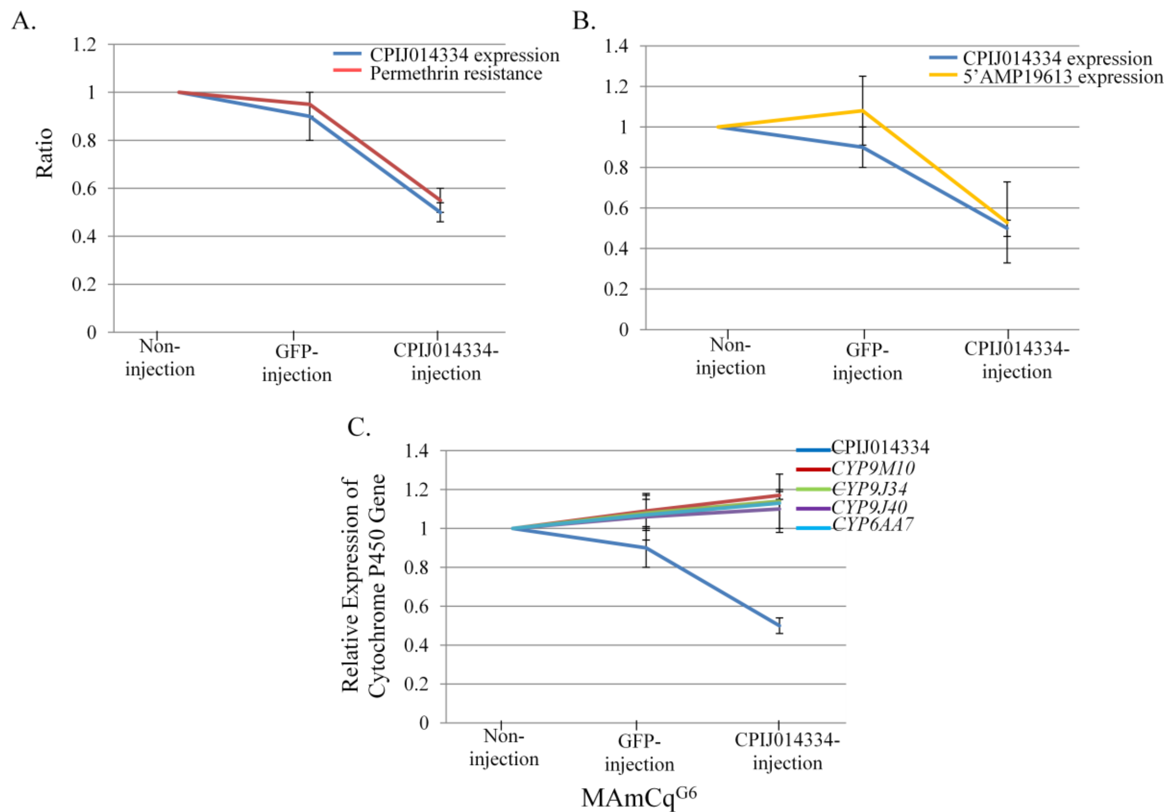

Fig. 9.6. Functional study of up-regulated CPIJ014334 gene in adult of permethrin resistance of MAmCq^{G6} strain. A. knockdown of CPIJ014334 gene associated with decreased resistance to permethrin in dsRNA of CPIJ014334-injected MAmCq^{G6} mosquitoes compared with non-injection and GFP-injection ones. B. knockdown expression of CPIJ014334 gene caused decreased expression of 5'AMP gene. C. four cytochromeP450 genes were down-regulated in CPIJ014334 knockdown mosquitoes.

Discussion

Multiple genes or mechanisms have been demonstrated in insecticide resistance in various insect species (Adelman, et al. 2011; Yewhalaw, et al. 2011). Cytochrome P450 gene up-regulation involved in metabolism of insect can develop insecticide resistance in insects (Zhu, et al. 2010; Liu, et al. 2011; Stevenson, et al. 2011). However, the P450 regulation in insecticide resistance is still unclear. Guanine nucleotide-binding proteins (G-proteins) and GPCRs play pivotal roles on switch of chemical signals and trigger of several specific downstream signaling pathways in cell membrane, cytoplasmic and nuclear target activity to response to environment stimulations (Rosenbaum, et al. 2009; Shen, et al. 2012). In insects, GPCRs for the visual signal, neuronal and endocrine peptides, biogenic amines, trehalose, glycoprotein hormone, orphan, diuretic hormone, metabotropic glutamate, frizzled-like, bride of sevenless (Boss), and odorant binding have been characterized (Broeck, V. J. 2001). A GPCR present in female moth, *Helicoverpa zea*, was related to pheromone biosynthesis via binding with pheromone biosynthesis-activating neuropeptide (PBAN) and increased cAMP production (Choi, et al. 2003). The GPCR for neuropeptide binding was involved in insect metabolic pathways by regulating ion and water balance for insect gut system and hemolymph homeostasis (Gäde, 2004). Moreover, several GPCRs bind with neurotransimtters, which include GABA, biogenic amines, glutamate, neuropeptides and others in central nerves system (CNS), to control some critical metabolic processes, reproduction, development, and feeding (Baumann, et al. 2003; Nässel, et al. 2006). The importance of GPCRs for neurohormone binding also has been studied in many insects (Hauser, et al. 2006, 2008; Honegger, et al. 2008). Bai et al. have used RNAi technique to determine the functions of non-sensory GPCR genes in the red flour beetle, *Tribolium castaneum*, including 6 GPCRs involved in larval and pupal molting and mortality

(Bai, et al, 2011). The insects also use specific receptor, like DmX GPCR receptor in *Drosophila*, to taste repellent, which causes a behavioral avoidance (Mitri, et al. 2009). As some insects, such as *Aedes aegypti* (135 GPCRs) (Vishvanath Nene, et al), *Anopheles gambiae* (122 GPCRs) (Catherine A. Hill, et al), and *Drosophila melanogaster* (~200 GPCRs) (Adams, et al. 2000), *Cx. quinquefasciatus* has 60 named GPCRs and more than 200 GPCR-related hypothetical genes, indicating that GPCRs may play important roles in physiological and cell biological pathways of *Culex* mosquitoes. Our group has found that a GPCR gene was up-regulated in the resistant strain, HAmCq^{G6} (Liu, et al. 2007). However, whether the GPCRs involve in insecticide resistance or not is a blank. Thus, we tested the expression of 120 GPCR and GPCR-related genes, including 60 GPCRs with rhodopsin-like class A (48 GPCRs), Secretin-like class B (9 GPCRs), GABAB class C (3 GPCRs), and 60 GPCR-related genes between resistant strains and susceptible strain to determine the potential role of GPCRs in insecticide resistance in *Culex* mosquitoes.

To demonstrate the function of the up-regulated GPCR and GPCR-related genes, we used RNAi technique, which is a powerful tool to silence the gene expression post-transcriptionally (Montgomery, et al. 1998; Mello, et al. 2004), to determine the function of these up-regulated genes in permethrin-resistance. The result showed silence of up-regulated GPCR-related genes (CPIJ019111,-007717, -014419, -14413) was associated with decreased resistance tolerance in HAmCq^{G8} and MAmCq^{G6}, indicating the important function of these GPCR-related genes in permethrin resistance. Since the PKA (Rosenbaum, et al. 2009) and AMPK (Merlin, et al. 2010) played critical role in the GPCR regulatory pathway, we tested the expression of PKA and AMPK expression in GPCR-regulated gene knockdown mosquitoes and found the deceased expression of PKA genes (CPIJ018257, -000798) and an AMPK (CPIJ019613), indicating the

involvement of PKA and AMPK genes in these up-regulated GPCR signaling pathway. Furthermore, as the regulatory factors of P450 gene expression, inhibition or activation of PKA (Deb and Bandiera, 2011) or AMPK (Blättler, et al. 2007; Hsu, et al. 2011) could regulate the P450 gene expression. We characterize the regulatory function of GPCR in P450 gene expression through the PKA and AMPK pathway by identification of the P450 gene expression, which were up-regulated in resistant mosquitoes, in GPCR-regulated gene knockdown mosquito and the result showed the decreased expression of P450 genes in GPCR-regulated gene (CPIJ019111, -007717, and -14419) injected mosquito. However, knockdown of the GPCR-related gene (CPIJ014413), which was up-regulated in adult of MAmCq^{G6} mosquito, caused decreased resistant tolerance to permethrin and decreased expression of AMPK (CPIJ019613) gene expression, but no effects on these 4 P450 gene expression, suggesting that either more resistance-related P450 genes may be regulated by GPCR signal pathway or other regulatory pathway of GPCR involved in insecticide resistance.

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Chapter 10 Future Study

The multiple mechanisms of insecticide resistance in insects have been studied for several years. However, the regulation factors and pathways of resistance are still unclear. In my current studies, we are interested in the function of the up-regulated GPCR and GPCR-related genes in permethrin-resistance mosquitoes compared with the susceptible ones. Our results provide some new evidences that GPCR signaling transduction pathways may play a pivotal role in the regulation of insecticide resistance by regulating the resistance-related P450 gene expression. According to these interested discoveries, in future, I will focus on the possible factors, which may be involved in permethrin resistance of *Culex quinquefasciatus*, in GPCR signaling transduction pathway, via various methods.

1. Transgenic mosquito with resistance-related GPCR gene

Transgenesis technology has been developed for the mosquito studies, such as *Anopheles* mosquito species (Ito, et al. 2002), and *Aedes aegypti* (Moreira, et al. 2000). Successful integration of exogenous DNA into the germline of the mosquitoes has been achieved with several class II transposable elements (Tu, et al. 2004). The marker gene and fluorescent protein genes have been used to monitor the expression of transgenic genes in mosquitoes (Pinkerton, et al. 2000). The trangenesis technology is commonly used in mosquito for control of the transmission of disease by expression the antiparasitic genes in the mosquito genome (Isaacs, et al. 2012). However, there is no evidence show the transgenesis technology applied in insecticide resistance study. In our study, we will use this technique to transfer the resistance-related GPCR

genes into mosquito and identify the regulatory pathway in P450 gene expression and other factors in insecticide resistance, for example the protein kinase genes. Moreover, the inhibitor of resistance-related genes will be studied using this technique to provide an anti-resistance germline for developing a novel and genetics-based resistance control strategy.

2. Protein kinase study

In human disease studies, protein kinases have been developed as a medicine target, because of their regulatory function in cellular cascades (Hardie, et al. 2012). Protein kinases also work on the regulation of P450 gene expression in several reports (Deb, et al. 2011; Hsu, et al. 2011). Our previous studies reveal the important function of protein kinases in insecticide resistance and P450 gene regulation. Thus, the functional study of protein kinases will be conducted by using RNAi technique to knockdown the resistance-related protein kinase genes. The inhibitor and activator of protein kinases will be used to exam the protein kinase activity at the biochemistry level. We hypothesize that the inhibited or activated protein kinase in cell probably would mediate the P450 gene expression in order to involve in insecticide resistance. Furthermore, to identify the GPCR regulatory function in P450 gene expression through PKA pathway, I would use the fluorescence cAMP assay to monitor the PKA activity and P450 gene expression in GPCR inhibited cells or mosquitoes. These studies will clarify the crucial role of GPCR signaling transduction pathway in insecticide resistance.

3. Cytochrome P450 metabolisms of permethrin

The role of Cytochrome P450 enzyme detoxification to insecticide in insects have been characterized, such as CYP6BQ9 metabolizes deltamethrin (Zhu, et al. 2010), CYP6M2 metabolizes permethrin and deltamethrin (Stevenson, et al. 2011), and CYP6G1 metabolizes methoxychlor (Joußen, et al. 2010). In my further studies, even the function of GPCR-regulatory

pathway in P450 gene expression and insecticide resistance could be demonstrated, the resistance-related P450 genes, especially the GPCR regulated P450s, would be candidates to test their metabolism function, eventually complete the blueprint of GPCR signaling transduction pathway roles in permethrin resistance of *Culex quinquefasciatus*.

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