The Sodium Channel Gene Expression on the Kdr Locus in the House Fly, Musca domestica and the Mosquito, Culex quinquefasciatus

by

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Abstract

A point mutation of leucine to phenylalanine (L-to-F), resulting from a single nucleotide substitution in the sodium-channel of insects, is associated with pyrethroid resistance. In this study, to understand genetic basis of sodium channel mutations in resistance, 14 house fly lines with different autosomal combinations were generated from crosses of resistant and susceptible house flies. This study, for the first time, mapped the sodium channel gene on autosome 3, factors on which were demonstrated to be involved in the elevated levels of resistance. In addition, two copies of the sodium channel gene of mosquitoes were identified. A strong correlation among the frequency of the resistant allele at the L-to-F site at the DNA level, the frequency of L-to-F allelic expression at the RNA level, and the levels of pyrethroid resistance suggests that one of the two copies is a functional sodium channel gene in mosquitoes, which is involved in insecticide resistance.
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Chapter 1 Literature Review

1.1 Insecticide resistance and its problems

Insecticide resistance is a serious problem resulting from wide and heavy application of insecticides. It was defined by World Health Organization (WHO) as “The development of an ability in a strain of some organisms to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal population of the same species” (WHO, 1957). Insecticide resistance is described by some scientists as a “pre-adaptive phenomenon” (WHO, 1957), because individuals bearing resistance factors may be present in a population prior to its exposure to an insecticide (WHO, 1957). Under selection pressure from insecticides, these pre-existing resistant individuals are able to survive exposure to toxins and pass resistance factors (i.e., genes) to their offspring (WHO, 1957). Consequently, the frequency of individuals with resistant genes increases in the population, making the population more resistant to certain toxicants.

To date, insecticide resistance has been documented in more than 500 insect species (Georghiou, 1990). The resistance phenomenon has been reported in most classes of insecticides, such as carbamates, pyrethroids, organophosphates and DDT. It is also found in some new insecticides, such as fipronil (Liu and Yue, 2000) and spinosad (Moulton et al., 2000). Insecticide resistance may have several important impacts on humans. First of all, rapid development of insecticide resistance makes it incredibly difficult to control agriculturally and medically important pest species through the using of pesticides. Secondly, insecticide resistance decreases the effectiveness of insecticides, resulting in pesticide dosage application increases, which may cause serious contamination to the environment
Moreover, many species have developed cross resistance to two or more insecticides, thus, accelerating the decrease in the number of available effective insecticides (Golenda and Forgash, 1985; Scott, 1989; Bisset et al., 1997). Finally, insecticide resistance results in resurgence of agriculturally important pests and insect vectors, which, in turn, results in reduced crop yields and outbreaks in humans and animals diseases (Hemingway and Ranson, 2000).

1.2 Resistance mechanisms

Diverse resistance mechanisms have been documented in various insect species. Some insects might survive insecticides by avoiding exposure, referred to as behavioral change mechanism; others might have changes in the chemical component of their cuticles that reduce the penetration rate of toxin into their bodies. More importantly, significant resistance occurs when some biochemical resistance mechanisms are present. Two major classes of biochemical resistance mechanisms are: 1) increased metabolic detoxification and sequestration and 2) decreased target site sensitivity (Hemingway and Ranson, 2000). Enhanced metabolic detoxification and sequestration causes resistance by decreasing the effective dose of insecticide that reaches the target site. This is often completed by elevating the amount or activity of detoxification enzymes, such as Cytochrome P450s, esterases and glutathione-s transferases (GSTs). The target site insensitivity confers resistance by reducing the ability of insecticide molecules to bind effectively to their targets, which are typically located in the nervous system. Reduced sensitivity of sodium channels, acetylcholinesterase (AChE) and gamma-aminobutyric acid (GABA) receptors is a major mechanism of biochemical resistance (Liu et al., 2006).
1.2.1 Behavioral resistance

Insects may survive pressure from insecticide selection by successfully escaping from harmful chemicals, repelling insecticides, or behaviorally avoiding contact with insecticides. Primary behavioral resistance mechanisms include increased irritability, increased repellency and stimulus-independent behavioral resistance (Georghiou, 1972). Irritability to insecticides stands for the tendency of insects to escape from insecticidal chemicals after short contact with insecticide-treated residues. One case of increased irritability is documented in the mosquito *Anopheles atroparvus*. After selection by DDT for 10 generations (Gerold and Laarman, 1964), the selected strains possess high irritability, enabling them to escape DDT residues more rapidly than the normal populations. Repellency stands for the ability of insects to detect certain repellent components of an insecticide. One typical case of this mechanism was documented in the state of Georgia, where fly control using applications of malathion-sugar baits failed (Kilpatrick and Schoof, 1958). It was observed that the flies obtained a high level of repellency to malathion (organophosphate insecticide), thus, most of flies did not alight on the treated sugar, despite approaching the bait (Kilpatrick and Schoof, 1958).

Stimulus-independent behavioral resistance occurs in insects with naturally exophilic or zoophilic behaviors. Insects with these behaviors prefer to remain in natural habitats distant from human dwellings or feeding on wild animals instead of humans. Exophilic and zoophilic behavior are manifestations of neither irritability nor repellency and are examples of situations not requiring chemical control. This natural habit makes insects stay far away from human living places that are always treated by pesticides, reducing their chance of exposure to insecticides. If exophilic or zoophilic individuals are present in a certain population, we would expect that the percentage of such individuals in the population would increase under the pressure of insecticides because those staying or frequently visiting pesticide-treated human place would be more likely to be killed. Such a case of behavioral resistance was
observed in the control of *Anopheles gambiae* in Swaziland and Rhodesia. Prior to pesticide control, this species was abundant and often observed in human living places. Treatment using the organochlorine insecticide hexachlorocyclohexane (HCH) caused extirpation of this species in treated and untreated anthropogenic habitats; populations did not rebound, even after chemical control had stopped. However, healthy populations that preferentially fed on non-human vertebrates were found in non-anthropogenic situations (Muirhead-Thomson, 1960). This may be explained as a case of stimulus-independent behavioral resistance because *A. gambiae* are not known to exhibit irritation or repellency to BHC (Davidson, 1953).

1.2.2 Reduced penetration rate

Reduction in the rate of insecticide penetration through insect cuticle also confers insects with a certain levels of resistance. Since the amount of insecticide getting into the insect’s body is reduced, it becomes easier for detoxification enzymes to cope with smaller amounts of the toxin entering the body per unit of time (Georghiou, 1972). Therefore, the amount of insecticide able to reach their target systems decreases, resulting in decreased kill rates. It has been documented that a decreased rate of insecticide penetration significantly enhances resistance through association with other resistance mechanisms, and that by itself, may only cause marginal resistance (Scott, 1991). A common method to examine reduced penetration rate is the use of $^{14}$C-labeled pesticides. A study on resistance mechanisms in the house fly, *Musca domestica*, indicate that penetration of $^{14}$C-labelled fipronil was slower in resistant strains than susceptible strains (Wen and Scott, 1999), suggesting that reduced penetration is one of the mechanisms in resistance to fipronil.
1.2.3 Increased detoxification and sequestration

1.2.3.1 Insect P450-based insecticide resistance

Cytochrome P450 is an important hemoprotein involved in monooxygenases systems. In many organisms, including mammals and arthropods, cytochrome P450 dependent monooxygenases play an essential role in regulating titers of hormones, as well as in metabolizing xenobiotics (Stegeman et al, 1998; Scott et al, 1999).

P450 monooxygenase detoxification is an extremely important mechanism for insects to survive insecticides because of the enzyme’s ability to detoxify a large variety of toxicants (Agosin, 1985). P450 monooxygenase has been systematically studied in both mammals and insects (Agosin, 1985). Work with P450 monooxygenase since the 1960s may be divided into several aspects: isolation and characterization of cytochrome P450s, biochemic determination of P450 enzymes, and functional diversity of cytochrome P450s and the P450 gene superfamily. In this review, I discuss recent literature with emphases on insect cytochrome P450s and their associations with insecticide resistance. Cytochrome P450s were first successfully purified from mammal livers in 1968 (Lu and Coon, 1968). Subsequently, a large number of cytochrome P450s from different mammal species were purified and characterized during the 1970s and 1980s (Scott, 1999). In the late 1980s, studies of P450 nomenclature were conducted (Nebert et al., 1987), and a standardized P450 nomenclature system was founded (Nebert et al., 1987), which facilitated classification of the isolated cytochrome P450s. The P450 gene superfamily contains 70 families comprising 127 subfamilies. In insects, there are as estimated 80-100 P450 genes in each species, which are assigned to six CYP families (Feyereisen, 1999, Scott, 1999).

The insect P450 was first detected by Ray in 1967 (Ray, 1967). Evidence for multiple P450 genes in insects came out of subsequent research (Agosin, 1985; Scott et al., 1994). In 1971, insect P450 reductase was first purified and characterized from the house fly, M.
domestica (Wilson and Hodgson, 1971), followed by the successful purification of insect cytochrome b5 from several insect species such as Ceratitis capitata and Spodoptera eridania during the 1970s (Mcfadden et al., 1979; Megias et al., 1986; Wheelock and Scott, 1992). Due to the importance of identifying insect cytochrome P450s in functional studies of these enzymes, many attempts were made to purify cytochrome P450s from insects (Fisher and Mayer, 1984; Schonbrod and Terriere, 1975). Two main methods were applied, including protein purification and gene cloning (Scott, 1999). Most purification protocols can only be considered to be partially successful because the quantity and quality of the purified protein was too low to be useful due to the instability of insect cytochrome P450s (Hodgson, 1985). This problem was not solved until 1989, when the first insect P450 of high quality (named CYP6D1 or P450_{lpr}) were purified from the house fly by the antisera-based method (Wheelock and Scott, 1989). The first successful cloning of the insect P450 gene, CYP6A1 was done from the Rugters strain of the house fly, Musca domestica, which processed resistance to diazinon (Feyereisen et al., 1989). Later studies identified and sequenced several insect cytochrome P450s from other species by similar methods. However, even though antiserum seems to have high effectiveness in isolating cytochrome P450s, there are still debates concerning the utility of antiserum-based method in characterization of single insect P450 because some of the antisera are able to cross-react with multiple cytochrome P450s, causing unreliable results (Feyereisen, 1999, Scott, 1999).

Success in gene cloning and protein purification of insect cytochrome P450s also facilitates the study on biochemistry of these enzymes. Common studies are the investigation of P450 though heterologous expression and reconstitution of P450 activities, where expression of P450 cDNA is accomplished in heterologous systems, such as Escherichia coli and Saccharomyces cerevisiae. In addition, the relationship and interactions of P450 with P450 reductase and cytochrome b5 has been well-described (Guzov et al., 1996; Murataliev
et al., 1999). P450 monooxygenases play various functional roles in metabolism. The knowledge of insect cytochrome P450s' function is largely based on assumptions of their homology to mammalian cytochrome P450s (Feyereisen, 1999). Besides the function of detoxification, other well-studied functions of insect cytochrome P450s include hormone regulation during insect development (Scott, 1999; Feyereisen, 1999). For example, cytochrome P450s were found to be involved in the synthesis and degradation of juvenile hormone in the cockroach, *Blaberus giganteus* (Hammock, 1975), the House fly, *M. domestica* (Hammock and Quistad, 1981) and locusts, *Locusta migratoria* (Feyereisen et al., 1981). Cytochrome P450s are also documented to be involved in the metabolism of 20-hydroxyecdysone, fatty acids and other endogenous substrates, as well as bioactivation of phosphorothioate insecticides (Feyereisen, 1999).

The developmental regulation of P450 has been well documented (Hadgson, 1985; Carino et al., 1994; Gandhi et al., 1992; Saner et al., 1996; Snyder et al., 1995). Insect cytochrome P450s may be detected in various tissues with different expression levels based on the tissue's metabolism requirement. High levels of cytochrome P450s are usually detected in the midgut and fat bodies, where they serve as the first defense system for absorbed foreign compounds, including insecticides (Scott, 1999; Feyereisen, 1999). The level of P450 expression may be different among different developmental stages. Generally, cytochrome P450s cannot be detected in eggs and pupae but are highly expressed in adults (Scott, 1999). Some of the cytochrome P450s might be expressed specifically in a particular stage. For instance, CYP6B2 in *Helicoverpa armigera* is only expressed during the larval stages (Ranasinghe et al., 1997), while CYP6D1 in the house fly is specific to adults (Scott, 1996).

The role of P450 in insecticide resistance was first demonstrated in the 1960s when Eldefawi (1960) found that the P450 inhibitor sesamin was able to abolish insect resistance to carbaryl. This was confirmed by later studies (Georghiou et al., 1961; Schonbrod et al.,
1965). Others found that P450 detoxification may confer cross-resistance to toxins having different target sites (Scott, 1991).

Some authors have focused on the mechanism of P450-mediated resistance. Investigation of cytochrome P450s in resistant and susceptible strains using aldrin showed a higher level of P450 monooxygenase in resistance strains compared to susceptible strains, indicating that resistance might be the result of increased metabolism of the insecticides by P450-monooxygenases (Plapp and Casida, 1969). However, the role of cytochrome P450s in insecticide resistance remained controversial (Scott, 1999) since no significant correlation was found between total P450 levels and insecticide resistance. The association of cytochrome cytochrome P450s with insecticide resistance was not clear until Wilkinson (1983) suggested that the substrates applied might not be measuring the P450 involved in insecticide resistance. This idea was corroborated with Scott’s (1991) suggestion that resistance might be due to the elevation of only select cytochrome P450s and that this may not result in a significant increase in total cytochrome P450s, Accordingly, one needs to find and isolate the particular cytochrome P450s that are involved in insecticide resistance from the total cytochrome P450s before investigating the relationship between cytochrome P450s and insecticide resistance (Scott, 1999).

To date, many insect cytochrome P450s have been isolated and characterized. However, the status of these cytochrome P450s as being involved in insecticide resistance remains unclear. Only a few of the cytochrome P450s might contribute to insecticide resistance, such as CYP6D1 isolated from house fly LPR strain (Wheelock and Scott, 1992c), CYP6A2 isolated from Drosophila melanogaster and P450MA from the German cockroach, Blattella germanica (Scharf et al., 1998b).

Some studies focus on the role of P450 reductase and cytochrome b5 in P450 mediated resistance. The P450 reductase and cytochrome b5 are required in the P450 metabolic cycle.
It is documented that the level of P450 reductase and cytochrome b5 are increased in P450-mediated resistance (Scott and Georghiou, 1986b), but genetic analysis suggests that b5 might play a role in P450-mediated resistance while P450 reductase might not (Liu and Scott, 1996).

The molecular basis of P450-mediated resistance was also conducted by several research groups (Zhang and Scott, 1994; Liu and Scott, 1998). It has already been demonstrated that P450-mediated resistance is conferred by an increased level of P450 gene expression, either by insecticide induction or constitutive overexpression. Some studies focus on constitutive overexpression of some cytochrome P450 genes in resistant insects (Zhu et al, 2008). For instance, cDNA fragments of two cytochrome P450s, CYP6A36 and CYP6A5v2, have been isolated from house flies (Zhu and Liu, 2008; Zhu et al, 2008a). Studies of these two genes revealed that they were constitutively overexpressed in a permethrin-resistant house fly strain, ALHF, suggesting an important role of constitutive overexpression of these two genes in the development of resistance to permethrin in the house fly (Zhu and Liu, 2008; Zhu et al, 2008a). Some studies, however, focus on up-regulation and genetic linkage of some P450 genes (Zhu et al, 2008b). For instance, three P450 genes, CYP4D4v2, CYP4G2, and CYP6A38 in the house fly, have been isolated and genetically studied. CYP4D4v2 and CYP6A38 have been mapped to autosome 5 of the house fly, while CYP4G2 has been mapped to autosome 3 of the house fly. CYP4D4v2 and CYP6A38 were identified to be highly inducible by permethrin and the induction of the two genes was showed to play an important role in permethrin resistance in the resistant house fly strain, ALHF (Zhu et al, 2008b). Regulation of resistance-related P450 genes was also studied, CYP6D1, was mapped to autosome 1 and the presence of regulatory factors on autosome 2 for CYP6D1 expression was suggested (Liu and Scott, 1998). However, additional work remains to be done to develop a more complete understanding of the regulatory factors on autosome 2.
1.2.3.2 Esterase-based insecticide resistance

Carboxyl esterase protects insects from organophosphates and carbamates by hydrolyzing or sequestering the insecticides before they reach their targets (Hemingway and Karunaratn, 1998). The esterase-based resistance is most extensively studied in mosquito Culex species and in the green peach aphid Myzus persicae (Hemingway and Karunaratn, 1998; Devonshire and Field, 1991). One mechanism of esterase-based insecticide resistance is the elevation of carboxyl esterase. Esterase is observed to be overproduced in resistant insect strains, resulting in an increase of the amount of available esterase for sequestering penetrated insecticides in a unit time, reducing the amount of pesticide to reach the target. It has been demonstrated that the enhanced carboxylesterase is a result of gene amplification (Small and Hemingway, 2000). Southern blot analysis, detected a 64-fold amplification in resistant green peach aphid, M. persicae esterase gene, E4. In addition, a 250-fold amplification of B1 esterase and co-elevation of A2/B2 esterase were detected in the Southern house mosquito Culex quinquefasciatus (Devonshire and Field, 1991; Vaughan and Hemingway, 1995). Furthermore, more mRNA encoding E4 esterase was detected in resistant aphid strains than in susceptible strains. The investigation of DNA and RNA provides strong evidence that increases of esterase levels results from amplification of the esterase gene (Devonshire and Field, 1991). However, the catalytic activity of the elevated E4 in resistant strain is low, suggesting that E4 provided resistance merely by sequestering, instead of enhancing the hydrolyzing activity (Devonshire and Moores, 1982).

There is another possible mechanism of esterase-based insecticide resistance called non-elevated esterase mechanism, which is documented to confer resistance to organophosphates (OP) in some species such as the house fly. In this mechanism, mutations of amino acids in carboxylesterase enhance the hydrolase activity of the enzyme. One example is the amino acid substitution from glycine to aspartic acid (Gly\textsuperscript{137} to Asp), which
enhances the hydrolase activity in ali-esterase in the blowfly *Lucilia cuprina* and in the housefly *M. domestica* (Campbell et al., 1998).

1.2.3.3 Glutathine S-transferase (GST)-based resistance

Glutathine S-transferases (GSTs) are one of the major detoxification enzymes in both invertebrates and vertebrates. These enzymes exert their functions of metabolizing insecticides, catalyzing dehydrochlorination of insecticidal molecules, or facilitate their conjugation with reduced glutathione (GSH) (Enayati et al, 2005). This makes the resultant products more soluble in water and hence more easily excreted (Prapanthadara et al, 1993).

Like cytochrome P450s, GSTs belongs to a multiple gene family. Enhanced activity of GSTs might result from overexpression of one or more GST genes (Grant et al, 1991). The elevation of GSTs confers resistance against organophosphates to a wide range of insect species. For instance, the high activity of GSTs provides resistance against organophosphates in the diamond back moth *Plutella xylostella* (Chiang and Sun, 1993). In brown plant hopper, *Nilaparvata lugens*, the enhanced activity of GSTs results in resistance against permethrin (Vontas et al., 2002). In the malaria vector *Anopheles gambiae*, resistance against DDT resulting from the presence of high levels of GSTs has also been documented (Ortelli et al., 2003; Habig et al., 1974). The same mechanism of has also been found in other Diptera, including *M. domestica* and *D. melanogaster* (Enayati et al., 2005).

1.2.4 Reduced sensitivity of target sites

1.2.4.1 Insensitivity of insect voltage-gated sodium channels

1.2.4.1.1 The voltage-gated sodium channel

The voltage-gated sodium channel is a trans-membrane protein complex that forms sodium ion channels. They are located on the membranes of electrically excitable cells in both vertebrates and invertebrates, being responsible for the initiation and propagation of
action potentials of electrically excitable cells in many systems such as nervous systems, skeletal and cardiac muscle (Zlotkin, 1999; Dong, 2007).

The pathways for the voltage-gated sodium channel to initiate an action potential have been revealed by the voltage clamp technique (Hodgkin and Huxley, 1952). When no signal arrives, the sodium channel is in a resting phase (deactivated), where the membrane of excitable cells possesses a selective permeability. As a result, sodium ions are kept outside the cell while chloride and potassium ions are kept inside, resulting in a resting potential on the polarized membrane. When the stimulus is strong enough, sodium channels open (activate), allowing for a sodium ion flux into the cell, therefore depolarizing the membrane. Within milliseconds after the activation, the sodium channel spontaneously closes (inactivates) when the action potential reaches its peak. The inactivation blocks the in-flux of sodium ions while allowing potassium ions to flux out, re-polarizing the cell membrane. Thus, the inactivation of the sodium channel contributes to the falling phase of the action potential (Dong, 2007). The further out-flux of potassium ion will then cause hyper-polarization (cell drops temporarily below the resting potential) (Zlotkin, 1999). After the hyper-polarization stage, the cell membrane gradually returns to the resting potential. The sodium channel is then available for the next stimulus.

The structure of voltage-gated sodium channels is best understood in mammals. Mammalian sodium channels contain a 260 kDa trans-membrane α subunit and one or more 33-36 kDa auxiliary β subunits (Yu and Catterall, 2003). The α subunit contains the functional domains and is believed to be responsible for the main function of the entire channel, as demonstrated by the finding that the α subunit alone could generate sodium currents when expressed in *Xenopus* oocytes without the assistance of β subunits (Catteral, 2000; Goldin, 2003). The α subunit consists of four homologous domains (I-IV). Within each domain, there are six putative trans-membrane helical segments (S1-S6), which are connected
by small extra or intracellular loops (Yu and Catterall, 2003). It has been demonstrated that
S4 is the most conserved and is reported to be involved in the voltage-sensing mechanism
(Cordon, 1997). The extracellular loop connecting S5 and S6 in each domain contains amino
acids that are demonstrated to determine the ion selectivity (Zlotkin, 1999), while several
amino acids on the intracellular loops between S4 and S5 serve as a docking receptor for fast
inactivation of the sodium channel (Eaholz et al., 1994). The α subunit is believed to play a
role in regulating the channel gating or protein expression (Zlotkin, 1999).

Current understanding of insect voltage gated-sodium channels is largely derived from
work with mammals (Dong, 2007). Currently, the realization that mutations on insect
voltage-gated sodium channels were involved in insect resistance to insecticides has attracted
more and more researchers to study the insect voltage-gated sodium channel. Also, the
successful heterologous expression of the sodium channel gene in Xenopus oocytes is
facilitating research into the functional, pharmacological, biochemical and molecular bases of
the insect sodium channel.

1.2.4.1.2 Structures of the insect voltage-gated sodium channel proteins

The structure of the insect voltage-gated sodium channel was not clear until the
successful isolation of sodium channel genes by different methods during 1980s (Dong,
2007). Insect voltage-gated sodium channels differ from those in mammals in that they only
possess a 241kDa α subunit and lack auxiliary β subunits (Zlotkin, 1999). The structure of the
α subunit is highly similar with that of mammalian sodium channels (Zlotkin, 1999). In
addition, a 50kDa regulatory subunit was found in the functional expression of Drosophila
sodium channel genes and was named TipE (Feng et al., 1995). The TipE subunit can increase
current amplitudes and the rate of inactivation, suggesting that this subunit might be
functionally analogous to sodium channel β subunits in vertebrates (Feng et al., 1995). A
similar structure was also found in the house fly and designated as Vsscβ, which is considered to be orthologous to the *D. melanogaster* TipE (Lee et al., 2000a).

1.2.4.1.3 The insect voltage-gated sodium channel gene and its functional expression

Two putative sodium channel genes were isolated from *Drosophila melanogaster* in the 1980s, and were named *DSC1* and *para*, respectively. *DSC1* was isolated by screening a *D. melanogaster* genomic DNA library using an eel sodium channel gene as the probe (Salkoff et al., 1987). The *para* gene was isolated from mutants that are responsible for the phenotype of “temperature sensitive paralyze” (Loughney et al., 1989). Later on, another sodium channel gene resembling *DSC1*, named *BSC1*, were isolated from *B. germanica* (Liu et al., 2001). However, a recent study showed that the *DSC1* and *BSC1* actually encode a novel calcium ion channel instead of the sodium channel (Zhou et al., 2004). Protein sequence analysis shows a high similarity in primary and overall structure between predicted proteins coded by *para* and the rat brain sodium channel protein (Ramaswami and Tanouye, 1989; Salkoff et al., 1987). Functional expression of *para* further confirmed this result (Feng et al., 1995). These studies provided sufficient evidence that *para* is the structural gene encoding the voltage gated sodium channel in *D. melanogaster*.

Because of the importance of the insect voltage-gated sodium channel in the development of insecticide resistance, much effort has been placed on isolating *para*-type sodium channel genes from other species, but only a few genes have had their full-length cDNA successfully cloned. These genes include *Vssc1* from the house fly (Ingles et al., 1997; Williams et al., 1996; 1997), *BgNa* from German cockroach (*Blattella germanica*) (Dong, 1997) and *VmNa* from the varroa mite (*Varroa destructor*) (Wang et al., 2003). Thus, only these genes could be successfully expressed in *Xenopus* oocytes and demonstrated to be functional sodium channel genes (Ingles et al., 1996; Dong, 2007).
1.4.2.1.4 The insect voltage-gated sodium channel as target of insecticides

According to Zlotkin (1999), there are at least nine distinct classes of neurotoxins that are designed to target the sodium channel based on different receptor sites required for their binding. These include chemicals produced by plants and animals for self-defense or predation, such as tetrodotoxin (TTX), batrachotoxin (BTX), scorpion toxins, and insecticidal chemicals such as pyrethroids and DDT. There are four major classes of insecticides primarily targeting the insect sodium channel: DDT; pyrethroids; N-alkylamides and dihydropyrazoles (Bloomquist, 1996; Soderlund and Kniple, 1995). Moreover, indoxacarb, an oxadiazines insecticide, has also been demonstrated to target sodium channels (Silver and Soderlund, 2005).

Action of these neurotoxins affects the sodium channel in different ways. Most of these chemicals inhibit the inactivation phase of the sodium channel, causing persistent activation. This will cause overexcitation, rapid paralysis and eventual death of the target insects (Xu et al., 2006). Some chemicals, like β-scorpion toxins, poison the sodium channel by shifting the voltage-dependence of activation (Zlotkin, 1999), making activation of the sodium channel more difficult. Much effort has been made investigating different binding sites on the sodium channel for different toxins (Catteral, 1992; Cestele and Catterall, 2000; Fainzilber et al., 1994; Pauron et al., 1989; Pichon, 1984). Based on this research, Zlotkin (1999) summarized nine different binding sites. Furthermore, molecular mechanisms of these neurotoxins were investigated. According to Cestele and Catterall (2000), the mechanisms of action of neurotoxins can be categorized into three classes, including pore blocking, allosteric modulation and voltage sensor trapping.

1.2.4.1.5 The insect voltage-gated sodium channel and knockdown resistance.

Insect voltage-gated sodium channels, same as mammalian sodium channels, are responsible for generating and propagating action potentials in the insect central nerve system
and excitatory cells and are the target for pyrethroids and DDT (Zlotkin, 1999). Insensitivity of the sodium channel on its binding site results in insects’ resistance to DDT and pyrethroids, known as knockdown resistance (Kdr) (Soderlund and Knipple, 2003). The knockdown resistance was first identified in the house fly *Musca domestica* in the 1950s (Busvine, 1950). Since DDT and pyrethroid insecticides exert their function by altering the function of sodium channels (Sattelle and Yamamoto, 1988; Soderlund, 1995), studies on the mechanisms of kdr were primarily focused on point modifications of the insect voltage-gated sodium channel (Soderlund and Bloomquist, 1990). During the early 1990s, two significant studies were made that genetically linked kdr and super-kdr phenotypes to insect voltage-gated sodium channels. Williamson et al. (1993) identified the genetic linkage between super-kdr traits and the house fly *para*-type sodium channel gene (*Vssc1*) by applying restriction fragment length polymorphisms (RFLP) coupled with a DDT mortality bioassay. Knipple (1994) identified the genetic linkage between *kdr* and *Vssc1* by polymerase chain reaction and RFLP. Similar studies were also conducted on other species such as German cockroach (*Blattella germanica*), tobacco budworm (*Heliothis virescens*), and Colorado potato beetle (*Leptinotarsa decemlineata*) (Dong and Scott, 1994; Taylor et al., 1993; Lee et al., 1999b).

1.2.4.1.6 *Mutations of insect voltage-gated sodium channel in knockdown resistance*

Strong genetic linkage between the kdr resistance trait and the insect voltage-gated sodium channel gene promote the research on the analysis of entire sequence of the gene (Soderlund and Knipple, 2003). In 1996, the complete coding sequence of the insect voltage-gated sodium channel in the house fly was identified (Ingles et al., 1996; Williamson et al., 1996). Comparison of the coding sequences of susceptible, kdr and super-kdr strains revealed two consistent point mutations associated with resistance; L1014F and M918T, which occur at the 1014 and 918 amino acid residues respectively. The L1014F, also called L-to-F mutation, was identified in all cases of resistance, while the M918T occurred only in the
super-kdr phenotype. Moreover, Williamson et al (1996) observed that the M918T does not occur in the absence of L-to-F (Williamson et al., 1996). The identification of the L-to-F mutation not only provides evidence that the insect sodium channel is indeed associated with knockdown resistance, but also stimulated more studies to identify mutations corresponding to L1014F in other species. The L1014F mutation has since been identified in seven additional species, including malaria mosquito An. gambiae; German cockroach B. germanica; Colorado potato beetle L. decemlineata; the horn fly Haematobia irritans; the diamondback moth Plutella xylostella; the green peach aphid M. persicae and the common house mosquito Cx. pipiens (Soderlund and Knipple, 2003; Dong, 1997; Guerrero et al., 1997; Martinez-Torres et al., 1998; Schuler et al., 1998; Martinez-Torres et al., 1999a; 1999b; Lee et al., 1999b). Some studies were also conducted to identify M918T in other species. Besides in the house fly, this mutation only can be found in H. irritans so far (Guerrero et al., 1997).

L1014F and M918T are not the only two mutations on sodium channel that are associated with knockdown species. To date, at least 20 resistance-associated mutations on the sodium channels of 15 insect species were identified (Soderlund and Knipple, 2003). At least 10 mutations have been functionally expressed in Xenopus oocytes and are confirmed to be associated with kdr resistance (Dong, 2007). Besides L1014F and M918T, other mutations include L1014H (leucine to histidine) and V410M (valine to methionine) mutation in H. virescens, T929I (threonine to isoleucine) mutation on P. xylostella, F1519I (phenylalanine to isoleucine) in Boophilus microplus; E434K (glutamic acid to lysine) and C764R (cysteine to arginine) in B. germanica; L932F in the louse Pediculus capitis; L1770P (leucine to proline) in the mite Varroa destructor (Soderlund and Knipple, 2003; Dong, 2007).

Functional expression analysis revealed that kdr-associated mutations can change the function of the sodium channel in different ways. Wild type insect sodium channel genes
were modified by site-directed mutagenesis to contain desired mutations. The modified genes were then functionally expressed in *Xenopus* oocytes and investigated by the two-electrode voltage-clamp technique. Some tested mutations were found to significantly reduce the sensitivity of the sodium channel to insecticides, and “accelerate the rate of insecticide-reduced tail current decay” (Soderlund and Knipple, 2003). Some mutations, however, resulted in resistant sodium channel by affecting gating, making activation of the channel more difficult or inactivation much easier. Mutations L1014F and V410M, for instance, were documented to enhance the threshold for sodium channel activation, therefore reducing the sensitivity of the sodium channel to insecticides (Lee et al., 1999; Soderlund and Knipple, 2003). Mutations L1014F and T929I, however, contribute to the insensitivity of sodium channel by increasing the steady state inactivation of the sodium channel. As a result, the mutant sodium channel inactivates more rapidly than the wild type, resulting in a sodium channel with reduced sensitivity to pesticides such as deltamethrin (Vais et al., 2001).

1.2.4.1.7 Molecular basis of sodium channel mutations and its association with kdr-mediated resistance

Recently, the molecular basis of the L1014F kdr mutation was investigated for a better understanding of the regulation of sodium channel gene expression. It has been demonstrated that the L1014F mutation results from a single nucleotide polymorphism on the sodium channel gene. For instance, in *M. domestica*, L1014F mutation results from a substitution of cytosine to thymine (C-to-T) on the sodium channel gene, causing a change of corresponding codon from CTT (coding leucine) to TTT (coding phenylalanine) (Xu et al., 2006a) In the mosquito *Cx. quinquefasciatus*, a mutation from adenine to thymine (A-to-T) was detected on the sodium channel gene of the resistant strain (Xu et al., 2006b). Correspondingly, the codon coding amino acid residue 1014 was change from TTA (coding leucine) to TTT (coding phenylalanine) (Xu et al., 2006b). The L1014F mutation was also investigated in *B.*
**germanica**, where the single nucleotide on the L1014F mutant site changed from guanine to cytosine (G-to-C) (Xu et al., 2006a).

The regulation of the sodium channel gene was recently investigated (Xu et al., 2006a; 2006b). Genomic DNA and RNA sequences were compared within the same house fly and mosquito individuals from different strains. A strong relationship was observed between *kdr* allelic expression at the RNA level and levels of insecticide resistance and susceptibility. However, no correlation was found for the *kdr* allele at the genomic DNA level with levels of resistance to insecticides, indicating that the correlation between the sodium channel gene mutation and the *kdr* phenotype is probably regulated through RNA variation and RNA editing. Thus, a hypothesis was proposed that posttranscriptional regulation might play an important role in the connection of the sodium channel genotype and the mutation-mediated resistance phenotype in the house fly (Xu et al., 2006a; 2006b).

1.2.4.2 *Insensitivity of the gamma-aminobutyric acid (GABA) receptor*

Ionotropic gamma-aminobutyric acid (GABA) is the principle inhibitory transmitter in the central nerve system and nerve/muscle junctions (Usherwood and Grundfest, 1965). It is released in response to a wave of excitation and binds with receptors on the postsynaptic membrane, enhancing permeability to chloride ions. More chloride ions with negative charge (Cl\(^-\)) will then be allowed to enter the postsynaptic membrane, inducing hyperpolarization. Consequently, the hyperpolarized postsynaptic membrane requires a stimulus greater than normal to change the membrane potential enough to exceed the threshold for generation of a membrane depolarization (Watanabe et al, 2002).

GABA receptor, the receptor for \(\gamma\) -amniobutyric acid, forms Cl\(^-\) channels that contain four trans-membrane regions (M1-M4). The residue located on the extracellular domain provides the neurotransmitter binding site while the second trans-membrane region (M2) contains residues that contribute to the integral chloride channel (Buckingham et al., 2005,
Whiting, 2003). GABA receptors of insect are the targets of a number of important classes of insecticides, including cyclodienes, trioxabicyclooctanes, and picrotoxinin (PTX). These insecticides inhibit the flow of chloride ions through the receptor channel complex by binding to it (Le Goff et al., 2005, Bloomquist, 2001). It is reported that the amino acid alanine on 302 amino acid residue (A302) of the receptor occupies the binding site directly and is very important for toxin binding.

The molecular mechanism of the GABA receptor-based resistance was most extensively studied in D. melanogaster. The first invertebrate GABA receptor subunit was cloned from D. melanogaster (ffrench-Constant, 1994). Functional expression of the cDNA in Xenopus oocytes indicated that the gene encodes a functional, homo-oligomeric chloride channel that is gated by GABA (Feyereisen, 1995). Rdl (resistance to dieldrin) gene, the GABA analogues, has been associated with 4000-fold resistance in a D. melanogaster strain and has been mapped on the left arm of autosome III and D. melanogaster (ffrench-Constant, 2000). Sequencing analysis of Rdl indicated that resistance to dieldrin in D. melanogaster was associated with a point mutation on the Rdl locus which results in the amino acid substitution of alanine by serine at 302 amino acid residue (A302S) on RDL GABA-receptors (Feyereisen, 1995). This mutation is located in second trans-membrane region (M2) of the receptor subunit (Le Goff et al., 2005; ffrance-Constant et al., 1993a; 1993b). Site-directed mutagenesis and functional expression in Xenopus oocytes indicated that the mutation causes insensitivity of the chloride channel to dieldrin (ffrench-Constant et al., 1993a).

In addition to the Ala302 to Ser mutation, another mutation, Ala302 to Gly was found in other species (ffrench-Constant, 1994). Partial or full length of cDNA encoding subunits homologous to Drosophila GABA receptors subunits have been isolated from various species including Aedes aegypti (Thompson et al., 1993a), German cockroach, Blattella germanica Kaku and Matsumura, 1994), the house fly M. domestica (Thompson et al., 1993b) flour
beetle *Tribolium castaneum* (Andreev et al., 1994), Coffee berry borer *Hypothenemus hampei* (Ffrench-Constant, 1994), and whitefly *Bemisia tabaci* (Anthony et al., 1995).

1.2.4.3 **Ininsitivity of acetylcholinesterase**

Acetylcholine (ACh) is an important excitatory transmitter in the insect nervous system. Acetylcholinesterase (AChE) serves to hydrolyze acetylcholine into acetic acid and choline after their function is completed. As a result, the signal transmission at the synapse will be terminated since the AChE molecules are hydrolyzed and lose their physiological function. Inhibition of AChE by organophosphorus and carbamate insecticides (Hemingway and Ranson, 2000), prevents the hydrolysis of ACh, resulting in prolonged stimulation at synapses, which will cause uncontrolled action, paralysis and finally death of insects (Hemingway and Ranson, 2000; Mutero et al., 1994; Fournier et al., 1992).

Resistance against organophosphorus and carbamate insecticides occurs when the sensitivity of AChE to these xenobiotic is reduced. Insensitivity of AChE is usually a result of an alteration of its conformation, which is caused by multiple point mutations in its structural genes (Mutero et al., 1994; Ayad and Georghiou, 1975; Fournier et al., 1992). AChE based resistance is extensively studied in *D. melanogaster*. The gene *Ace*, coding for AChE in the central nervous system of *Drosophila*, has been cloned and characterized. Five *Ace* mutations have been identified in *D. melanogaster* by comparing sequences of resistant and susceptible strains (Feyereisen, 1995; Mutero et al., 1994; Fournier et al., 1992). These mutations include Phe115Ser, Ileu199Val, Ileu199Thr, Gly303Ala and Phe368Tyr (Mutero et al., 1994). The mutations were expressed in *Xenopus* oocytes and functionally characterized (Feyereisen, 1995). Each of these point mutations affects the sensitivity of the enzyme by acting on substrate binding, increasing the Km and lowering the Ki values for insecticides. Also, some results suggested that combination among these point mutations might result in a higher level of resistance (Feyereisen, 1995; Mutero et al., 1994).
Besides *Drosophila*, insensitivity of AChE has been identified in other species. In the house fly, resistance-related AChE mutations have been observed and identified on four sites of AChE gene. In the mosquitoes *Culex pipiens*, and *An. gambiae*, mutations such as F331W (phenylalanine to tryptophan) and G119S (glycine to serine) have also been identified (Russell et al., 2004). AChE mutations have also been identified in other species, such as the diamondback moth *Plutella xylostella* (Lee et al., 2007) and Colorado potato beetle (*Leptinotarsa decemlineata*) (Zhu et al., 1996).

1.2.5 Interactions among multiple resistance mechanisms

The co-presence of multiple resistance mechanisms is found in various species such as the house fly (Liu and Pridgeon, 2002), and the mosquito (Xu et al., 2005). The interaction of multiple resistance mechanisms confers some insect species with high levels of resistance. As previously discussed, reduced penetration alone might only confer marginal resistance against DDT in the house fly. But the combination of reduced penetration rate and enhanced activity of detoxification enzyme enhances its resistance significantly.

Some resistance mechanisms, however, might have a negative correlation with others so that they may not be able to co-exist with other mechanisms. For instance, a negative correlation was found between behavioral resistance and biochemical-physiological resistance mechanisms, including reduced penetration rate, enhanced detoxification and insensitivity of the target site (Georghiou, 1972). Increased irritability requires enhanced sensitivity of the nervous system to the insecticide, or a reduction of the penetration barriers. In addition, it requires a decreased detoxification rate so that a smaller dose of insecticide is required to reach the nervous system to induce the avoidance behavior. This negative correlation was evidenced by Hooper and Brown (1965). In their study, picture-winged flies, *Euxesta notata* (Diptera, Ulidiidae) selection by malathion through facultative exposure lead to the development of increased irritability to insecticide. As a result, the selected strains
possessed higher tendencies to avoid exposure to malathion. However, when topical application was performed on this highly irritable population with the same insecticide, an increase in susceptibility was detected, implying a decrease in biochemical-physiological resistance.

1.3 The mosquito as a pest

1.3.1 The mosquito and their medical importance

Mosquitoes belong to family Culicidae (Diptera) and have more than 3000 documented species (Munstermann and Conn, 1997). They are holometabolous insects, which have four different stages in their life cycle, including egg, larva, pupa, and adult. Adult mosquitoes have distinct feeding habits between the two sexes. Adult male mosquitoes mainly feed on sugar, while most adult females need to feed on blood from animals and humans for viable egg production.

The repeated blood feeding habit of females makes them important carriers and transmitters of various disease agents. Three important genera of mosquito disease vectors to humans are Culex, Aedes and Anopheles. Culex (such as C. quinquefasciatus and C. pipiens), transmit West Nile virus (WNV) (Sardelis et al., 2001), filariasis and Japanese encephalitis (Hemingway and Ranson, 2000). Aedes, (such as A. albopictus, and A. aegypti) transmit yellow fever and dengue fever (Liu et al., 2006). Anopheles gambiae is the major transmitter of the malaria causative agent Plasmodium falciparum (Hemingway and Ranson, 2000). Some of these mosquito vectors are globally distributed, increasing the scale and risk of disease transmission as well as the difficulties of work for vector control (Hemingway and Ranson, 2000).
1.3.2 Insecticide resistance in the mosquito

Mosquito-borne diseases have been primarily controlled by chemical means. However, development of insecticide resistance is amazingly rapid, resulting in resurgence of vectors and their transmitted disease. Mosquito management by DDT began in 1946 to eradicate malaria. The first case of resistance to DDT was documented one year later (Hemingway and Ranson, 2000). Since then, resistance to insecticides has been reported in >100 mosquito species (Hemingway and Ranson, 2000). For instance, resistance against organophosphates and carbamates occurs commonly in *Culex quinquefasciatus*, and resistance to pyrethroids has been documented in *Culex, Anopheles*, and *Aedes* (Hemingway and Ranson, 2000). Multiple resistance mechanisms have been found in these mosquitoes.

1.3.2.1 Target site insensitivity

Insecticide resistance conferred by target site insensitivity is common in mosquito species. Point mutations (such as L1014F mutation) in the mosquito *para*-type voltage-gated sodium channel results in a high level of resistance to DDT and pyrethroid insecticides in many species such as *Culex pipiens* (Martinez-Torres et al., 1999a), *Culex quinquefasciatus* (Xu et al., 2003), *Aedes albopictus* (Liu et al., 2006a), *Aedes aegypti* (Brengues et al., 2003), and *Anopheles gambiae* (Martinez-Torres et al., 1998). Point mutations on acetylcholinesterase (AChE) are associated with resistance against organophosphate and carbamate insecticides. One example is the mutation from glycine to serine on amino acid residue 119 of AChE in *Culex quinquefasciatus*, which confers resistance to a crystalline organophosphate insecticide, Chlorpyrifos (Liu et al., 2005). Insensitivity of GABA receptors is also found in resistant mosquitoes, which confers resistance to cyclodiene. The cyclodiene insecticide resistance gene has been cloned and sequenced in *Aedes aegypti* and the mutation of alanine to serine in amino acid residue 302 has been found to be associated with cyclodiene resistance (Thompson et al., 1993a).
1.3.2.2 Increased detoxification and sequestration

Increased detoxification and sequestration is another important resistance mechanism in mosquitoes. The increase in the quantity of insect cytochrome P450s due to gene over expression has been detected in Culex and Anopheles mosquitoes (Nikou et al., 2003; Kasai et al., 2000). Enhancement of esterase activity resulting from gene amplification has been documented in three genera: Culex, Anopheles and Aedes (Hemingway and Karunaratne, 1998; Brogdon and Barber, 1990; Mourya et al., 1993). The overproduction of GST has been reported in Anopheles and Aedes (Ortelli et al., 2003; Grant and Hammock, 1992).

1.4 The house fly as a pest
1.4.1 The house fly

The house fly, Musca domestica, belongs to Musca (Diptera: Muscidae). It is a holometabolous insect. The lifecycle of the house fly consists of four stages including egg, larva, pupa and adult. The house fly is an important pest of livestock, poultry facilities and human. Adult flies are responsible for the phoretic transmission of various disease agents to humans and domesticated animals. They vector bacteria and viruses in their excreta and filth when they alight on surfaces and indirectly transmit these etiological agents to humans through their interaction with human food and transmit pathogens include bacterial agents of Typhoid fever, Bacillary dysentery and trachoma, and protozoan amoebic dysentery (West, 1951). Current studies indicate that the house fly carries more than 100 different pathogens, and is able to transmit over 65 of them to livestock and humans (Service, 1996).

1.4.2 Insecticide resistance in the house fly

House fly control is an important issue in dairy farms and public places regarding its impact on animal and human health (Scott et al., 2000). Since chemical control is the primary control method, rapid development of its resistance against a wide variety of insecticides
makes control increasingly difficult (Learmount, 2002). To date, a high level of resistance has been documented in the house fly for major insecticides including pyrethroids, fipronil, spinosad, carbamates and organophosphates (Scott et al., 2000). Multiple resistance mechanisms have also been documented in resistant house fly strains (Scott, 1999).

1.4.2.1 Target site insensitivity

Insensitivity of voltage-gated sodium channels is common in house fly strains that are resistant to DDT and pyrethroid insecticides. The association of the sodium channel and knockdown resistance (kdr) has been extensively studied in the house fly. To date, the kdr and super-kdr trait have been genetically mapped to autosome 3 in resistant house flies (Soderlund and Knipple, 1999). The house fly para-type sodium channel gene has been isolated and characterized (Ingles et al, 1996), and two important mutations, L1014 F and M918T, have been identified (Williamson et al., 1996).

1.4.2.2 Increased detoxification and sequestration

P450-mediated resistance in the house fly has been extensively studied in several resistant strains through an increased quantity of cytochrome P450s (Feyereisen et al, 1989, Liu and Scott, 1996, Liu and Yue, 2000). Several P450 genes, such as CYP6D1 and CYP6A1 have been cloned and characterized (Feyereisen et al, 1989; Liu and Yue, 2000). Esterase-based resistance has also been documented in the house fly. One esterase gene named MdaE7 was cloned and characterized (Claudianos, et al., 1999). A single mutation from glycine to aspartic acid at amino acid residue 137 of ali-esterase has been identified to enhance the hydrolase activity of the enzyme and to be associated with organophosphate resistance (Van and Oppenoorth., 1959; Oppenoorth and Van., 1960). The GST-based resistance is another resistance mechanism reported in the house fly. Two classes of GSTs have been identified in
house fly strains with resistance to organophosphate and carbamate insecticides (Fournier et al., 1992)
Chapter 2 Research Goal and Specific Objectives

2.1 Introduction

Mosquitoes are responsible for transmitting various deadly human diseases. The mosquito species, *Culex quinquefasciatus*, is a vector of West Nile virus (WNV), filariasis, Japanese encephalitis virus (JEV) and Saint Louis encephalitis virus (SLEV) (Hemingway and Ranson, 2000). The house fly, *Musca domestica* is also a major pest in livestock and poultry facilities. House flies are medically important as well since they carries over 100 different pathogens and are responsible for transmitting more than 65 diseases to human and livestock (Service, 1996). Currently, pyrethroid insecticides are most widely applied insecticides for the control of mosquitoes and house flies because of their high insecticidal effect to target pests and low toxicity to humans and other animals (Hougard et al., 2002). However, insecticide resistance to pyrethroids has developed in both mosquitoes and house flies (Hemingway and Ranson, 2000). Pyrethroid insecticides are known to exert their insecticidal effects by altering the function of the voltage-gated sodium channel in the nerve membranes of insects. Insensitivity of the insect voltage-gated sodium channel resulting from point mutations in its amino acid sequence has been demonstrated to be closely associated with resistance to pyrethroids, termed knockdown resistance (kdr). The molecular basis of kdr-mediated resistance has been investigated in several studies (Soderlund and Knipple, 2003). Yet the genetic linkage the insect voltage-gated sodium channel gene, as well as the regulation of sodium channel gene expression and their connetion with insecticide resistance remains to be further studied.
2.2 The research goal and specific objectives

The goal of my research is to understand the molecular and genetic basis of sodium channel insensitivity-mediated resistance in the mosquito and the house fly. Four specific objectives will be addressed in my research in order to reach this goal: 1) Investigating the genetic inheritance of pyrethroid resistance in house flies; 2) Mapping the voltage-gated sodium channel gene in house flies; 3) Investigating the regulation for gene expression of L1014F mutations in house flies; and 4) Investigating the genomic organization of the sodium channel gene in the mosquito, *Cx. quinquefasciatus*.

2.2.1 Investigating the genetic inheritance of pyrethroid resistance in house flies

In my first objective, I will investigate genetic linkage and inheritance of pyrethroid resistance. I will also characterize the chromosomal contribution to the evolution of pyrethroid resistance under the pressure of pesticide selection in the house flies. Two house fly strains, resistant ALHF and susceptible aabys, will be used in the study. ALHF a wild type house fly strain, was originally collected in 1998 from a poultry farm in Alabama after a failure to control the flies (Liu and Yue 2000). This strain obtained a high level of resistance after subsequent selection with permethrin for six generations. It is biannually selected with permethrin to maintain the resistance level (Liu and Yue, 2000). aabys is a susceptible marker strain with five recessive and visible morphological markers: *ac* (alicurve, the wings are curved upwards on their tips), *ar* (aristapedia, leg like aristae of antennae), *bwb* (brown body), *ye* (yellow eyes), and *sw* (snipped wings), on autosomes 1, 2, 3, 4, and 5, respectively. Nine house fly lines with different autosomal combinations will be generated by crossing ALHF with aabys. Bioassays will be conducted to investigate the level of resistance to permethrin for each line. The LD₅₀ values for each house fly line will be calculated using POLO probit analysis software. The relative resistance level of the nine lines before and after permethrin
selection will be compared. Accomplishment of this objective will provide important materials and essential information for subsequent research in the second and third objectives.

2.2.2 Mapping the insect voltage-gated sodium channel in the house fly.

I will map the insect voltage-gated sodium channel gene to a specific autosome in the house fly. Previous research has linked the kdr trait to autosome 3 of the house fly (Soderlund and Knipple, 1999). Also, L1014F mutation in the sodium channel gene of the house fly has been demonstrated to be closely associated with the kdr phenotype in resistant house flies (Williamson et al, 1993). Thus, my hypothesis is that the structural gene of the house fly voltage-gated sodium channel is located on autosome 3. To test my hypothesis, five house fly backcross lines (section 4.2.2) will be used. RNA will be extracted from individual flies and reverse transcript (RT)-PCR will be performed to amplify sodium channel cDNA fragments containing the L-to-F locus. Single nucleotide polymorphism (SNP) determination with an ABI Prism SNaPshot Multiplex Kit will be used to investigate the allelic variation of L-to-F locus among the five different house fly lines. The results of the SNP determination will be analyzed on the ABI Prism® 3100 Genetic Analyzer (A&B Applied Biosystems). This is the first attempt to map the sodium channel gene at the genomic level. Moreover, successful mapping of the sodium channel gene in the house fly will allow me to conduct further study to characterize whether the transcriptional regulation is involved in the sodium channel gene expression (Objective 3).

2.2.3 Investigate the regulation for gene expression of L-to-F mutations in the house fly

Based on the genetic linkage results, regulation of the gene expression of the L-to-F mutation in the house fly will be investigated. I hypothesize that the L-to-F allelic expression in the house fly undergoes post-transcriptional regulation, and that there are regulatory
factor(s) that can be mapped to certain autosomes. To test my hypothesis, nine house fly lines (section 5.2.2) and parental strains of ALHF and aabys will be used in the study. Both SNP determination and bioassay experiments will be conducted on the nine lines. This proposed study will provide evidence of whether posttranscriptional regulation plays an important role in the correlation of the genotype of the L-to-F locus in the sodium channel gene and the phenotype of kdr-mediated resistance.

2.2.4 Investigating the differential genotype at the L-to-F locus of multiple sodium channel genes in the mosquito, Culex quinquefasciatus

Previous work in our lab has detected two copies of the sodium channel gene in the mosquito, Cx. quinquefasciatus by Southern blot analysis (Xu, 2006). Yet, a single sodium channel transcript was identified by Northern blot analysis (Xu, 2006). Based on these result, I hypothesize that multiple sodium channel genes are present in the genome of the mosquito Cx. quinquefasciatus, but only one of the copies can be functionally expressed. To test my hypothesis, I will calculate the frequency of L-to-F (A/T) genotype at genomic DNA (gDNA) level as well as the frequency of its allelic expression at RNA level, and compare the data with the level of resistance of tested mosquito strains. Five strains will be used in this study: HAmCq\textsuperscript{G0} and MAmCq\textsuperscript{G0} were collected from Huntsville and Mobile, Alabama, in 2002, respectively (Liu et al, 2004). HAmCq\textsuperscript{G8} is the HamCq strain that has been continuously selected with permethrin for eight generations after collection. MAmCq\textsuperscript{G6} is the MAmCq strain that has been continuously selected with permethrin for six generations after collection. S-Lab is a laboratory susceptible strain. My first step is to confirm the presence of multiple sodium channel genes by amplifying and analyzing sodium channel gene genomic DNA fragments in all tested strains through polymerase chain reaction (PCR) and nucleotide sequencing. My next step is to identify the frequency of the allelic expression at the L-to-F in each of the gDNA fragments. Genotypes of the L-to-F locus on different sodium channel
gene gDNA fragment, as well as the allelic expression of L-to-F locus on sodium channel gene cDNA fragment will be investigated by SNP determination. This study will, for the first time, confirm the presence of multiple sodium channel genes in an insect. In addition, results of my study will help us to understand the complexity of the sodium channel gene expression at the L-to-F locus.

2.3 Significance of research

Studies of the genetic and molecular basis of kdr-mediated pyrethroid resistance are fundamental since it will help us to understand the causal link between insect voltage-gated sodium channel and kdr-mediated insecticide resistance in both mosquitoes and house flies. A more comprehensive understanding of knockdown resistance provided by this study might facilitate the development of new, more effective pesticides as well as novel pest control strategies, which will contribute to successful control of the mosquito and the house fly as well as the human diseases transmitted by these two pests.
Chapter 3 Genetic Studies on the Evolution of Pyrethroid Resistance Development in House Flies under the Pressure of Pesticide Selection

3.1 Introduction

The house fly *Musca domestica* is an agriculturally and medically important insect pest since it is responsible for transmitting both animal and human pathogens (Burgess, 1990). Pyrethroid insecticides are one of the most widely applied insecticides for control of the house fly because of their high insecticidal effectiveness and low toxicity to humans and other animals. However, resistance to pyrethroids has been developing so rapidly in the house fly that it poses a severe threat to house fly control (Scott et al, 1989).

The house fly stain, ALHF, exhibits high level of resistance to pyrethroids (Liu and Yue, 2000). According to our previous study, the main mechanisms of pyrethroid resistance in this strain include increased detoxification mediated by P450 monoxygenases and decreased sensitivity of voltage-gated sodium channels (kdr) (Liu and Yue, 2001). Our previous study have been isolated and mapped multiple P450 genes to autosome 5 in the ALHF strain (Zhu et al, 2008a, b). Furthermore, the genetics of kdr-mediated resistance has also been studied in ALHF strain. The kdr, as well as another resistant mechanism, pen (ie. decreased rate of cuticular penetration) have been linked to autosome 3 in ALHF (Liu and Yue 2001). Although the autosomal linkages of pyrethroid resistance in the ALHF have been studied, the contribution of each autosome to the evolution of pyrethroid resistance is unclear.

Previous studies have also linked the factors responsible for the over-expression of P450 genes to autosomes 1 and 2 (Liu and Yue, 2001, Ahu et al. 2008 a,b) in house flies,
suggested that factors on autosome 2, and probably on autosome 1, have regulatory functions in P450-mediated pyrethroid resistance. However, whether or not the same regulation factors are involved in the regulation of the kdr-mediated resistance was not explored.

This study focuses on the genetic linkage and inheritance of pyrethroid resistance to characterize the autosomal contribution to the evolution of pyrethroid resistance. Nine house fly lines with different combinations among the 5 autosomes were isolated from crosses of strains ALHF and aabys. Pesticide selection by permethrin was conducted on each of 9 resulting lines. The level of resistance to permethrin was examined for each line before and after permethrin selection through bioassay (topical application) and the resistance factors were investigated. This study is the first step to examine the relative contribution of sodium channel insensitivity to pyrethroid resistance. It is also fundamental for genetic mapping for sodium channel gene as well as the regulation of sodium channel gene expression at the kdr locus.

3.2 Material and methods
3.2.1 House fly strains

Two house fly parental strains, ALHF and aabys, were used as parental strains in this study. ALHF was originally collected in 1998 from a poultry farm in Alabama. This strain obtained a high level of resistance after subsequent selection with permethrin for six generations. It is biannually selected with permethrin to maintain the resistance level (Liu and Yue, 2000). The other strain, aabys, is a susceptible lab marker strain homozygous for five recessive morphological markers, which are ac (alicurve, the wings are curved upwards on their tips), ar (aristapedia, leg like aristae of antennae), bwb (brown body), ye (yellow eyes), and sw (snipped wings), on autosomes 1, 2, 3, 4, and 5, respectively. The aabys strain was obtained from Dr. J. G. Scott (Cornell University).
3.2.2 Genetic crosses

To examine the genetic basis of kdr-mediated resistance, nine homozygous house fly lines were generated (Figs. 3.1. and 3.2) by genetic crosses of ALHF and aabys house flies. Briefly, virgin females for crossing were collected within 8 hrs after emergence. Crosses between ALHF and aabys were conducted. Males of the F1 generation were backcrossed with virgin females of aabys. Nine phenotypes were isolated from the backcross generation (BC1), with genotypes: ac/ac, +/ar, +/bwb, +/ye, +/sw (A2345); +/ac, ar/ar, +/bwb, +/ye, +/sw (A1345); +/ac, +/ar, bwb/bwb, +/ye, +/sw (A1245); +/ac, +/ar, +/bwb, ye/ye, +/sw (A1235), +/ac, +/ar, +/bwb, +/ye, sw/sw (A1234), ac/ac, +/ar, +/bwb, ye/ye, sw/sw (A23), +/ac, ar/ar, +/bwb, ye/ye, sw/sw (A13), +/ac, +/ar, +/bwb, ye/ye, sw/sw (A123), and ac/ac, ar/ar, +/bwb, ye/ye, sw/sw (A3). Each line bears a unique combination of visible mutant phenotypes, indicating that the respective autosomes are homozygous for the recessive mutant-type markers derived from aabys and the other autosomes are heterozygous for the dominant wild-type markers from ALHF since crossing-over rarely occur in male house flies (Liu and Yue, 2000). The name of each line was given according to the four autosomes bearing wild-type markers from ALHF. For instance, the A2345 strain had wild-type markers on autosomes 2, 3, 4, 5 from ALHF and the mutant marker on autosome 1 from aabys.

To make the nine lines completely homozygous for all of their 5 autosomes, additional genetic isolation methods were applied. Generally, the nine BC1 lines were saved and then mass-crossed for four generations. For each generation, flies were sorted for appropriate phenotypes and selected with permethrin at a dose that killed approximately 70% of treated individuals. One hundred single-pair crosses were then set up for each line as described by Liu and Scott (Liu and Scott, 1995). Families (offspring of individual single pair crosses) from each single pair cross that showed only individuals of the desired phenotype for three consecutive generations were combined and then selected with permethrin for one generation.
3.2.3 Bioassays

Topical application was performed on two parental strains, ALHF and aabys, and nine house fly lines, A2345, A1345, A1245, A1235, A1234, A3, A13, A23, A123, by dropping 0.5-µl of insecticide (dissolved in acetone) to the thoracic notum of 2-3-day old female flies (Liu and Scott, 1995). Each bioassay consisted of 15 flies per dose and four or five doses that give >0 and <100% mortality. Control groups received acetone alone. Treated flies were put into 6 oz. Sweetheart ice cream cups (Sweetheart Cup Co., Owings Mills, MD) with a piece of dental wick saturated in 15% sugar water. Mortality was assessed after 24 h. Flies that did not move were scored as dead. All tests were performed at 25±2°C and replicated at least three times on different days for one generation. Bioassays were replicated for each line for two consecutive generations using the same methodology as described above. Bioassay data were pooled and probit analysis was conducted by POLO probit analysis software. Significant differences in resistance level of house fly lines were based on nonoverlap of 95% confidence intervals.

3.3 Results and discussion

The level of resistance to permethrin in the ALHF strain was 1054-fold compared to the susceptible aabys strain (Table 3.1). Resistance level in the A1235 strain (flies with mutant-type markers on autosome 4) was 932.5-fold (Table 3.1), which was similar to the ALHF (1054-fold). This indicated that factors on autosome 4 are not involved in pyrethroid resistance, which agrees with our previous work (Liu and Yue, 2001).

Strains A1245 and A1234 (flies with mutant-type markers on autosomes 3 and 5 from aabys) possess a much lower resistance level to permethrin (101.5- and 201-fold resistance, respectively, Table 3.1), indicating that factors on autosome 3 and 5 are involved in permethrin resistance in the ALHF strain (Table 3.1). Strains A2345 and A1345 (flies with mutant-type markers on autosome 1 and 2 from aabys) had 389- and 117.5-fold resistance.
respectively (Table 3.1), which is much lower than A1235 and ALHF but higher than strains A1245 and A1234, suggesting that factors on autosomes 1 and 2 also have a role in pyrethroid resistance, although they were not as important as those on autosomes 3 and 5. These results further confirm the previous work by Liu and Yue (2001), but contrast with the work on LPR, which suggested that factors on autosome 5 are not involved in pyrethroid resistance and that factors on autosome 1 and 2 are more important (Liu and Scott, 1995). In addition, the resistance level in A1345 was much lower than A2345, indicating that factors on autosome 2 might be relatively more important to pyrethroid resistance than those on autosome 1. Thus, factors on autosomes 1, 2, 3 and 5 combine together to confer pyrethroid resistance in the ALHF strain while factors on autosome 4 are not involved. These results indicate the presence of multiplicative interactions between resistance factors on different autosomes in the ALHF strain, a finding which was also identified in the LPR strain (Georghiou, 1969; Liu and Scott, 1995).

Since kdr-mediated resistance has been linked on autosome 3, I also compared the levels of resistance in house fly line with autosome 3 from ALHF, A3, and lines with the combination of the autosome 3 with other autosomes from ALHF, A13, A23, and A123, to characterize the importance of factors on autosome 3. I found that when factors on autosomes 1, 2 and 3 of ALHF (A123) are combined together, they had a higher contribution to resistance compared with the factors on autosome 3 alone, i.e., A3 (Table 3.1), suggesting that interaction of factors on autosomes 1 and 2 with the factors on autosome 3 plays the role in the kdr-mediated insecticide resistance. I further investigated the developmental rate of permethrin resistance among all 9 house flies lines (Table 3.1) by comparing the levels of insecticide resistance between permethrin selected lines and their non-selected counterparts. Non-selected house fly lines were house fly lines that did not undergo the processes of permethrin selection and single-pair crosses. These lines had a lower level of resistance than
their offspring after permethrin selection (Table 3.1). This discovery may suggest 1) the resistance factors are incompletely recessive. In the non-selected house flies, their genotypes were not homozygous for the alleles of resistant factors. 2) a evolution phenomenon of resistance development following the insecticide selection.

To examine the contribution of each autosome to the evolution of pyrethroid resistance, we compared the rate of pyrethroid resistance development under the pressure of permethrin selection. After permethrin selection, the resistance level in the A1235 line was dramatically increased from 212-fold to 932-fold, which is as similar as that in ALHF parental strain. The significant increase in the level of resistance in this line indicated that factors on autosome 4 were not involved in pyrethroid resistance, nor was there any contribution to the development of resistance under selection, since in this line, autosome 4 bearing the yellow-eye mutant marker was derived from the susceptible aabys strain. The rate of resistance development for A2345, A1345 and A1234 was much slower than that of A1245, indicating that the lack of selectable resistance factors on autosomes 1, 2, and 5 results in a much slower evolution of resistance than does the lack of selectable factors on autosome 3. Recently, Zhu et al. (2008a, b) have isolated and mapped multiple P450 genes to autosome 5 in the ALHF strain. Furthermore, the factors controlling the overexpression of these p450 genes have been genetically linked to autosomes 1 and 2 (Liu and Yue, 2001, Ahu et al. 2008 a,b). My finding may suggest that P450-monoxygenases (encoded by the genes on autosome 5) may serve as the major mechanism in permethrin resistance. Lacking them, the resistance developed very slow. This finding also suggests that the P450 factors on autosome 5 might be more important for the insect's adaptation to pyrethroids than the kdr factors on autosome 3, which are responsible for decreasing the sensitivity of the target site. Whereas, the finding of slow rate of resistance development in the permethrin selected A2345 and A1345 lines may suggest that lack of resistance factors on autosomes 1 and 2 might slow the evolution of pyrethroid
resistance. Factors on autosome 2 might be more importantly involved in the resistance phenomenon than the factors on autosome 1 since the house fly line (A1345) lacking the factors on autosome 2 had much lower level of resistance than the line lacking the factors on autosome 1 from ALHF. The involvement of factors on autosomes 1 and 2 in insecticide resistance in the house fly has been identified in previous studies (Liu and Scott, 1996; Carino et al., 1994). In the pyrethroid resistant LPR strain, the overexpression of P450 gene CYP6D1 on autosome 1, which is responsible for high level of pyrethroid resistance, was reported to be regulated by trans-regulatory factor(s) on autosome 2 (Liu and Scott, 1996). In the organophosphate-resistant Rutgers strain, the elevated level of the P450, CYP6A1 on autosome 5 was also reported to be trans-regulated by factors on autosome 2 (Carino et al., 1994). In a previous study on ALHF (Liu and Yue, 2001), factors on autosomes 1 and 2 were reported to be involved in pyrethroid resistance, indicating a strong possibility that these factors might be involved in regulating the overexpression of P450 genes in ALHF as they do in the LPR and Rutgers strains.

In conclusion, autosome 4 was not involved in pyrethroid resistance in the ALHF strain, which supports our previous findings. Factors on autosomes 3 and 5 (kdr and P450-monoxygenases, respectively) contributed the most to pyrethroid resistance. Under the pressure of selection, the factors on autosome 5 might contribute more to the development, or evolution, of pyrethroid resistance than the factors on autosome 3. Factors on autosomes 1 and 2 also play a role in pyrethroid resistance. Three possible roles for the factors on autosomes 1 and 2 in pyrethroid resistance in the ALHF strain are proposed: 1) regulation of the over-expression of P450 genes; 2) regulation of sodium channel gene expression at the kdr locus; 3) facilitate multiplicative interaction between different resistance mechanisms.
Table 3.1 Toxicity of permethrin to ALHF, aabys, and nine selected and non-selected house fly lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>n(^a)</th>
<th>LD50(^b) (95%CI)</th>
<th>Slope (SE)</th>
<th>RR(^c)</th>
<th>Strain</th>
<th>n(^a)</th>
<th>LD50(^b) (95%CI)</th>
<th>Slope (SE)</th>
<th>RR(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aabys</td>
<td>280</td>
<td>2(1-2)</td>
<td>3.0(0.3)</td>
<td>_</td>
<td>ALHF</td>
<td>284</td>
<td>2108(1640-2553)</td>
<td>2.0(0.3)</td>
<td>1054</td>
</tr>
<tr>
<td>A2345</td>
<td>80</td>
<td>466(287-973)</td>
<td>1.6(0.4)</td>
<td>233</td>
<td>A2345</td>
<td>315</td>
<td>778(682-902)</td>
<td>3.0(0.3)</td>
<td>389</td>
</tr>
<tr>
<td>A1345</td>
<td>62</td>
<td>232(98-555)</td>
<td>1.1(0.3)</td>
<td>116</td>
<td>A1345</td>
<td>250</td>
<td>235(204-265)</td>
<td>3.8(0.5)</td>
<td>117.5</td>
</tr>
<tr>
<td>A1245</td>
<td>80</td>
<td>57(38-86)</td>
<td>2.0(0.4)</td>
<td>28.5</td>
<td>A1245</td>
<td>377</td>
<td>203(84-639)</td>
<td>1.9(0.2)</td>
<td>101.5</td>
</tr>
<tr>
<td>A1235</td>
<td>80</td>
<td>424(290-680)</td>
<td>2.2(0.5)</td>
<td>212</td>
<td>A1235</td>
<td>407</td>
<td>1865(1159-3158)</td>
<td>1.4(0.1)</td>
<td>932.5</td>
</tr>
<tr>
<td>A1234</td>
<td>80</td>
<td>223(161-307)</td>
<td>3.2(0.6)</td>
<td>112</td>
<td>A1234</td>
<td>392</td>
<td>402(305-561)</td>
<td>2.0(0.2)</td>
<td>201</td>
</tr>
<tr>
<td>A3</td>
<td>100</td>
<td>57(36-91)</td>
<td>1.4(0.3)</td>
<td>28.5</td>
<td>A3</td>
<td>315</td>
<td>74(62-89)</td>
<td>2.1(0.2)</td>
<td>37</td>
</tr>
<tr>
<td>A13</td>
<td>60</td>
<td>66(48-93)</td>
<td>3.7(0.9)</td>
<td>33</td>
<td>A13</td>
<td>752</td>
<td>92(80-105)</td>
<td>2.7(0.2)</td>
<td>46</td>
</tr>
<tr>
<td>A23</td>
<td>100</td>
<td>75(45-121)</td>
<td>1.5(0.3)</td>
<td>37.5</td>
<td>A23</td>
<td>225</td>
<td>112(84-145)</td>
<td>1.9(0.2)</td>
<td>56</td>
</tr>
<tr>
<td>A123</td>
<td>100</td>
<td>71(51-97)</td>
<td>3.1(0.6)</td>
<td>35.5</td>
<td>A123</td>
<td>446</td>
<td>124(85-182)</td>
<td>1.9(0.2)</td>
<td>62</td>
</tr>
</tbody>
</table>

\(^a\) Number of house flies tested.

\(^b\) LD50 values in nanograms per house fly.

\(^c\) RR, LD50 of the resistant strain/LD50 of the, susceptible aabys strain.
Figure Legends

Fig.3.1 Image exhibition of the nine house fly strains used in the experiments and the two parental strains: ALHF and aabys.

Fig.3.2 Diagrammatic representation of the genetic isolation of nine house fly lines generated by crossing ALHF and aabys. House fly lines were named according to the autosomes bearing the wild-type marker derived from ALHF
Fig. 3.1
Fig. 3.2

1. Each line was mass crossed and appropriate markers were sorted for 4 generations with/without permethrin selection.
2. One hundred single pairs were then set up for lines that had undergone morphology and permethrin selection, yielding the following lines:

- A2345
- A1345
- A1245
- A1235
- A1234
- A3
- A23
- A13
- A123
Chapter 4 Genetic Mapping of the Sodium Channel Gene in the House Fly

4.1 Introduction

Pyrethroid insecticides exert their function by affecting insect voltage-gated sodium channels in the nerve membranes. The insensitivity of the sodium channel to pyrethroid insecticides resulting from amino acid mutations is an important mechanism of pyrethroid resistance, which is known as knockdown resistance (kdr). A substitution of leucine to phenylalanine at amino acid residue 1014 of the sodium channel (termed L1014F, or L-to-F mutation), has been demonstrated to be closely associated with kdr-mediated resistance (Williamson et al, 1996).

The house fly, Musca domestica is an agriculturally and medically important pest since it is responsible for transmitting both animal and human pathogens (Burgess, 1990). The species possesses a strong capability to develop resistance against pyrethroids, making pyrethroid control of house fly increasingly difficult (Scott et al, 1989). Mechanisms of pyrethroid resistance in M. domestica include P450-monoxygenases mediated detoxification, insensitivity of sodium channels (target site insensitivity), and a decreased rate of cuticular penetration (pen) (Scott and Georgiou, 1986a). The L-to-F mutation-mediated sodium channel insensitivity is an important mechanism of pyrethroid insecticide resistance in many insect species, including house flies (Williams et al., 1996). The L-to-F mutation, resulting from a substitution of cytosine by thymine (CTT-to-TTT) in the house fly, has been recently studied by our group. For the first time, the mutation has been compared at both the genomic DNA and RNA levels within the same individuals from different strains of house flies. No significant differences were found in the frequency of the L-to-F genotypes (CTT,
TTT or C/TTT) at the L-to-F locus between resistant and susceptible strains of the house fly. However, a strong correlation between kdr allele expression and the levels of insecticide resistance were detected at RNA level. This study suggested that posttranscriptional regulation, such as RNA variation and RNA editing, was responsible for the mutation-mediated resistance phenotype in the house fly. Thus, characterizing the regulation factors or passways are necessary. However, without information on the genetic location of the sodium channel gene, it is difficult to evaluate the factors involved regulation of the mutation-mediated sodium channel insensitivity. In the current study, I conducted a genetic linkage study to map the sodium channel gene of house flies using the C-to-T SNP as a marker, which will facilitate the next study on regulation of the gene expression of L-to-F mutation.

4.2 Material and methods

4.2.1 House fly strains

Two parental strains of house flies, wild-type ALHF and the susceptible, morphological-marker aabys, were used as parental strains in this study. The ALHF is a wild-type resistant strain, originally collected in 1998 from a poultry farm in Alabama. This strain obtained a high level of resistance after subsequent selection with permethrin for six generations. It is biannually selected with permethrin to maintain the resistance level (Liu and Yue, 2000). The aabys strain is a susceptible morphological marker one with five recessive visible markers: ac (alicurve, the wings are curved upwards on their tips), ar (aristapedia, leg like aristae of antennae), bwb (brown body), ye (yellow eyes), and sw (snipped wings), on autosomes 1, 2, 3, 4, and 5, respectively. The aabys strain was obtained from Dr. J. G. Scott (Cornell University).
4.2.2 Genetic crossing

To examine genetic basis of kdr-mediated resistance, a genetic crossing experiment was conducted (Fig. 4.1). Virgin females for crossing were collected within 8 hrs after emergence. Crosses between ALHF and aabys were conducted. Males of the F1 generation were backcrossed with virgin females of the aabys strain. Five phenotypes were isolated from the backcross generation (BC1), with genotypes (Fig 4.1): ac/ac, +/ar, +/-bwb, +/-ye, +/-sw (A2345); +/ac, ar/ar, +/-bwb, +/-ye, +/-sw (A1345); +/ac, +/-ar, bwb/bwb, +/-ye, +/-sw (A1245); +/-ac, +/-ar, +/-bwb, ye/ye, +/-sw (A1235) and +/-ac, +/-ar, +/-bwb, +/-ye, sw/sw (A1234). Each line bears only one visible mutant phenotype indicating that the respective autosome is homozygous for the recessive mutant-type marker derived from aabys and the other four autosomes are heterozygous for the dominant wild-type markers from ALHF since crossing-over rarely occur in male house flies (Liu and Yue, 2000). The name of each line was given according to the four autosomes bearing wild-type markers from ALHF. For instance, the A2345 strain had wild-type markers on autosomes 2, 3, 4, 5 from ALHF and the mutant marker on autosome 1 from aabys (Fig. 3.1).

4.2.3 Preparation of RNA and cDNA

RNA was extracted from 20 individuals of each house fly strain for each experiment using the acidic guanidine thiocyanate and phenol-chloroform method (Sambrook et al., 1989; Liu and Scott, 1997). Complementary DNA (cDNA) synthesis was carried out by reverse transcription - polymerase chain reaction (RT-PCR). First strand cDNA synthesis was prepared from total RNA using the SuperScript II reverse transcriptase kit (Invitrogen) according the the manufacturer's instructions using the antisense 5’-anchored oligo(dT) primer (5’TAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTT3’) (Tomita and Scott, 1995).
4.2.4 Amplification of the sodium channel gene fragment in the house fly

The sodium channel cDNA fragments were amplified by the primer pair Kdr (HF) F3 (5’-TCATCTTTGCCGTGATGGGAATGC-3’) and Kdr (HF) R3 (5’AATGATTGTCACCATCCAGCTG). The amplicons generated using this primer pair span the intron/extron boundaries to avoid amplification of genomic DNA, excluding genomic DNA contamination. The PCR solution with JumpStart™ Taq DNA Polymerase (Sigma), cDNA template, and the primer pair was heated to 95°C 2min, followed by 40 cycles of PCR reaction (94°C 30s, 60°C 30s and 72°C for 2min) and a final extension of 72°C for 10 min.

4.2.5 SNP determination for kdr allelic expression variation in house flies, Musca domestica

The frequency and heterozygosity of the kdr allele at the L-to-F site in house flies were investigated by Single Nucleotide Polymorphism (SNP) determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacturer’s instructions (A&B Applied Biosystems). SNP determination is based on a single-base extension with fluorescently labeled ddNTPs. The sodium channel cDNA fragment from each of individuals was used as template. The primer for the SNP determination was designed according to the sequence immediately upstream of the kdr allele. Three replications of the SNP determination were carried out using the same cDNA for each individual, with different preparations of the PCR templates. To confirm that our PCR products were in fact the sodium channel gene fragments, the PCR products from each sample were sequenced.

4.3 Results

4.3.1 Kdr allelic expression in house flies.

We investigated the correlation between the kdr allelic expression at the L-to-F site and the levels of pyrethroid resistance in house flies by SNP determination. We found a strong correlation between the kdr allelic expression and the levels of pyrethroid resistance in house
flies. We found that 100% (20/20) of individuals expressed the susceptible allele C (Cytosine) in aabys (Table. 4.1), generating a codon encoding Leu corresponding to the susceptibility to insecticides in these two strains. In contrast, in the highly resistant house fly strain ALHF, 100% (20/20) of individuals expression the kdr allele T (Thymine) (Table. 4.1), resulting in a change of Leu to Phe, which is strongly correlated with a high level of resistance in this house fly population.

4.3.2 Autosomal linkage of the sodium channel gene

To investigate the autosomal linkage of the sodium channel gene, we examined the allelic expression variation at the L-to-F locus in the sodium channel gene in the F1 generation and five BC1 lines, A2345, A1345, A1245, A1235, and A1234, each of which was homozygous for one autosome bearing a recessive morphological marker from the aabys strain and heterozygous for the other four autosomes (i.e., from both ALHF and aabys strains). We investigated the allelic expression at the L-to-F site of the sodium channel in 20 individuals of each of house fly strains, lines, and F1 progeny by SNP determination. Our results indicated that F1 progeny expressed both A and T alleles, corresponding to its heterozygous nature at the RNA level. The house fly BC1 lines of A2345, A1345, A1235 and A1234 with morphological markers on autosomes 1, 2, 4, and 5 from aabys, respectively, and heterozygous for the remaining autosomes (i.e., from both aabys and ALHF) expressed both alleles (C/T), suggesting not only these strains are heterozygous for the alleles of sodium channel gene, i.e., C allele is from aabys and T allele is from ALHF, but also the sodium channel gene is not located on autosomes 1, 2, 4, and 5. Whereas, the BC1 line of A1245 with the genotype +/-ac, +/-ar, bwb/bwb, +/-ye, +/-sw, bearing a recessive morphological marker from autosome 3 of aabys and heterozagus for the other 4 autosomes (i.e., from both aabys and ALHF), expressed the C allele in all individuals tested, which was the same as the expression in the aabys and CS susceptible strains. This result suggested not only is this strain
homozygous for the susceptible allele C of sodium channel gene, i.e., C allele is from aabys, but also the sodium channel gene is located on autosome 3.

4.4 Discussion

Target site insensitivity resulting from point mutations within the voltage-gated sodium channel of the insect nervous system is known to be of primary importance in the development of resistance to pyrethroid insecticides. The substitution of leucine to phenylalanine (L-to-F), termed the kdr mutation, resulting from a single nucleotide polymorphism (SNP) in the insect sodium channel domain II segment 6 (IIS6), has been clearly associated with resistance to pyrethroids and DDT in many insect species. Recently, the role of posttranscriptional regulation has been connected with the sodium channel L-to-F genotype and its mutation-mediated resistance phenotype in house flies (Xu et al, 2006a), suggesting that there are regulatory factors involved in kdr allelic expression. Nevertheless, little research has focused on the identification and characterization of regulatory factors for the kdr mutation-mediated insecticide resistance. The long term goal of my research is to characterize the regulatory factors and regulation pathways involved in the kdr-mediated resistance in insects. To do this, we have, for the first time, mapped the sodium channel gene on autosome 3 in house flies using back cross lines (BC1) generated from crossing of the pyrethroid resistant ALHF (wild-type) and susceptible aabys (bearing recessive morphological markers on each of five autosomes). Previous work with the ALHF strain revealed that the L-to-F kdr locus may undergo transcriptional regulation (Xu et al., 2006a). A question is raised by this: are there also regulatory factors on autosomes 1 and 2 that serve to regulate the expression of the sodium channel gene at the L1014F locus, which contribute to kdr-mediated resistance? Moreover, multiplicative interactions between resistance factors have been reported in different strains in previous studies (Liu and Scott, 1995, Liu and Yue, 2001). Thus, factors on autosomes 1 and 2 play important roles in the evolution of pyrethroid
resistance. As such, we cannot exclude the possibility that autosomes 1 and 2 also bear factors that serve to regulate the interaction between different resistance factors in addition to the regulatory factors for a single resistance mechanism. The knowledge of the sodium channel gene location will allow us to conduct future studies on genetic mapping and characterization of the regulatory factors of sodium channel mutations in insecticide resistance.
Table 4.1 Expression of L-to-F kdr locus in ALHF, aabys, F1 generation and five BC1 (backcross) house fly lines, *M. domestica*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>n^a</th>
<th>Phenoype</th>
<th>Nucleotide Changes^b</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CTT</td>
</tr>
<tr>
<td>aabys</td>
<td>20</td>
<td>mutant type</td>
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</tr>
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</tr>
<tr>
<td>F1</td>
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<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>A2345</td>
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<td>ali-curve</td>
<td>0</td>
</tr>
<tr>
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<td>20</td>
<td>aristapedia</td>
<td>0</td>
</tr>
<tr>
<td>A1245</td>
<td>20</td>
<td>brown body</td>
<td>20</td>
</tr>
<tr>
<td>A1235</td>
<td>20</td>
<td>yellow eyes</td>
<td>0</td>
</tr>
<tr>
<td>A1234</td>
<td>20</td>
<td>snipped wings</td>
<td>0</td>
</tr>
</tbody>
</table>

^a The total number of tested flies.

^b The nucleotides changed in the L-to-F kdr locus are underlined.
Figure Legends

Fig. 4.1 Diagrammatic representation of the genetic isolation of five house fly BC1 (backcross) lines generated by crossing ALHF and aabys. House fly lines were named according to the autosomes bearing the wild-type markers derived from ALHF.
Fig 4.1

32 phenotypes are produced and 5 lines are saved.
Chapter 5  Molecular Evolution of Kdr-Mediated Resistance and Regulation of Sodium Channel Gene Expression at L-to-F Kdr Locus in the House Fly

5.1 Introduction

Knockdown resistance (kdr) refers to resistance to pyrethroids and DDT (Soderlund and Knipple, 2003) caused by reduced neuronal sensitivity to these insecticides. Pyrethroid insecticides and DDT are known to exert their insecticidal effects by altering the function of the voltage-gated sodium channels in the nerve membranes of insects. A substitution of leucine to phenylalanine (L-to-F) in the sodium channel protein resulting from a single nucleotide polymorphism, termed the \textit{kdr} mutation, has been demonstrated to be associated with kdr-mediated resistance (Ingles et al, 1996).

The house fly, \textit{Musca domestica} is an agriculturally and medically important pest since it is responsible for transmitting both animal and human pathogens (Burgess, 1990). The species possesses a strong capability to develop resistance against pyrethroids, and is the species in which the \textit{kdr}-mediated resistance was first indentified (Busvine, 1951). The L-to-F mutation can be commonly found in pyrethroid-resistant house fly strains (Williams et al., 1996). This mutation results from a substitution of cytosine by thymine (\textit{CTT-to-TTT}) (Ingles, 1996). The house fly strain ALHF has developed a high level of resistance to pyrethroids through two mechanisms, including P450-mediated detoxification and insensitivity of the sodium channels \textit{(kdr)} (Liu and Yue, 2001). The L-to-F mutation resulting from the \textit{CTT-to-TTT} single nucleotide polymorphism is present in the ALHF strain (Xu et al., 2006a). Recently, our group has, for the first time, compared genomic DNA to RNA sequences within the same individuals from different strains of house flies (Xu et al, 2006a).
No correlation was detected between the \( kdr \) allele at the genomic DNA level with levels of resistance to insecticides. No significant differences were found in the frequency of the \( kdr \) genotypes (\( C\)TT, \( T\)TT or \( C/T\)TT) at the L-to-F locus between resistant and susceptible strains of the house fly. However, a strong correlation between \( kdr \) allele expression and the levels of insecticide resistance were detected at RNA level. This suggested that posttranscriptional regulation such as RNA variation and RNA editing was responsible for the mutation-mediated resistance phenotype in the house fly.

In addition, previous work with the ALHF strain revealed that gene expression of the L-to-F locus may undergo transcriptional regulation (Xu et al., 2006a). Thus, a question was raised: are there also regulatory factors on autosomes 1 and 2 that serve to regulate the expression of the sodium channel gene at the L-to-F locus, which contribute to \( kdr \)-mediated resistance? Moreover, multiplicative interactions between resistance factors have been reported in different strains in previous studies (Liu and Scott, 1995, Liu and Yue, 2001). Thus, factors on autosomes 1 and 2 may also play important roles in the evolution of pyrethroid resistance. As such, we cannot exclude the possibility that autosomes 1 and 2 also bear factors that serve to regulate the interaction between different resistance factors in addition to the regulatory factors for a single resistance mechanism.

In my previous study (Chapter 3), I investigated the evolution of pyrethroid resistance in house fly strains and lines and I mapped the sodium channel gene to autosome 3 in the house flies (Chapter 4). These studies provide the basis to further investigate whether the regulation is involved in the L-to-F \( kdr \) allelic expression in house flies and to examine the evolution of insecticide resistance following the insecticide selection. This current study is to investigate allele frequency changes at the L-to-F locus in each of the house fly strains and lines following permethrin selection.
5.2 Material and Methods

5.2.1 House fly strains

Two house fly parental strains, ALHF and aabys, were used as parental strains in this study. ALHF was originally collected in 1998 from a poultry farm in Alabama. This strain obtained a high level of resistance after subsequent selection with permethrin for six generations. It is biannually selected with permethrin to maintain the resistance level (Liu and Yue, 2000). The other strain, aabys, is a susceptible lab marker strain homozygous for five recessive morphological markers, which are: ac (alicurve, the wings are curved upwards on their tips), ar (aristapedia, leg like aristae of antennae), bwb (brown body), ye (yellow eyes), and sw (snipped wings), on autosomes 1, 2, 3, 4, and 5, respectively. The aabys strain was obtained from Dr. J. G. Scott (Cornell University).

5.2.2 Genetic crosses

To examine the genetic basis of kdr-mediated resistance, nine homozygous house fly lines were generated (Figs. 3.1 and 3.2) by genetic crosses of ALHF and aabys house flies. Briefly, virgin females for crossing were collected within 8 hrs after emergence. Males of the F1 generation were backcrossed with virgin females of aabys. Nine phenotypes were isolated from the backcross generation (BC1), with genotypes: ac/ac, +/ar, +/bwb, +/ye, +/sw (A2345); +/ac, ar/ar, +/bwb, +/ye, +/sw (A1345); +/ac, +/ar, bwb/bwb, +/ye, +/sw (A1245); +/ac, +/ar, +/bwb, ye/ye, +/sw (A1235), +/ac, +/ar, +/bwb, +/ye, sw/sw (A1234), ac/ac, +/ar, +/bwb, ye/ye, sw/sw (A23), +/ac, ar/ar, +/bwb, ye/ye, sw/sw (A13), +/ac, +/ar, +/bwb, ye/ye, sw/sw (A123), and ac/ac, ar/ar, +/bwb, ye/ye, sw/sw (A3). Each line bears a unique combination of visible mutant phenotypes indicating that the respective autosomes are homozygous for the recessive mutant-type markers derived from aabys and other autosomes are heterozygous for the dominant wild-type markers from ALHF since crossing-over rarely occur in male house flies (Liu and Yue, 2000). The name of each line was given according to
the four autosomes bearing wild-type markers from ALHF. For instance, the A2345 strain had wild-type markers on autosomes 2, 3, 4, 5 from ALHF and the mutant marker on autosome 1 from aabys.

To make the nine lines completely homozygous for all of their 5 autosomes, additional genetic isolation methods were applied. Generally, the nine BC1 lines were saved and then mass-crossed for four generations. For each generation, flies were sorted for appropriate phenotype and selected with permethrin at a dose that killed approximately 70% of treated individuals. One hundred single-pair crosses were then set up for each line as described previously (Liu and Scott, 1995). Families (offspring of individual single pair crosses) from each single pair cross that showed only individuals of the desired phenotype for three consecutive generations were combined and then selected with permethrin for one generation.

5.2.3 Preparation of RNA and cDNA

Total RNA was extracted from 30 individuals from each strain using the acidic guanidine thiocyanate and phenol-chloroform method (Sambrook et al., 1989; Liu and Scott, 1997). Complementary DNA (cDNA) synthesis was carried out by reverse transcription followed by polymerase chain reaction amplification (RT-PCR). First strand cDNA synthesis was prepared from total RNA using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions using the antisense 5’-anchored oligo(dT) primer (5’TAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTT3’) (Tomita and Scott, 1995)

5.2.4 Amplification of the sodium channel gene fragment in the house fly

The sodium channel cDNA fragments were amplified by the primer pair Kdr (HF) F3 (5’-TCATCTTTGCCGTGATGGGAATGC-3’) and Kdr (HF) R3 (5’ATGATGATGTCACCATCCAGCTG). The amplicons generated using this primer pair spanned the
intron/extron boundaries but did not amplify genomic DNA, thus this approach excluded genomic DNA contamination. The PCR solution with JumpStart™ Taq DNA Polymerase (Sigma), cDNA template, and the primer pair was heated to 95°C 2min, followed by 40cycles of PCR reaction (94°C 30s, 60°C 30s and 72°C for 2min ) and a final extension of 72°C for 10 min.

5.2.5 SNP determination for L-to-F kdr allelic expression in the house fly

The frequency and heterozygosity of the kdr allele at the L-to-F site in house flies were investigated by Single Nucleotide Polymorphism (SNP) determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacture’s instructions (A&B Applied Biosystems). SNP determination was based on a single-base extension with fluorescent labeled ddNTPs. The sodium channel cDNA fragment from each individual was used as template. The primer for the SNP determination was designed according to the sequence immediately upstream of the kdr allele. Three replications of the SNP determination were carried out using the same cDNA for each individual, with different preparations of the PCR templates. PCR products were sequenced to confirm that they were, in fact, the sodium channel gene fragments.

5.3 Results

5.3.1 L-to-F kdr allelic expression of the sodium channel in the house fly

To investigate whether the expression of the L-to-F mutation was regulated by regulation factors on certain autosomes, we assessed the RNA expression variation of the alleles at L-to-F site of the sodium channel in different house fly strains and lines. In the susceptible aabys strain, 100% (30/30) of the tested individuals expressed the C allele (susceptible). In the resistant strain, ALHF, 100% (30/30) of the tested individuals expressed
the T allele (resistant). In the selected, homozygous strains of A2345, A1345, A1235 and A1234, 100% (30/30) tested individuals of each expressed the resistance T allele (Table 5.1). In the strain A1245, 100% (30/30) expressed the C allele (Table 5.1). The strains A2345, A1345, A1235 and A1234 are homozygous for the wild-type markers on autosome 3 derived from ALHF. This indicates that the kdr allele is on autosome 3 and derived from ALHF. That is, as long as their kdr allele on autosome 3 was derived from the resistant ALHF strain, the tested individuals expressed the T kdr allele, which was the same as in ALHF, regardless of the variation of the factors on the other autosomes. In contrast, in the strain A1245, which has a recessive morphological marker (brown body) on autosome 3 provided by aabys, all tested individuals expressed susceptible C allele (Table 5.1), the same as in the aabys strain. This result indicated that there may be no trans-regulation factors on certain autosomes (autosome 1, 2, 4 or 5) that serve to influence the L-to-F kdr allelic expression on autosome 3.

To further investigate whether factors on autosomes 1 or 2 were involved in the regulation of kdr allelic expression, we examined the RNA expression variation of the kdr allele in strains A3, A23, A13 and A123. In selected strains, all individuals tested expressed the T allele (Table 5.1), which further confirmed that no trans-regulatory factors are involved in kdr-allelic expression.

5.3.2 The evolution of kdr allelic expression with the insecticide selection

To investigate the importance of the L-to-F mutation in pyrethroid resistance, we compared the L-to-F kdr allelic expression following permethrin selection. The non-selected strains, except A1245 and F1, exhibited an irregular expression of the kdr allele. The alleles C and C/T were observed in addition to the allele T although these strains bore the wild-type morphological marker on autosome 3 from ALHF (Table 5.1). This was probably caused by the presence of heterozygous kdr alleles or crossing over between homologous autosomes. The pyrethroid resistance levels of non-selected strains were lower than selected strains,
indicating that the presence of the homozygous allele C and heterozygous allele C/T in a population lowered the level of resistance in the population. Thus the L-to-F mutation did affect the level of resistance to some extent. However, in all selected strains (except A1245) where all individuals expressed the T allele (Table 5.1), the resistance level could not be restored to the level of ALHF unless the factors on autosome 1, 2, 3 and 5 were derived from ALHF. This indicated that the mutation from the kdr mechanism could not confer the full level of resistance without the presence of the other resistance factors. This, again, suggests the importance of the multiplicative interaction between resistance factors.

The resistance levels in A3, A13, A23, and A123, however, are slightly different among these four strains (Table 5.1). The strain, A3, had only a 37-fold resistance (Table 5.1), indicating that kdr factors on autosome 3 alone could only provide a marginal level of resistance. Strains A23, A13 and A123 had a relatively higher resistance level compared to A3 (Table 5.1), suggesting that factors on autosomes 1 and 2 serve to enhance kdr-mediated resistance, although they did not affect the L-to-F kdr allelic expression. This further confirms the importance of the multiplicative interaction between resistance factors on autosomes 1, 2, 3, and 5 in resistance.

5.4 Discussion

Target site insensitivity resulting from point mutations within the voltage-gated sodium channel of the insect nervous system is known to be of primary importance in the development of resistance to pyrethroid insecticides. The substitution of leucine to phenylalanine (L-to-F), termed the kdr mutation, results from a single nucleotide polymorphism (SNP) in the insect sodium channel domain II segment 6 (IIS6), and has been clearly associated with resistance to pyrethroids and DDT in many insect species. Recent studies in our group have indicated that posttranscriptional regulation is connected with the sodium channel genotype and the mutation-mediated resistance phenotype in house flies (Xu
et al., 2006). This suggests that post-transcriptional modification of kdr allelic expression is regulated by regulatory factor(s). Nevertheless, little research has focused on identification and characterization of regulatory factors of the kdr mutation-mediated insecticide resistance. My current study investigated the relative importance of the L-to-F mutation in pyrethroid resistance and took the first step in investigating the location of the regulation factors for the L-to-F mutation. The results indicated that the L-to-F mutation on autosome 3 alone cannot account for the high levels of resistance without the presence of the resistance factors on autosomes 1, 2, and 5. This agrees with previous studies on ALHF (Liu and Yue, 2001), which suggested that the interaction between multiple resistance mechanisms were responsible for the high level of resistance. The results have shown that no trans-regulatory factors can be found to influence the L1014F kdr allelic expression. However, since both trans and/or cis genes (factors) are likely to account for the resistance mechanism (Liu and Scott, 1995; 1996), we cannot exclude the possibility that some cis factors, located on autosome 3 are involved in regulation of the L-to-F kdr allelic expression. The factors on autosomes 1 and 2 were not involved in the L-to-F kdr allelic expression regulation. However, the presence of these factors from ALHF enhances the resistance level when combined with factors on autosome 3 (ie. A23, A13 and A123). This might be explained by silent mutations, which have been previously identified in pyrethroid-resistant house flies (Ingles et al., 1996). Usually silent mutations do not change the amino acid composition of the proteins, however, some have been reported to inactivate genes by inducing an RNA splicing mechanism to skip the mutant exons, resulting in the modification of protein function (Cartegni et al., 2002). Current studies on a pyrethroid-resistant Culex mosquito strain in our group (Xu., 2006 unpublished) found that allelic expression of several silent mutations in the sodium channel gene are strongly correlated with the level of pyrethroid resistance. Thus, since only the L-to-F mutation has been investigated in this study, we cannot exclude the
possibility that the ALHF strain may also have kdr-related silent mutations on the sodium channel gene that might change the function of sodium channels. These proposed mutations, if present, might be regulated by factors on autosome 1 or 2. Moreover, since multiplicative interactions between multiple resistant mechanisms are present in ALHF, factors on autosomes 1 and 2 might also serve to facilitate the interaction between the different resistance mechanisms. More effort is needed to study the kdr mutations and regulatory factors in the sodium channel gene in the ALHF strain. The knowledge gained from this work will allow for further studies on the characterization of the regulatory factors for the expression of kdr-related sodium channel gene mutations as well as the factors for the interaction between multiple resistance mechanisms, which are believed to confer high levels of resistance in the house fly.
Table 5.1 Single nucleotide polymorphisms (SNPs) in the house fly, *M. domestica* at the RNA level

<table>
<thead>
<tr>
<th>Strain</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C</th>
<th>C/T</th>
<th>T</th>
<th>RR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Strain</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C</th>
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<th>T</th>
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<td>0</td>
<td>0</td>
<td>_</td>
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</table>

<sup>a</sup> The total number of tested mosquitoes
<sup>b</sup> RR, LD<sub>50</sub> of the resistant strain/LD<sub>50</sub> of the susceptible, aabys strain
Chapter 6  Multiple Sodium Channel Genes and Their Differential Genotype at the L-to-F Locus in the Mosquitoes, *Culex quinquefasciatus*

6.1 Introduction

Voltage-gated sodium channels are responsible for the depolarization phase of action potentials in the membranes of neurons and in all types of excitable cells (Catterall, 2005). The *para*-type sodium channel in insects is the primary target of pyrethroid and DDT insecticides (Narahashi, 1996). Pyrethroids and DDT deliver their toxic, insecticidal effects primarily by binding onto the sodium channel, altering its gating properties, and keeping the sodium channel open for unusually long times, thereby causing a prolonged flow of the sodium current. This prolonged sodium current elevates and prolongs the depolarizing phase of the action potential of the neuron membrane, which initiates repetitive discharges and prevents the repolarization phase of action potentials (Narahashi, 1988). However, modifications in the sodium channel structure (i.e., point mutations or substitutions, resulting from single nucleotide polymorphisms [SNP]), cause insensitivity to DDT and pyrethroids in the sodium channels of the insect nervous system, via a reduction in, or an elimination of, the binding affinity of the insecticides to the protein (Narahashi, 1988), thus diminishing the toxic effects of the insecticides and resulting in insecticide resistance (Soderlund, 2005; Dong, 2007). Reduced target-site sensitivity of the sodium channel is one of the major mechanisms of pyrethroid resistance, known as knockdown resistance (kdr) (Soderlund and Knipple, 2003).
Molecular and pharmacological studies in the past decade provided cumulative evidence for the involvement of point mutations in voltage-gated sodium channels in kdr and kdr-like resistance in many medically or agriculturally important pest species, including the mosquito (Williamson et al., 1996; Soderlund, 2005; Davies et al., 2007; Dong, 2007, Liu, 2008). Among these kdr mutations, substitution of leucine to phenylalanine [L to F] in the 6th segment of domain II (IIS6), results from a single nucleotide polymorphism (change from TTA to TTT in mosquitoes). This mutation was first detected in pyrethroid-resistant house flies and cockroaches, and was also found in many other pyrethroid-resistant insect species, while other kdr mutations appeared to be unique and specific to many other insect species (Knipple et al., 1994; Dong, 1997; Ingles et al., 1996, Liu et al. 2009). Recently, our group investigated the frequency of alleles (A or T) at the L-to-F locus at the genomic DNA and RNA levels within the same individuals of different mosquito strains of Cx. quinquefasciatus, bearing different resistant phenotypes (Xu et al., 2005; 2006a). We generated a 341 bp PCR fragment from the mosquito sodium channel genomic DNA, where the L-to-F mutation resides, and found no correlation between the L-to-F allele (T) at the genomic DNA level and the level of permethrin resistance. We did, however, find a strong correlation between the prevalence of the L-to-F allelic (T) expression at the RNA level and the level of resistance (Xu et al., 2006a). Interestingly, we did not detect any introns in this 341 bp sodium channel genomic DNA region as previously reported (Martinez-Torres et al., 1998; Rinkevich et al., 2006). In addition, more than one sodium channel gene copy has been identified in the genome of Cx. quinquefasciatus through Southern blot analysis. Yet, only one transcript, albeit multiple sodium channel gene copies, has been identified by Northern blot analysis (Xu, 2006). Given this, I propose that multiple copies of the sodium channel gene are present in the genome of the Culex quinquefasciatus.
The current study was to further characterize the genomic organization, gene expression patterns, and the allelic expression at the L-to-F site of the sodium channel gene in *Cx. quinquefasciatus*. We identified multiple copies of the *para*-type sodium channel gene in the mosquito *Cx. quinquefasciatus* through polymerase chain reaction (PCR) analysis and DNA sequencing, which further confirmed our previous results from the Southern blot analysis. The allele frequency at the L-to-F site of sodium channel gene at DNA level, as well as the allelic expression variation at the L-to-F site at RNA level were examined in this study.

6.2 Materials and methods

6.2.1 Mosquitoes

Five strains of *Cx. quinquefasciatus* were used in this study: two field collected strains HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup> from Madison and Mobile County, Alabama, respectively (Liu et al. 2004a); two permethrin selected resistant strains HAmCq<sup>G8</sup> and MAmCq<sup>G6</sup>, the 8<sup>th</sup> and the 6<sup>th</sup> generation of permethrin-selected offspring of HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup>, respectively (Xu et al., 2006 a; Li and Liu, 2010); and a susceptible strain S-Lab, 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).

6.2.2 Amplification of sodium channel gene fragments from genomic DNA and RNA of *Culex quinquefasciatus*

Genomic DNA and RNA were extracted from both males and females for each experiment. Three replications were performed, each on different days, for a total of 50 individual mosquitoes from each population. The sodium channel cDNA fragments were amplified by the primer pair KDR S#8 (5’-ATTTCATCATCGTGGCCCTTTCGC-3’) and KDR AS#1 (5’-TTGTTTCGTTTCTGTTGCGGTGCTGTG-3’), which generated amplicons that
spanned the intron/exon boundaries but did not amplify genomic DNA. This approach eliminated contamination by genomic DNA. PCR was conducted using the cDNA template and a primer pair (KDR S#8/KDR AS#1) with JumpStart™ Taq DNA Polymerase (Sigma). The reaction was heated to 95°C for 2 min, followed by 40 cycles of (94°C for 45 s, 65°C for 45 s and 72°C for 2 min) and a final extension of 72°C for 10 min.

The sodium channel genomic DNA fragments were amplified using a primer pair KDR S14 (5′ GGAACCTTCACCGACTTCATGCACTC 3′) and KDR AS1 (5′ GACAAAAG CAAGGCTAAGAAAAGG 3′). The primers were designed based on exonic sequences (Martinez-Torres et al. 1999) that did not generate products in RNA samples without a reverse transcription step. The PCR reactions were heated at 95 °C for 5 min, followed by 42 cycles of PCR (94°C for 30 s, 57°C for 30 s, and 72°C for 1 min) and a final extension of 72°C for 10 min.

6.2.3 SNP determination for the L-to-F kdr allele in Culex quinquefasciatus

The frequency and heterozygosity of the kdr allele in mosquitoes were investigated further by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to manufacturer’s instructions (A&B Applied Biosystems, Xu et al., 2006a; b). The primer Cx_SNP2 (5′ GCCACCGTATGTATGGAAATTT 3′), used for SNP determination, was designed according to the sequence immediately upstream of the kdr allele mutation site. Three replications of the SNP determination were conducted with different preparations of the PCR templates. Representative samples of the PCR products from both the cDNA and genomic DNA used for the SNP determination were then sequenced to confirm that they were, in fact, the para-type sodium channel gene fragments.
6.3 Results

6.3.1 Sequencing analysis of multiple sodium channel genes in *Culex quinquefasciatus*

Two genomic DNA products (~500 bp and ~200 bp) were generated by PCR from all 50 individuals in each of five mosquito strains (susceptible [S-Lab], intermediate [HAMCq\textsuperscript{G0} and MAMCq\textsuperscript{G0}] to highly resistant [HAMCq\textsuperscript{G8} and MAMCq\textsuperscript{G8}]) (Fig. 6.1) when only a single primer pair, KDR S14/KDR AS1, was used. Both the ~500 and ~200 bp PCR products from the five individual samples of each strain were sequenced. Sequencing analyses indicated that the ~500 bp PCR product of the sodium channel gDNA was the mosquito sodium channel gene sequence with an intron sequence near the L-to-F site (Fig. 6.2) as previously reported in mosquitoes (Martinez-Torres et al., 1998). The ~200 bp PCR product, in contrast, did not contain the intron sequence (Fig. 6.2). This was consistent with the previous report by Xu et al. (2005; 2006a). This discovery provided strong support for our previous Southern blot analyses, i.e., there were at least two copies of the sodium channel gene presented in the genome of mosquitoes, *Cx. quinquefasciatus*. In addition, it helps explain the conflicting results reported by Martinez-Torres et al. (1998) and Xu et al. (2005; 2006a) where each of these studies identified only one of the two copies of the sodium channel gene - one with an intron near the L-to-F site (Martinez-Torres et al., 1998) and the other without (Xu et al. 2005; 2006a).

6.3.2 Comparison of L-to-F *kdr* allelic variation at the genomic DNA and RNA levels of *Culex quinquefasciatus*

The frequency of alleles (A or T) at the L-to-F locus at the genomic DNA (i.e., two DNA fragments) and RNA levels was investigated in 50 individuals from each of the five strains. We first assessed the RNA expression variation of the A or T alleles at the L-to-F locus. In all strains, a strong correlation existed between the prevalence of the L-to-F allelic expression at the RNA level and the level of susceptibility or resistance to permethrin (Table.
All individuals in susceptible S-Lab strain expressed the susceptible allele A at the L-to-F locus of the sodium channel gene at the RNA level (Table. 6.1), resulting in a codon TTA encoding Leu, which corresponded to the permethrin susceptibility of this strain. In contrast, in the highly resistant *Culex* strains, HAmCq\(^{G8}\) and MAmCq\(^{G6}\), 84 and 100% of the individuals showed monoallelic expression of the T allele, respectively, resulting in a change of Leu to Phe (Table.6.1). This correlated strongly with the high level of resistance in these two populations. Furthermore, we identified a pattern of allelic variation in individuals of the two field collected parental strains (Table.6. 1). Frequencies ranged from 0.02 and 0.04 for HAmCq\(^{GO}\) and MAmCq\(^{GO}\) respectively, for individuals that expressed only the susceptible allele A, 0.48 and 0.38 for those that expressed both alleles, and 0.50 and 0.58 for those that expressed the *kdr* T allele, respectively. The allelic variation pattern for the L-to-F locus in these two field collected mosquito populations correlated strongly with their intermediate levels of resistance (Liu et al., 2004a) and the proportions of both susceptible and resistant individuals, as suggested by Georghiou and Taylor (1997), and Xu et al. (2006a).

We next compared the frequency of alleles (A or T) at the L-to-F site at the genomic DNA level of both the ~200 and ~500 bp gDNA fragments in all five mosquito populations. We found different genotypes for the L-to-F site among individuals in the same population of all five populations, including individuals that were heterozygous for both susceptible (A) and resistant (T) alleles or homozygous for either the A or T allele (Table.6.1). This finding was consistent with our previous report (Xu et al., 2006a). No correlation was found between the frequency of L-to-F allelic variation at the L-to-F site of the ~200 bp sodium channel DNA fragment and the prevalence of the L-to-F allelic expression at the RNA level (Table.6.1). This also did not correlate to the level of permethrin resistance for any of the populations (Table. 6.1). This was consistent with the previous report of Xu et al. (2006a). In contrast, there was a strong correlation between the frequency of allelic variation at the L-to-
F site of the ~500 bp genomic DNA fragment (with the intron) and the prevalence of L-to-F allelic expression at the RNA level (Table. 6.1). This also correlated strongly with the level of permethrin resistance in all five populations (Table.6.1). In the susceptible S-Lab strain, all individuals had a genotype homozygous for the A allele at the L-to-F site. This corresponded with their L-to-F allelic expression at the RNA level, and all individuals expressed the susceptible allele A (Table.6.1). In the highly resistant HAmCq<sup>G8</sup> and MAmCq<sup>G6</sup> populations, 84 and 100% of the individuals were homozygous for the T allele at the L-to-F site, respectively. This correlated strongly with the frequency of their L-to-F allelic expression at the RNA level (Table.6.1) and their levels of resistance to permethrin. The field parental HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup> populations had individuals that were homozygous for susceptible A allele (28 and 42%, respectively), heterozygous for both (A/T) alleles (48 and 42%), and homozygous for resistant (T) alleles (24 and 16%) (Table.6.1). These are also strongly correlated with the frequency of their L-to-F allelic expression at the RNA level and their intermediate level of resistance. The data from the field parental mosquitoes HAmCq<sup>G0</sup> and MAmCq<sup>G0</sup> clearly represent a transitional status between susceptible and highly resistant through their intermediate genotype (~500 DNA fragment) and expression condition for the L-to-F locus, which closely corresponded to their relatively low levels of resistance.

6.4 Discussion

Voltage-gated sodium channels belong to a superfamily of ion channels, which play an important function in membrane excitability (Yu et al., 2005) and are responsible for the depolarization phase of action potentials in membranes of neurons and in all types of excitable cells (Catterall, 2005). Molecular characterization has revealed that nine voltage-gated sodium channel genes and one related sodium channel gene are present in the mammalian sodium channel gene family (Catterall, 2000; 2005; Goldin, 2002; Yu and Catterall, 2003); eight sodium channel genes have been documented in zebra fish (Novak et
al., 2006); and six sodium channel genes have been cloned from electric fish (Lopreato et al., 2001). Multiple sodium channel genes have also been characterized in some invertebrate species; for example, *Hirudo medicinalis* (leech) and *Holocynthia roretzi* (ascidia) have four and two characterized sodium channel genes, respectively (Blackshaw et al., 2003; Nagahora et al., 2000; Okamura et al., 1994). In comparison to the fairly well defined multiple vertebrate sodium channel genes, it appears that only one sodium channel gene, homologues to *para* (currently *DmNaV*) of *Drosophila melanogaster*, has been well characterized in insects (Shao et al., 2009). This gene encodes the equivalent of the α-subunit of the mammalian sodium channels (Shao et al., 2009). A putative sodium channel gene, DSC1 has been described in *D. melanogaster* (Salkoff et al. 1987) and its homologous has been found in other insect species (Park et al., 1999; Liu et al., 2001). The DSC1-orthologous, BSC1, in the German cockroach, *Blattella germanica*, has been recently characterized as a voltage-gated channel permeable to both Na+ and Ca2+ ions, and proposed as a novel family of Ca2+-selective cation channel genes (Zhou et al., 2004). A recent study by Moignot et al. (2009) revealed two sodium channel cDNAs, a *para*-like sodium channel and a novel sodium channel that is evolutionarily closely related to the *para*-type sodium channel, presented in the cockroach *Periplaneta americana*. This finding suggests the possibility that insects may also have multiple sodium channels in their genome.

My study has, for the first time, revealed multiple (or at least two) sodium channel genes existing in the genome of the mosquito, *Cx. quinquefasciatus* through PCR analysis. This coincided with the previous Southern blot analysis, in which more than one sodium channel gene copies was detected (Xu, 2006). However, in our previous study, the identification of a single sodium channel transcript by Northern blot analysis in all five mosquito strains (susceptible [S-Lab], intermediate [HAmCqG0 and MAmCqG0] to highly resistant [HAmCqG8 and MAmCqG8]) suggested that there might be only one sodium channel
gene transcript presented in mosquitoes. Furthermore, similar expression levels of the sodium channel transcript were identified in all five tested mosquito strains, suggesting that the up-regulation of sodium channel gene expression is not a possible factor involved in the pyrethroid resistance in mosquito strains. This contrasts with the regulation of other resistance genes, such as cytochrome P450 genes (Liu and Scott, 1998; Feyereisen, 2005), hydrolase genes (Hemingway et al., 2004), and glutathione S-transferase genes (Ranson et al., 2001), whose expression are overexpressed in resistant insects.

Comparison of the L-to-F kdr genotypes in two sodium channel genes (~500 and ~200 bp fragments) in five mosquito strains with their corresponding transcriptional expression and their levels of resistance clearly revealed a strong correlation between the frequency of the resistant allele (T genotype at the L-to-F site of the ~500 bp DNA fragment (with an intron sequence), the frequency of L-to-F allelic (T) expression at the RNA level, and their levels of pyrethroid resistance. Nevertheless, no-correlation among genotype, transcriptional expression, and resistance phenotype has been identified for the ~200 bp sodium channel DNA fragment lacking the intron sequence. These results strongly suggest that the sodium channel gene containing the intron might be a functional transcript of the sodium channel gene in mosquitoes, and in turn, is involved in insecticide resistance in Cx. quinquefasciatus.

One hypothesis is that the sodium channel gene without the intron may be a non-functional gene or a pseudogene. Recent studies have proposed that pseudogenes are presented in the genome of Cx. quinquefasciatus and are non-functional (Zheng et al., 2005; Harrison and Gerstein, 2002). Pseudogenes can be generated by either direct DNA duplication or retrotransposition. A pseudogene resulting from the latter is called a processed pseudogene (or retro-pseudogene). Processed pseudogenes arise from reverse-transcription of mRNA and re-integration into the genome. In the human genome, it is been proposed that processed pseudogenes are generated from reverse transcription of spliced mRNAs into
cDNAs by the reverse transcriptase from the long interspersed nuclear element (LINE) with subsequent re-integration into the human genome (Esnault et al., 2000; Weiner, 2000). About 8000 processed pseudogenes have been reported in the human genome through exhaustive sequence comparisons with known human proteins (Zhang et al., 2003). The characteristics of processed pseudogenes include the absence of introns, the presence of flanking direct repeats, and a 3’-polyadenylation tract (Zheng et al., 2005). Thus, whether the non-intron para-type sodium channel gene identified in the current study is a pseudogene or a functional gene with a post transcriptional regulation (Xu et al., 2006a) remains a subject for further investigation.

In conclusion, our study, for the first time, revealed multiple sodium channel genes in the mosquito Cx. quinquefasciatus. Only the L-to-F kdr genotypes in the sodium channel gene containing the intron sequence were consistent with the expression of their transcripts and levels of pyrethroid resistance. This research identified a complexity of the sodium channel gene expression and its role in pyrethroid resistance in insects. It provides a new framework for the study of sodium channels, the channel gene expression regulation, and its importance in insecticide resistance in insects.
Acknowledgments

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Table 6.1 Comparisons of the frequency of alleles (A or T) at the L-to-F site at the genomic DNA (i.e., two DNA fragments) and RNA levels of *Cx. quinquefasciatus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Phenotype</th>
<th>Template source</th>
<th>Individual allele&lt;sup&gt;c&lt;/sup&gt; (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/o intron</td>
<td>TTA</td>
</tr>
<tr>
<td>S-Lab</td>
<td>54</td>
<td>Susceptible</td>
<td>gDNA w/o intron</td>
<td>4 (0.07)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/ intron</td>
<td>54 (1.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cDNA</td>
<td>54 (1.0)</td>
</tr>
<tr>
<td>HAmCq&lt;sub&gt;G0a&lt;/sub&gt;</td>
<td>54</td>
<td>100-fold resistance</td>
<td>gDNA w/o intron</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/ intron</td>
<td>15 (0.28)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>cDNA</td>
<td>14 (0.26)</td>
</tr>
<tr>
<td>HAmCq&lt;sub&gt;G9&lt;/sub&gt;</td>
<td>50</td>
<td>3,100-fold resistance</td>
<td>gDNA w/o intron</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/ intron</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cDNA</td>
<td>0</td>
</tr>
<tr>
<td>MAmCq&lt;sub&gt;G0a&lt;/sub&gt;</td>
<td>24</td>
<td>40-old resistance</td>
<td>gDNA w/o intron</td>
<td>1 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/ intron</td>
<td>10 (0.42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cDNA</td>
<td>10 (0.42)</td>
</tr>
<tr>
<td>MAmCq&lt;sub&gt;G6&lt;/sub&gt;</td>
<td>24</td>
<td>583-fold resistance</td>
<td>gDNA w/o intron</td>
<td>1 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gDNA w/ intron</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cDNA</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>G0: The parental insects were collected directly from the field and the numeral indicates the generation (s) of selection with permethrin

<sup>b</sup>The total number of tested mosquitoes

<sup>c</sup>The nucleotides in the L-to-F *kdr* locus changed in the genotypes are underlined
Figure Legends

Figure 6.1  Polymerase chain reaction (PCR) amplification of the sodium channel gene fragments from the genomic DNA of *Culex quinquefasciatus*. The sodium channel genomic DNA fragments were amplified from individual mosquitoes using the single primer pair KDR S14/KDR AS1. The primers were designed based on exonic sequences that did not generate products in RNA samples without a reverse transcription step.

Figure 6.2  Sequence analyses of ~500 bp and ~200 bp sodium channel fragments. The ~200 bp sodium channel fragment spanned an intron sequence that was located between two exons (exon 20 and 21) compared with the ~500 bp fragment.
Fig. 6.1
Fig. 6.2
CHAPTER 7 Research Summary and Future Research Perspectives

7.1 Research Summary

My study has indicated that factors on autosomes 1, 2, 3 and 5 are responsible for the high level of pyrethroid resistance in the ALHF strain. Although multiple resistance mechanisms were present, these mechanisms, when present alone, provide only marginal resistance. Multiplicative interactions among different mechanisms are required for the insect to obtain a high level of resistance. I have, for the first time, mapped the voltage-gated sodium channel gene to autosome 3 in the ALHF strain, which will facilitate future research to study the regulation of kdr-mutations in the sodium channel gene. My study suggested that there may be no trans-regulatory factors that affect the expression of the sodium channel at the L-to-F kdr locus, but my study did not exclude the role of cis-regulatory factors in resistance, which will need further testing. Furthermore, there is still a strong possibility that other kdr-mutations (Xu et al. 2010 in preparation), or even silent mutations, are present in the ALHF strain. Thus, whether the expression of the proposed mutations is regulated by certain regulatory factors remains to be studied. In addition, since multiplicative interactions among multiple resistance mechanisms are responsible for the high level of pyrethroid resistance, we cannot exclude the possibility that there are factors that serve to regulate or facilitate this interaction between the different mechanisms. More interestingly, my study, for the first time, identified multiple copies of the sodium channel gene
in *Culex quinquefasciatus*. The identification of a single sodium channel transcript and its similar expression level in all tested resistant and susceptible mosquitoes suggested that not only there might be only one sodium channel gene transcript presented in mosquitoes but also that the expression of the sodium channel gene was a factor involved in the resistance of *Culex quinquefasciatus* to pyrethroids. There was a strong correlation between the frequency of the resistant allele (T) and the L-to-F site of the ~500 bp gDNA fragment (intron-containing), but not in the ~200 bp fragment, and the frequency of L-to-F allelic (T) expression at the RNA level. Based on this, we suggest that the sodium channel gene containing the intron may be a functional sodium channel transcript in *Cx. quinquefasciatus*.

These studies provide a more comprehensive understanding of the complexity behind sodium channel gene expression and its regulation, as well as its association with kdr-mediated resistance. This opens new opportunities for the further study of insect voltage-gated sodium channels and knockdown resistance in the house fly and mosquitoes.

7.2 Future research

In the current study, we found no regulatory factors that influenced the expression of the sodium channel gene at the L-to-F *kdr* locus. However, we observed a slight enhancement of resistance when we compared strains of A23 and A13 with A3. Thus, there is a strong possibility that other kdr-related mutations or even silent mutations are present in the sodium channel gene of the ALHF and these proposed mutations might contribute to kdr under the regulation of certain regulatory factors. It would be very interesting to compare the sodium channel gene of the ALHF strain to that of the susceptible strain to identify potentially more kdr-related SNPs. To do this, full length sodium channel cDNA should be isolated from both the ALHF and the susceptible strains and sequenced. Then, the amino acid sequence should be deduced and compared between the resistant ALHF strain and susceptible strains such as
aabys or CS to determine if there are other mutations in addition to the L-to-F kdr mutation. Then, SNP determination experiments for these mutations should be conducted to determine whether these mutations are correlated with the level of pyrethroid resistance. SNPs that are correlated with level of resistance could be further investigated through genetic and molecular methods as described in my previous chapters to determine whether regulation is involved in the expression of these mutations. To further investigate how SNPs affect the protein function, functional expression for SNPs on the sodium channel gene in *Xenopus* oocytes, followed by functional analysis experiments, could be conducted.

It would also be interesting to study the synergism effect between factors on autosomes 3 and 5. To do this, one could genetically isolate new fly lines with different combinations among autosomes 1, 2, 3, 5. For instance, comparison of resistance levels in the fly lines with the autosome combinations of 2, 3 and 5, 2 and 5, 2 and 3, and 3 and 5 from ALHF will enable one to evaluate the impact of factor-interaction among autosomes 2, 3, and 5. Comparison between the resistance levels between A125 and A1235 might provide a further understanding of the importance of factors on autosome 3 in the reconstitution of the resistance level.

Moreover, since multiple sodium channel gene copies were identified, these copies (w/wo intron) could be expressed in *Xenopus* oocyte functional system. The functional analysis for the different gene copies could then be conducted to further investigate which of the multiple copies is/are involved in the sodium channel function in the insecticide resistance. It would be more interesting if one compares all mutations in the entire sodium channel of insects, correlating which with pyrethroid resistance levels. In addition, it would be better to functionally analyze all the SNPs in *Xenopus* oocyte functional system, which would provide global information facilitating the functional analysis for these multiple copies.
Figure Legends

Fig. 7.1 Parental strains, ALHF and aabys and seven house fly strains derived from crossing ALHF and aabys for the future studies.
Fig. 7.1
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