RESISTANCE TO PYRETHROID INSECTICIDES IN *HELICOVERPA ZEA* (BODDIE) (LEPIDOPTERA: NOCTUIDAE): BIOASSAY VALIDATION, VOLTAGE-GATED SODIUM CHANNEL MUTATIONS AND *CYP6B* OVEREXPRESSION ANALYSIS

A Dissertation

by

BRADLEY WAYNE HOPKINS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Entomology

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Approved by:

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May 2010

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ABSTRACT

Resistance to Pyrethroid Insecticides in *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae): Bioassay Validation, Voltage-Gated Sodium Channel Mutations and *CYP6B* Overexpression Analysis. (May 2010)

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Helicoverpa zea is one of the most costly insect pests of food and fiber crops throughout the Americas. Pyrethroid insecticides are widely applied for control as they are effective and relatively inexpensive; however, resistance threatens sustainability because alternative insecticides are often more expensive or less effective. Pyrethroid resistance has been identified since 1990 and monitoring has utilized cypermethrin in the adult vial test, but resistance mechanisms have not yet been elucidated at the molecular level. Here we examined field-collected *H. zea* males resistant to cypermethrin for target site and metabolic resistance mechanisms.

We report the cDNA sequence of the *H. zea* sodium channel α-subunit homologous to the *Drosophila para* gene and identified known resistance-conferring mutations L1029H and V421M, along with two novel mutations at the V421 residue, V421A and V421G. An additional mutation, I951V, may be the first example of a pyrethroid resistance mutation caused by RNA-editing. We identified other specimens with significantly higher transcriptional expression levels of cytochrome P450 genes

CYP6B8 and *CYP6B9* compared to the susceptible, ranging from a factor of 3.7 to 34.9 and 5.6 to 39.6, respectively.

In addition, we investigated if differences in insect growth stage and pyrethroid structure affect our ability to predict resistance in the adult vial test. Vial bioassays with cypermethrin, esfenvalerate, and bifenthrin were conducted on third instars and male moths from a susceptible laboratory colony and the F1 generation of a resistant field population. For the resistant population, vial assays using either growth stage gave similar resistance ratios for each of the three pyrethroids, respectively, proving the adult vial test accurately reflects larval resistance. However, resistance ratios varied considerably depending on the pyrethroid used, so values obtained with one pyrethroid may not be predictive of another.

This dissertation is the first to identify molecular mechanisms associated with *H. zea* pyrethroid resistance. Our results suggest carefully chosen pyrethroid structures diagnostic for specific resistance mechanisms could improve regional monitoring programs and development of high throughput assays to detect the resistance mechanisms used in tandem with traditional monitoring may greatly improve our ability to identify and predict resistance and make better control recommendations.

ACKNOWLEDGMENTS

I would like to thank my committee chair, Dr. Pietrantonio, as well as my committee members, Dr. Coates, Dr. Medina, and Dr. Longnecker, for their guidance and support during the course of this research. The funding for my research provided by the C. Everette Salyer Fellowship in Cotton Research, as well as from the Insecticide Resistance Action Committee (IRAC) and Cotton, Incorporated, is greatly appreciated.

Thanks also to all my lab mates, friends, colleagues, and Department of Entomology faculty, staff, and students for making my time at Texas A&M so enjoyable. Finally, I would like to thank my parents, Sid and Holly Hopkins, and my colleague Mike Treacy for their help, ideas, and support, and most of all, my wife, Deanna Hopkins, for her continued patience and support as I completed my Ph.D.

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CHAPTER I

INTRODUCTION: *HELICOVERPA ZEA* AND PYRETHROID RESISTANCE

Helicoverpa zea

The corn earworm or bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is a highly polyphagous pest that feeds on over 100 species of plants throughout the United States including important agricultural crops such as cotton, corn, grain sorghum, soybeans, alfalfa, sunflowers, and vegetable crops such as sweet corn, tomatoes, peppers, and snap peas (Harding 1976; Martin et al. 1976; Stadelbacher 1981; Fitt 1989). Larvae feed upon plant fruiting structures, causing significant economic losses even at low population densities. *Helicoverpa zea* is a multivoltine species that occurs throughout the year in both North and South America (Fitt 1989). Pupae overwinter in the soil, and each female can typically lay from 500 to 3000 eggs at a rate of about 35 per day (King and Coleman 1989; Capinera 2001). Adults are highly mobile and, in the US, northern migration occurs during the spring and summer and reverse migration towards the south occurs in the fall, as this pest does not overwinter in northern states. The complete life-cycle typically lasts from 30 to 35 days (King and Coleman 1989; Bohmfalk et al. 1982).

This pest, along with the tobacco budworm, *Heliothis virescens* (Fabricius), comprises the heliothine complex in the Americas, while other heliothine species include *Helicoverpa armigera* (Hübner), the most common heliothine throughout the rest of the

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This dissertation follows the style of Insect Biochemistry and Molecular Biology.

world, and *Helicoverpa punctigera* (Wallengren) in Australia (Fitt 1989). Although there are many successful biological and cultural control methods used to manage *H. zea*, one of the most effective forms of control is the use of synthetic insecticides. These have proven extremely effective for controlling *H. zea*; however, extensive use of insecticides has led to problems with resistance to many classes of chemicals (Sparks 1981; McCaffery 1998; Whalon et al. 2010).

Pyrethroids

One of the most common classes of insecticide used to control heliothines is pyrethroids. Pyrethroids are synthetic derivatives of the naturally occurring toxin pyrethrum, found in the flowers of *Chrysanthemum* spp. (Elliot 1977). Extracts of pyrethrum contain six pyrethrin esters that are slightly different based upon chirality and the makeup of their acid and alcohol moieties. The basic structure of pyrethrins was modified to give rise to synthetic pyrethroids. The mode of action of pyrethroid insecticides is interference with inactivation of the sodium channel, which allows excess sodium to leak into the nerve cell, causing hyperstimulation of the nerve, and eventually paralysis and death. Pyrethroids are classified into two groups, Type I and Type II, based on the presence of an α-cyano group on the alcohol moiety of Type II compounds, and the different physiological response elicited by the two. Type I pyrethroids typically cause hyperactivity, tremors, and convulsions, while Type II compounds cause convulsions and rapid paralysis (Narahashi 2002; Soderlund and Knipple 2003). Early commercial pyrethroids were mixtures of isomers with differing levels of insecticidal

activity; however, more recent products have resolved isomers that increase efficacy and allow for a reduction in application rates.

Although the introduction of transgenic *Bt* (*Bacillus thuringiensis*) crops in the US has reduced the amount of insecticide applied for controlling *H. zea*, pyrethroid applications are still recommended for high density populations of *H. zea* in *Bt* cotton (Seibert et al. 2008; Sivasupramaniam et al. 2008) and on average in 2008, three-quarters of the *Bt* cotton acres in the US were treated once for bollworms (Williams 2009). The recent report of resistance to *Bt* CryIAc toxin in *H. zea* field populations in Arkansas and Mississippi (Tabashnik et al. 2008) also increases the likelihood that growers may continue to apply pyrethroids to control *H. zea* in *Bt* crops, although there is debate on if this is truly field resistance (Moar et al. 2008). An increased deployment of *Bt* crops and boll weevil eradication programs have caused hemipterans such as stink bugs and *Lygus* spp. to emerge as primary pests of cotton, resulting in increased additional non-targeted pyrethroid exposure of *H. zea* because pyrethroids are one of the main choices for control, especially for stink bugs (Jackson et al. 2004; Snodgrass et al. 2008).

Sodium channels

Activity

The target site of pyrethroids is the voltage-gated sodium channel (Narahashi 1987). This transmembrane protein is responsible for increased sodium permeability in excitable cells during the rapidly rising phase of action potential in the nerve (Catterall

1992). The inside of the nerve cell contains a high concentration of potassium ions and a low concentration of sodium ions. This is opposite to the area surrounding the axonal membrane, and at rest, the axonal membrane is relatively permeable to potassium, but not sodium, creating an approximately -60 mV difference in the inside of the cell with respect to the outside (Narahashi 1987; Catterall 1992). Upon nervous stimulation, depolarization causes a conformational change to occur in the sodium channel that allows sodium ions to flow into the cell (Narahashi 1987; Catterall 1992). Sodium permeability rises rapidly, changing the inside of the cell to become positive, resulting in the rising phase of the action potential. After ~1 millisecond the channel closes or inactivates (fast inactivation) and potassium effluxes from the cell, causing the falling phase of the action potential (Catterall 1992; Zlotkin 1999). In the long term, an ATP-driven sodium-potassium pump maintains the ion gradient across the axonal membrane to restore resting potential (Catterall 1992).

Toxins/Toxicants

There is an enormous variety of toxins/toxicants that affect the function of the sodium channel. At least ten different classes of neurotoxins have been identified based upon binding sites and the range of physiological effects caused by binding (Catterall 1992; Zlotkin 1999; Soderlund 2005). Four different classes of synthetic insecticides, DDT and its analogs, *N*-alkylamides, dihydropyrazoles, and pyrethroids, are known to target the sodium channel (Soderlund 2005).

Insecticide resistance

In many cases, extensive use or overuse of insecticides for pest control have led to the development of resistance. Insecticide resistance has been defined by the Insecticide Resistance Action Committee (IRAC) as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species.' Sawicki (1987) defined resistance as 'a genetic change in response to selection by toxicants that may impair control in the field.' One important distinction that must be made is whether or not resistance is defined based upon conditions in the laboratory or from the field (Moar et al. 2008). Even if a pest population may be shown as statistically more resistant to a compound relative to a susceptible strain of that pest, this still may be no indication that there would be any change in field efficacy of that compound. Although determining the presence of laboratory-based resistance is an important measure for establishing resistance monitoring programs, one must use caution when extrapolating the results of laboratory studies to what may occur in real-world field populations.

Insecticide resistance mechanisms

Target-site resistance

Alterations in the insecticide target site cause reduced sensitivity to that insecticide. Single nucleotide polymorphisms in structural genes coding for target site proteins (Taylor and Feyereisen 1996; Khambay and Jewess 2005; Davies et al. 2007b),

alternative splicing of gene transcripts (Dong et al. 2007), and RNA editing (Dong et al. 2007) can be responsible for the resistant phenotype. Many of the relative locations of these changes have been shown to be conserved across insect species (Soderlund 2005; Davies et al. 2007b; Dong 2007).

Metabolic resistance

The increased ability to degrade or sequester insecticidal compounds to non-toxic forms is termed metabolic resistance. Cytochrome P450 monooxygenases (oxidation), esterases (hydrolysis), and glutathione-S-transferases (conjugation) are the three main families of enzymes responsible for the metabolism of natural and synthetic xenobiotics (Sun et al. 2001; Feyereisen 2005; Li et al. 2007). Polyphagous insects such as *H. zea* have an inherently large number of detoxifying enzymes present in their system due to the high level of toxic plant compounds encountered while feeding, and expression of these enzymes can be quickly induced in the presence of novel toxins (Schoonhoven et al. 2005). Metabolic resistance can develop in the organism through gene amplification (i.e., selection for insects with more copies of the gene encoding the enzyme), enhanced transcription of that gene (i.e., more transcript is made, thus resulting in overexpression of the enzyme), or by mutations in enzymes that give a greater capacity for metabolism. Insecticide sequestration has also been implicated in resistance and is caused by gene amplification of esterases, resulting in considerable overexpression of enzymes that serves as an insecticide sink (Gunning et al. 2005).

Reduced cuticular penetration

Reduced penetration causes resistance by modifications in the cuticle and/or epidermis that reduce the rate of absorption of pesticides, delaying the time to lethality (Khambay and Jewess 2005). This mechanism alone is thought to play a minor role in resistance. In concert with other mechanisms, however, especially enhanced metabolism, reduced penetration can have a synergistic effect on resistance by increasing the period of time the insect has available to detoxify the insecticide (Ahmad and McCaffery 1999). This mechanism has been studied in heliothines and was always found to be present with other resistance mechanisms (Little et al. 1989; Gunning et al. 1991; Abd-Elghafar and Knowles 1996; Ahmad and McCaffery 1999).

Documented resistance mechanisms in heliothines

The two heliothine pests with the largest number of documented reports of insecticide resistance are *H. armigera* and *H. virescens*. These pests have developed resistance to all of the major classes of insecticides used for their control (e.g., organochlorines, cyclodienes, organophosphates, carbamates, pyrethroids), and there is also potential for resistance to pesticidal plants containing *Bt* toxins (Tabashnik et al. 2008). Insecticide resistance also occurs in *H. zea* (Sparks et al. 1981; Hsu and Yu 1991; Abd-Elghafar et al. 1993), however, it has taken considerably longer for resistance to develop in this species. More recent studies of resistance have focused upon determining mechanisms and the underlying genetics contributing to resistance in order to best develop a resistance management program and recommend appropriate

alternative active ingredients with differing modes of action. Multiple mechanisms have been implicated in heliothine resistance, and their roles have fluctuated with time and different selection pressures. Individual cases of documented heliothine insecticide resistance have been reviewed extensively (Georghiou and Lagunes-Tejada 1991; McCaffery 1998; Kranthi et al. 2005; Whalon et al. 2010).

Wolfe and Wingate (1998) reported an alanine to serine (A285S) mutation in the *rdl* (resistance to dieldrin)–like gene of cyclodiene-resistant *H. virescens*. This mutation was present in the M2 pore forming region of the GABA receptor. A leucine to histidine (L1029H) (Park and Taylor 1997) and valine to methionine (V421M) (Park et al. 1997) mutation in the α-subunit of the *Drosophila para*-homologous sodium channel gene *Heliothis sodium channel protein* (*hscp*) of *H. virescens*, present in the S6 transmembrane segment of homology domains II and I, respectively, was associated with pyrethroid resistance (i.e., knockdown resistance or *kdr*-like*)*. Functional expression analysis of the sodium channel in *Xenopus* oocytes implicated both mutations in channel insensitivity as well as modifications of voltage-dependent gating properties (Zhao et al. 2000). Head et al. (1998) identified an aspartatic acid to valine (D1561V) and a glutamic acid to glycine (E1565G) mutation in pyrethroid resistant *H. virescens* and *H. armigera* present in the intracellular linker located between domain III S6 and IV S1. Taylor et al. (1993) showed association linkage of pyrethroid resistance and the *hscp* gene of *H. virescens* through use of restriction fragment length polymorphism (RFLP) markers. Among the newer insecticide classes, spinosad resistance was selected in

laboratory colonies of *H. virescens,* with the putative mechanism being reduced target site sensitivity (Young et al. 2003).

Based on activity toward the model substrate, α-naphthyl acetate, increased larval esterase activity was documented in methyl parathion resistant (Konno et al. 1990), profenofos resistant (Harold and Ottea 2000), and thiodicarb and cypermethrin resistant (Goh et al. 1995) *H. virescens*, esfenvalerate resistant *H. armigera* (Gunning et al. 1996b), and fenvalerate resistant *H. punctigera* (Gunning et al. 1997). Goh et al. (1995) described three esterase isozymes (A1, B1, and C1) and identified an immunoresponse to esterase A1. Increased hydrolysis of acetylcholine iodide due to insensitive acetylcholinesterase was documented in thiodicarb-resistant *H. armigera* (Gunning et al. 1996a).

Although resistance to newer insecticidal compounds has yet to be documented in field populations of heliothines; in the diamondback moth, *Plutella xylostella* (L.), increased levels of esterases were associated with indoxacarb resistance (Sayyad and Wright 2006), and monooxidases and esterases with abamectin resistance (Liang et al. 2003). Monooxygenases were associated with spinosad resistance in *Spodoptera exigua* (Hübner) (Wang et al. 2006). Resistance to diacylhydrazines has been documented in *S. exigua* (Moulton et al. 2002).

Methods for detecting/monitoring resistance

Methods for assessing resistance depend upon whether the goal is early detection of resistance, or monitoring resistance that is already present. Two methods commonly

used are: (i) bioassay of insects exposed to a wide range of logarithmically increasing dosages of insecticide followed by probit analysis to compare median lethal dosages and obtain resistance ratios in relationship to a known susceptible strain and (ii) application of diagnostic dosages or doses to discriminate between susceptible and resistant individuals (ffrench-Constant and Roush 1990). Discriminating dosages allow for greater efficiency in the ability to detect resistance at low frequencies because in locations where test insects are limited, more individuals may be tested at a few dosages (ffrench-Constant and Roush 1990).

Topical assays

The initial standard technique to monitor for heliothine insecticide resistance was topical application of technical-grade insecticide in solvent to the first dorsal thoracic segment of third instar (35 ± 5 mg) larvae. A weight of 20 ± 5 mg was later adopted as the optimal testing weight, since most larvae molt to fourth instars before they attain this weight (Mullins and Pieters 1982). The topical application has also been used for adult moths. This assay can be conducted with extreme precision and is useful in monitoring susceptibility changes. However, this assay does not relate directly to field application rates and requires considerable time and labor (e.g., rearing larvae to test size) and equipment (e.g., micro-syringes, auto-applicators).

Vial assays

Plapp (1987) developed a monitoring method that utilized exposure to insecticide-coated liquid scintillation vials. This vial assay has been used for monitoring larvae as well as adults, and the adult vial test was accepted in 1987 as the principal monitoring test for resistance to pyrethroids in *H. virescens* and *H. zea* in the United States. Adult male moths are used because they are the easiest life stage to capture with use of an artificial sex pheromone trap. Vials can be produced quickly and shipped for widespread implementation of a resistance monitoring program. This methodology saves labor by eliminating the need to rear larvae to a specific test size or weight, however, a drawback is the inconsistency of exposure to a particular dosage due to variable contact and the unknown and varied age of moths.

Foliar assays

A third resistance monitoring method is a foliar bioassay. Leaves can be treated by using a leaf dip method as described in IRAC Test No. 7 (Anonymous 1990) or by foliar spray (Luttrell et al. 1987). In both methodologies, neonate or third instar larvae have been the targeted stages. Leaf assays allow for testing formulated compounds as well as technical grade ones, yet again, the exact insecticide dose acquired by the test insect is unknown and it can take considerable labor to field-collect enough insects for testing.

Synergism

Synergism studies used in combination with topical and vial assays provide insight into the mechanisms of resistance (Campanhola and Plapp 1989). Insecticide synergists (e.g., piperonyl butoxide, DEF, formamidines, etc.) are applied to resistant insects prior to insecticide exposure. Suppression of resistance with synergist addition implies that the enzymes or metabolic pathways being blocked are associated with resistance. Occurrence of synergism allows researchers to hypothesize about the resistance mechanism; however, such studies are unable to preclude other non-synergistic mechanisms.

Resistance monitoring programs

Insecticide resistance monitoring programs have been implemented for heliothines in many countries. For example, in the US, the *H. virescens* pyrethroid resistance monitoring program tested moths in glass vials coated with one of two discriminating pyrethroid dosages: one that should kill all susceptible adults (e.g., 5 µg cypermethrin per vial), or one that should allow only resistant individuals to survive (e.g., 10 µg cypermethrin per vial) (Plapp 1987). The same adult/vial technique is used to monitor pyrethroid resistance in *H. zea*; however, discriminating cypermethrin doses utilized for this species vary across the US cotton producing areas (Kanga et al. 1996; Payne et al. 2006; Pietrantonio et al. 2007; Temple et al. 2006). Kanga et al. (1996) established the dosage of 2.5 µg cypermethrin per vial as likely killing all susceptible *H. zea* adult male moths. However, the discriminating dosage for heterozygotes remains

unknown since a colony resistant to pyrethroids has not been available to perform crosses with a susceptible colony and obtain F1 bioassay results as well as investigate various mechanisms of resistance. The possibility of multiple mechanisms of resistance co-existing in field populations makes it difficult to establish a cut-off dosage for resistant heterozygous or homozygous individuals in monitoring programs.

An emphasis for any program should be to use a standardized method that closely relates data obtained in the laboratory to field exposure. This allows for increased accuracy in detection of resistance and enhances relationships between monitoring results and the prediction of field failures due to resistance (ffrench-Constant and Roush 1990).

Hypotheses

In heliothine species, mutations in the sodium channel gene have been associated with reduced sensitivity of the sodium channel protein to pyrethroid application (Park and Taylor 1997; Park et al. 1997; Head et al. 1998) and increased expression of cytochrome P450 genes have been associated with increased metabolism of pyrethroids (Xiao-Ping and Hobbs 1995; Pittendrigh et al. 1997; Rose et al. 1997; Ranasinghe and Hobbs 1998; Pimprale and Brown 1999a,b; Li et al. 2000; Sasabe et al. 2004; Yang et al. 2006; Chen and Li 2007). Therefore, we hypothesize that pyrethroid resistance in *H. zea* is likely due to reduced sensitivity of the sodium channel and/or the increased ability of the insect to metabolize pyrethroid insecticides.

Pyrethroid applications in the field are targeted specifically at eggs and neonate larvae; however, adults are also exposed if they are present in the field at the time of the application or move in while residues are present. Thus, as both larvae and adults are subjected to exposure, if alternative resistance mechanisms operate at different developmental stages, the adult vial test used for resistance monitoring may not be sufficient for detecting resistance in other life stages. In addition, many different pyrethroids are used for *H. zea* control, so monitoring with cypermethrin may not detect resistance development to other pyrethroid compounds.

The objectives of this research were the following: (i) determine if resistance to pyrethroids in *H. zea* is associated with mutations in candidate regions of the sodium channel gene, (ii) determine if resistance to pyrethroids in *H. zea* is associated with transcriptional overexpression of cytochrome P450 genes, and (iii) determine if the adult vial test using cypermethrin is a good indicator of pyrethroid resistance in *H. zea*.

The results of this research are important for crop production across the United States and the world. The bollworm is a pest of multiple crops, and pyrethroids are often the most cost-effective measure for its control. Understanding the mechanisms of resistance will enable the development of more effective resistance management strategies that will allow producers to make the most cost effective, environmentally sound decision for controlling bollworm and support the preservation of bollworm susceptibility to pyrethroids. Additionally, this research will add to the growing knowledge of the dynamics of insecticide resistance and how it can be best managed.

CHAPTER II

TARGET SITE RESISTANCE

Introduction

Pyrethroids are widely used for control of *H. zea* because they are extremely effective and relatively inexpensive; however, extensive use of these insecticides has led to some instances of resistance. Resistance to pyrethroids was first detected in *H. zea* in the early 1990s (Stadelbacher et al. 1990; Hsu and Yu 1991), and presently high levels of resistance are detected yearly in the US Cotton Belt (Pietrantonio et al. 2007). In *H. zea*, low frequencies of target site resistance were discovered in field insects using a neurophysiological assay (Ottea and Holloway 1998). Target site resistance to pyrethroids occurs when there are alterations of the pyrethroid target, the sodium channel.

The primary structural component of the sodium channel is the α-subunit, which is a large glycoprotein of ~270 kDa (Noda et al. 1984). Two putative sodium channel genes, *paralytic* (*para*) (Loughney *et al.*, 1989) and *Drosophila sodium channel 1* (*DSC1*) (Salkoff et al. 1987), were cloned from the fruit fly, *Drosophila melanogaster*, in the late 1980's. Until recently, *DSC1* and its German cockroach ortholog *BSC1* were thought to encode a second insect sodium channel; however, they were determined to encode a new family of ion-selective calcium channels, making *para* likely the single voltage-gated sodium channel gene present in insects (Zhou et al. 2004; Dong 2007). *para* is structurally and functionally similar to the mammalian sodium channel α-subunit

and is composed of four homologous domains (I-IV) connected by intracellular linkers, with domains containing six alpha-helical transmembrane segments (S1-S6) connected by intra- and extra-cellular loops (Catterall 1992). The four domains form a bell-shaped pore with many cavities (Sato et al. 2001). The S5 and S6 segments, along with the S5-S6 linkers form the selectivity filter and pore of the sodium channel and are rich in the amino acids aspartic acid, glutamic acid, lysine, and alanine. The S4 segments contain five to eight basic residues (arginine or lysine) that are separated from each other by neutral residues; these positively charged segments function as voltage sensors for the channel (Davies et al. 2007; Dong 2007). A hydrophobic motif in the intracellular linker connecting domains III and IV acts as an inactivation gate that causes fast inactivation by docking with the inactivation gate receptor, thought to be located in the IIIS4-S5 and IVS4-S5 intracellular loops (Catterall 2000; Davies et al. 2007; Dong 2007).

Partial *para*-orthologous cDNA clones have been obtained from several arthropods (Soderlund 2005; Dong 2007), but full length genomic DNA or cDNA sequences have been cloned from only a handful of species other than *Drosophila*, such as *Voltage-sensitive sodium channel 1* (*Vssc1*) from the house fly *Musca domestica* (Ingles et al. 1996; Williamson et al. 1996), $BgNa_v$ from the German cockroach *Blatella germanica* (Dong 1997), *hscp* from the tobacco budworm *Heliothis virescens* (Park et al. 1999), *para* from the head louse *Pediculus capitis* (Lee et al. 2003), $VmNa_v$ from the varroa mite *Varroa destructor* (Wang et al. 2003), Ag^{para} from the mosquito *Anopheles gambiae* (Davies et al. 2007), and $BmNa_v$ from the silkworm *Bombyx mori* (Shao et al. 2009). Full length clones have successfully been expressed in *Xenopus* oocytes;

however, coexpression with TipE, a small auxiliary insect protein, is required to obtain typical gating properties (Feng et al. 1995).

Sequence comparisons have been made between susceptible and pyrethroid-resistant insects to identify mutations responsible for resistance. The first knock down resistance (*kdr*) mutation that was identified was a Leu 1014 to Phe mutation from the house fly voltage-gated sodium channel in the Domain IIS6 transmembrane region (Williamson et al. 1996). Since this discovery, many other resistance-conferring mutations have been identified from multiple insect species, and extensive reviews of these mutations have been published (Davies et al. 2007b; Dong 2007; Soderlund 2008).

In heliothine moths, target site mutations have only been identified from *H. virescens* and *H. armigera* (Fig. 2.1). The homologous *kdr*-like mutation was found in resistant *H. virescens* (Park and Taylor 1997); however, this mutation was a Leu 1024 to His rather than the Leu to Phe that has been identified in many other insect species. In addition, a novel mutation, Val 421 to Met, was identified in the Domain IS6 transmembrane region of resistant *H. virescens* (Park et al. 1997) and further shown to confer resistance to pyrethroids in electrophysiology experiments using house fly channels expressed in *Xenopus* oocytes (Lee and Soderlund 2001). To date, *H. virescens* is the only insect that has been identified with this mutation. Head et al. (1998) found a double mutation in the intracellular linker between Domain III-IV of resistant *H. virescens* and *H. armigera*, D1561V and E1565G, that may be associated with resistance, but their potential effect on the sodium current have yet to be confirmed through electrophysiological studies.

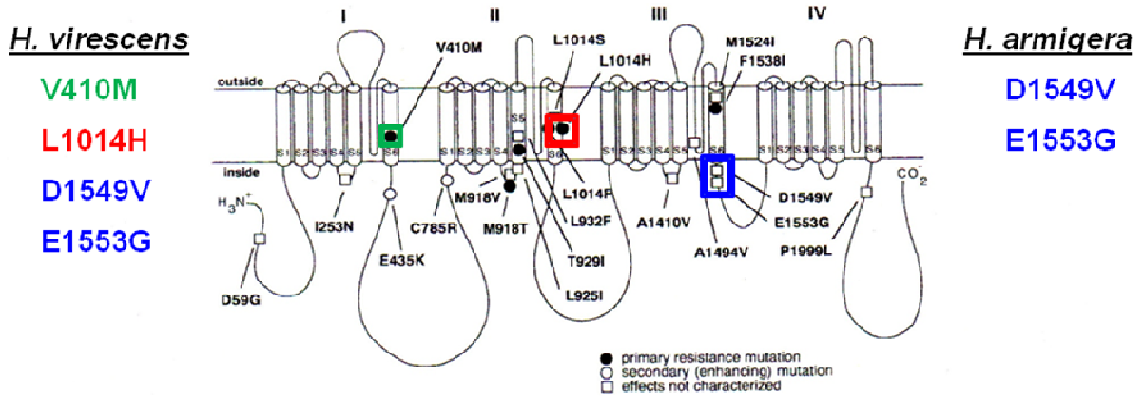

Figure 2.1. Schematic of the insect sodium channel α-subunit. There are four homologous domains (I-IV), each with six transmembrane regions (S1-S6). *kdr*-like mutations identified from *Heliothis virescens* and *Helicoverpa armigera* are boxed and the corresponding mutations listed (adapted from Soderlund and Knipple, 2003).

The objectives of this study were to first, clone and sequence the cDNA of the susceptible *H. zea* sodium channel gene and to compare this sequence to those of other insect species. Second, compare the susceptible sequence with that of pyrethroid-resistant *H. zea* specimens that had survived a discriminating dosage of cypermethrin to identify allelic variants that are associated with resistance to pyrethroids.

Materials and methods

Insects

Susceptible *H. zea* were from a colony generously provided by Nancy Adams of Monsanto (St. Louis, Missouri); this colony has been maintained insecticide-free in the laboratory for multiple generations at 27°C with a photoperiod of 16:8 (L:D) h. For rearing, larvae were provided with a pre-mixed synthetic diet (Stonefly *Heliothis* Diet, Ward's Natural Science, Rochester, NY), and adults with 10% sucrose solution.

Resistant moths were obtained through the Texas Bollworm Resistance Monitoring Program (Pietrantonio et al. 2007) and were exposed to different residual concentrations of cypermethrin in 20 ml scintillation vials using the adult vial test (Plapp et al. 1987). Moths surviving concentrations of 10, 30 and 60 µg cypermethrin per vial, respectively, were individually frozen for subsequent molecular analysis. The established discriminatory concentration for susceptible individuals is 2.5 µg cypermethrin per vial (Kanga and Plapp 1996).

cDNA synthesis

Messenger RNA from susceptible *H. zea* males was isolated from a pool of heads of 10 moths (DynaBead® mRNA Direct kit, Invitrogen, Carlsbad, CA, USA) and was used to synthesize full length cDNA through RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) (GeneRacer™ Kit, Invitrogen) according to manufacturer's directions in a final volume of 20 µL. This putative full length cDNA was used as template to amplify the *H. zea* sodium channel cDNA. Messenger RNA was also isolated from individual heads of pyrethroid resistant male moths with the DynaBead® mRNA Direct kit (Invitrogen) and was used as template to synthesize cDNA with the SuperScript® III First Strand Synthesis kit (Invitrogen) according to manufacturer's directions in a final volume of 20 µL. Resistant cDNA was used as template for PCR reactions to amplify three target fragments where mutations were expected and to compare these sequences to the susceptible sodium channel sequence.

Genomic DNA isolation

Genomic DNA was isolated from whole thoraces of individual male moths (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA, USA) according to manufacturer's directions using two consecutive 100 µL elutions. Genomic DNA was used as template for PCR reactions to compare fragments of sodium channel sequences of susceptible and resistant specimens. Targeted fragments were selected based on discovery of potential mutations in cDNA in *H. zea* and regions from other lepidopteran insects in which mutations conferring resistance have been reported.

PCR and cloning

PCR was performed with 5 µl 10X Buffer, 1 µl of 1:50 or 1:250 cDNA, 1 µl sense primer (20mM), 1 µl antisense primer (20mM), 1µl dNTPs (10 mM), and 1 µl polymerase in 50 µl total volume (Advantage® GC 2 PCR Kit, Clontech, Mountain View, CA, USA). Primer sequences are listed in Table 2.1. Primer pairs used to clone the susceptible sodium channel cDNA and regions amplified with their corresponding amino acid number (Table 2.1) were as follows: NaCh F14/NaCh R7 - the start codon to Domain IIS1 (residues 1 to 892); NaCh F5/NaCh R5 - Domain IIS1 to Domain IIIS5 (residues 760 to 1405); NaCh F6/NaCh R6 - Domain IIIS3 to Domain IVS6 (residues 1320 to 1840).

Only specific regions of the sodium channel were targeted for the analysis of resistance mutations. Primer pairs for resistant mutation analysis of cDNA and genomic DNA were as follows: DIS6 F/DIS6 R - the V421 region (residues 354 to 437); NaCh

17-18F/NaCh 18R - the I951 region (residues 861 to 951); NaCh F2a/NaCh R2 - the L1029 region (residues 952 to 1015). Residues in parentheses are internal to the primer sequences. The expected length of PCR products internal to the primer sequences were 267 bp from genomic DNA and 191 bp from cDNA for the V421 region; 202 bp from genomic DNA and 132 bp from cDNA for the L1029 region; 1311 bp from genomic DNA and 212 bp from cDNA for the I951 region.

PCR cycles were an initial 94^{o}C denaturation for 2 min, repeated cycles of 94^{o}C denaturation, 63^{o}C annealing, and 72^{o}C extension (40 cycles for reactions NaCh F14/NaCh R7, NaCh F5/NaCh R5, and NaCh F6/NaCh R6; 30 cycles for reactions DIS6 F/DIS6 R, NaCh 17-18F/NaCh 18R, and NaCh F2a/NaCh R2), followed by five minutes at 72^{o}C.

Products were visualized by agarose gel electrophoresis with GelStar™ dye (Lonza Group Ltd, Basel, Switzerland). PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen), and cloned using the TOPO TA Cloning® Kit (Invitrogen).

DNA sequencing and analysis

All sequencing reactions were set with ~400 ng DNA template and primers M13 Forward or M13 Reverse (Table 2.1), and proceeded for 50 cycles using ABI Big Dye® (Applied Biosystems, Foster City, CA, USA) with other details as described Pietrantonio et al. (2005). A minimum of three unique cDNA clones were sequenced from susceptible cDNA reactions for each of three regions overlapping the sodium channel

Table 2.1. Oligonucleotide primers used for PCR and sequencing reactions. Primer sequences are from 5' to 3' and listed with their corresponding nucleotide location in the *Helicoverpa zea* sodium channel (Fig. 2.2).

Sense Primers		
Name	Sequence	Nucleotide location
NaCh F14	ATGTCCGAGGACTTGGACTCGATCAGCGAGG	1 to 31
NaCh F5	GGGACTGCTGTTGGTTGTGGCTGGAGTTTC	2279 to 2308
NaCh F6	GAAATACTTCACAAATGCGTGGTGCTGGC	3960 to 3989
DIS6 F	CACGAGTTTCGATACGTTTGGATGGGCTTTC	1062 to 1092
Exon 17-18F	GGAAGGAGTTCAGGGTTTGTCAGTGTTG	2583 to 2610
NaCh F2a	TTCATGATTGTGTTCCGAGTGCTCTGCGGA	2854 to 2883
M13 Forward	GTAAAACGACGGCCAG	---
Antisense Primers		
Name	Sequence	Nucleotide location
NaCh R7	CGATACACAGGGTGATGAACAACTCCACG	2343 to 2371
NaCh R5	CGAAGATCAGCCAGAAGATAAGACACACC	4185 to 4213
NaCh R6	CGTAGTCGTCGTCCGTCAGACCTTCTTG	5491 to 5519
DIS6 R	CCTGAGAGCCTCCTCCTCAGCCTGTTCC	1284 to 1311
Exon 18R	CTGTGCATGAAATCCGTGAAGTTCCATCGC	2823 to 2853
NaCh R2	GCAGTCGGTGTCGACAAACTCGATGAGCC	3016 to 3044
M13 Reverse	CAGGAAACAGCTATGAC	---

open reading frame. A minimum of three unique genomic DNA clones were sequenced for each resistant specimen for the specific V421, I951 and L1029 regions to validate

consensus sequences, mutations and genotype, and mutations at each residue were confirmed from a minimum of three unique cDNA clones. Sequence data were analyzed using the DNASTAR software suite (DNASTAR Inc., Madison, WI, USA). Transmembrane region predictions were made using TMHMM (www.cbs.dtu.dk/services/). Sequences were aligned using MEGALIGN (DNASTAR) and CLUSTALW (DNASTAR).

Results

Gene structure

A sodium channel 5.49 kb cDNA sequence homologous to the *para* gene was cloned from a susceptible *H. zea* laboratory colony (GenBank accession no. GU574730) (Fig. 2.2). The cDNA sequence is combined from four overlapping sequences and spans the 5' UTR and past the Domain IV S6 transmembrane region, almost through the stop codon (Fig. 2.2). Repeated attempts to amplify the 3' end using a RACE strategy were unsuccessful.

The *H. zea* cDNA sequence was devoid of an 11 to 12 amino acid sequence expected for exon 2 (Fig. 2.3, *hscp* and $BmNa_v$ residues 49-59; *para* residues 49 to 60) and a 24 to 26 amino acid sequence expected for part of exon 11 (Fig. 2.3, *hscp* residues 526 to 549; $BmNa_v$ residues 527 to 552; *para* residues 530 to 554) that are present in other insect species. In addition, the *H. zea* cDNA sequence did not contain a 23 amino acid sequence expected for exon 20 from *D. melanogaster* (Fig. 2.3, *para* residues 1099 to 1121).

```
                                                        GAAAGAGCGCCCGCCGCGAGCGCGCTACACGCGCTGATACGCGTAA  -541
CGTCGCGCCCTTTACGCAACGCGCGACCACCGCGCGCACTCGCGCTCCTCACCGCCTTACCGAGGATTACTGTTGGAGTGATACGCTGTT  -451
AGTGTGCACGATATCGTCGAGCCCGGCTCGTGGACGTCGGAGCGGCCGGCCGCGGACCGCCGGAGCTGACGCAGCTCAGGGCTGACGCAG  -361
ATGTTGCGCGCGCGCAGAAGCTGGAGAGAGGAGCGCGCGCGGCCAGCGGGCGACGCGGCCACCATCCCGCGCGCCCTCTAGACCTACACA  -271
CACCCCTAGGCACTGCACTATATACGGTCGGTCATGAAAAATCTTGCCCATTTGCTGGCTGTCCCTATCAGCGATCACGTGGTAACTTTC  -181
ATCTCTCTAGTAGCCGCGATCACACTGCTCTCGCGGACAACAGAAGGGCGGTCCGCCCGTAGGTGGTCGGCGGGAGAACGCGGGCGTAGA   -91
GCGTGCTGCCGCGCGCGTTTCCCGCACCACACGCACCCGCCCATTCACTGTTAGCAGTAGGCAGTTCATTCGGGGGGAGTTCGATAGACA    -1
ATGTCCGAGGACTTGGACTCGATCAGCGAGGAAGAACGAAGCTTGTTCCGACCCTTCACAAGAGAGTCACTGGCCGCTATCGAAGCCCGC    90
 M  S  E  D  L  D  S  I  S  E  E  E  R  S  L  F  R  P  F  T  R  E  S  L  A  A  I  E  A  R     30

ATAGCTGAGGAGCATGCCAAGCAAAAGGAACTCGAGAAAAAACGAGCGGAAGGCGAGGTGCGTTATGATGACGAGGACGAGGACGAAGGT   180
 I  A  E  E  H  A  K  Q  K  E  L  E  K  K  R  A  E  G  E  V  R  Y  D  D  E  D  E  D  E  G      60

CCTCAGCCGGACGCGACCCTGGAGCAGGGCCTGCCGCTGCCGGTGCGAATGCAGGGCACCTTCCCGGCGGAAGTGTCCTCTATACCCCTC   270
 P  Q  P  D  A  T  L  E  Q  G  L  P  L  P  V  R  M  Q  G  T  F  P  A  E  V  S  S  I  P  L     90

GAGGACATCGATCCCTTCTATCATAACCAAAGAACCTTCGTAGTCATAAGCAAGGGTAAAGATATCTTCAGATTTTCGGCCACCAACGCC   360
 E  D  I  D  P  F  Y  H  N  Q  R  T  F  V  V  I  S  K  G  K  D  I  F  R  F  S  A  T  N  A    120

TTATGGATACTAGACCCATTCAATCCTATAAGAAGAGTGGCGATATACATTCTAGTACATCCTTTGTTCTCGTTGTTTATCATTACCACA   450
 L  W  I  L  D  P  F  N  P  I  R  R  V  A  I  Y  I  L  V  H  P  L  F  S  L  F  I  I  T  T    150

ATTCTAGTCAACTGTATTCTTATGATAATGCCTACGACGCCAACCGTCGAAAGTACTGAAGTTATCTTTACCGGGATCTACACGTTTGAA   540
 I  L  V  N  C  I  L  M  I  M  P  T  T  P  T  V  E  S  T  E  V  I  F  T  G  I  Y  T  F  E    180

TCAGCGGTGAAAGTAATGGCCAGGGGTTTCATACTACAGCCATTCACATACCTTAGAGATGCATGGAATTGGCTTGACTTCGTAGTTATA   630
 S  A  V  K  V  M  A  R  G  F  I  L  Q  P  F  T  Y  L  R  D  A  W  N  W  L  D  F  V  V  I    210

GCTTTAGCTTATGTGACGATGGGCATAGATCTCGGCAACTTGGCCGCTCTCAGAACGTTCAGGGTACTCCGAGCGCTCAAAACTGTGGCC   720
 A  L  A  Y  V  T  M  G  I  D  L  G  N  L  A  A  L  R  T  F  R  V  L  R  A  L  K  T  V  A    240

ATCATACCGGGTTTGAAAACGATCGTCGGCGCCGTCATCGAGTCAGTGAAGAATCTGAGAGATGTGATAATCTTGACGATGTTCTCACTG   810
 I  I  P  G  L  K  T  I  V  G  A  V  I  E  S  V  K  N  L  R  D  V  I  I  L  T  M  F  S  L    270

TCCGTGTTTGCGCTGATGGGCCTACAGATCTACATGGGAGTGTTAACGCAGAAGTGTATCAAGGTGTTCCCGGAAGACGGCTCCTGGGGT   900
 S  V  F  A  L  M  G  L  Q  I  Y  M  G  V  L  T  Q  K  C  I  K  V  F  P  E  D  G  S  W  G    300

AACCTCACCGATGAGAACTGGGAGAGGTTTTGCCAGAATGAGACCAACTGGTACGGGGATGGAGGGGAATATCCACTTTGTGGAAATTCA   990
 N  L  T  D  E  N  W  E  R  F  C  Q  N  E  T  N  W  Y  G  D  G  G  E  Y  P  L  C  G  N  S    330

TCAGGAGCAGGTCAATGTGAACCCGGATACGTCTGTCTGCAAGGCTATGGACCGAACCCTAACTACGGATACACGAGTTTCGATACGTTT  1080
 S  G  A  G  Q  C  E  P  G  Y  V  C  L  Q  G  Y  G  P  N  P  N  Y  G  Y  T  S  F  D  T  F    360

GGTTGGACTTTCTTGTCAGCTTTCCGCCTCATGACACAGGATTATTGGGAGAATCTCTATCAATTGGTGCTGAGGTCAGCGGGGTCATGG  1170
 G  W  T  F  L  S  A  F  R  L  M  T  Q  D  Y  W  E  N  L  Y  Q  L  V  L  R  S  A  G  S  W    390

CACGTGCTGTTCTTCGTAGTGATCATCTTCTTGGGCTCGTTCTATCTCGTGAACTTGATCTTAGCCATCGTCGCCATGTCGTACGACGAG  1260
 H  V  L  F  F  V  V  I  I  F  L  G  S  F  Y  L  V  N  L  I  L  A  I  V  A  M  S  Y  D  E    420

TTGCAGAAGAAAGCAGAAGAAGAGGAGCAAGCTGAGGAGGAGGCTCTCAGGGAAGCGGAACAGAAGGCAGCGGCGCGCGCGGACAAACAG  1350
 L  Q  K  K  A  E  E  E  E  Q  A  E  E  E  A  L  R  E  A  E  Q  K  A  A  A  R  A  D  K  Q    450

GAGGCACGAGAGGCGCACGCGCGCGAGGCGGCGGCGGCAGCACAGGCGGCCGCCTACGCCGAGGCACACCCCGCCAAGTCCCCCAGCGAC  1440
 E  A  R  E  A  H  A  R  E  A  A  A  A  A  Q  A  A  A  Y  A  E  A  H  P  A  K  S  P  S  D    480

TTCTCCTGTCAGAGCTACGAGCTGTTCGTCAACCAGGAGCGCGGCAACCAGGACGACAATACGCGCGAGCGCATGTCCCTCCGTAGCGAC  1530
 F  S  C  Q  S  Y  E  L  F  V  N  Q  E  R  G  N  Q  D  D  N  T  R  E  R  M  S  L  R  S  D    510

CCCTTCCAGGACTCGGCCTCGTTATCACTGCCTGGATCGCCGTTCAATTTGCGGCGAGGTTCGCGGGGCTCGCACCAGATGGCGCTGCGG  1620
 P  F  Q  D  S  A  S  L  S  L  P  G  S  P  F  N  L  R  R  G  S  R  G  S  H  Q  M  A  L  R    540

CCGAACGGGCGGGCTCGCTACCCGCCCGGCGCGGACCGCAAGCCGCTCGTGCTGTCCACCTACCTGGATGCGCAAGAGCATCTGCCTTAC  1710
 P  N  G  R  A  R  Y  P  P  G  A  D  R  K  P  L  V  L  S  T  Y  L  D  A  Q  E  H  L  P  Y    570
```

Fig. 2.2. Sequence and deduced translation of the voltage-gated sodium channel cDNA (cDNA GenBank accession no. GU574730; 6076 bp: 586 5'UTR and 5490 bp of open reading frame) from heads of pyrethroid-susceptible *Helicoverpa zea* male moths. The sequence includes the 5' untranslated region and ~90% of the open reading frame. Bold, underlined amino acids represent residue positions where polymorphisms associated with pyrethroid insecticide resistance have been identified.

```
GCTGACGACTCCAACGCCGTCACCCCCATGTCTGAAGAGAGCGGTGCTATCATTATTCCAGTTTACTACGCTAATTTAGGTTCTCGTCAC  1800
 A  D  D  S  N  A  V  T  P  M  S  E  E  S  G  A  I  I  I  P  V  Y  Y  A  N  L  G  S  R  H    600

TCGTCGTACACGTCGCATCAGTCGCGGCTGTCGTACACGTCGCACGGTGACCTGCTGGGCGGCGGCAAGGCGCAGACCAAGGAGGCGCGC  1890
 S  S  Y  T  S  H  Q  S  R  L  S  Y  T  S  H  G  D  L  L  G  G  G  K  A  Q  T  K  E  A  R    630

CTACGCAACCGGTCCGCCTCCCGGAACCATAGTGTGACGTCACAGCCGCACGGGTACCCGCTGCCCGGGCAGGATTTCTCGCTAGCCACC  1980
 L  R  N  R  S  A  S  R  N  H  S  V  T  S  Q  P  H  G  Y  P  L  P  G  Q  D  F  S  L  A  T    660

CGCCCACTTAGAGAATATGAAATGAGTACGACTGAATGCACAGACGAAGCTGGAAAAGTGTTAAAACCGTCGACTGACAACCCGTTCATA  2070
 R  P  L  R  E  Y  E  M  S  T  T  E  C  T  D  E  A  G  K  V  L  K  P  S  T  D  N  P  F  I    690

GAATCTTCGCAACAACCAAACGTAGTGGACATGAGAGATGTTATGGTACTGAATGAAATCATCGAGCAAGCGGGCCGCCAGAGTCGAGCG  2160
 E  S  S  Q  Q  P  N  V  V  D  M  R  D  V  M  V  L  N  E  I  I  E  Q  A  G  R  Q  S  R  A    720

AGTGATCAGAACGTGTCAGTGTACTACTTCCCAACAGCGGAAGACGATGAGGATGGGCCCACGTTCAAGGAGAAACTCCTCGAGTGCCTG  2250
 S  D  Q  N  V  S  V  Y  Y  F  P  T  A  E  D  D  E  D  G  P  T  F  K  E  K  L  L  E  C  L    750

ATGAAGGCGATAGACTTCTTCTGTGTGTGGGACTGCTGTTGGTTGTGGCTGGAGTTTCAGAAATACGTGGCGCTACTTGTGTTCGATCCC  2340
 M  K  A  I  D  F  F  C  V  W  D  C  C  W  L  W  L  E  F  Q  K  Y  V  A  L  L  V  F  D  P    780

TTCGTGGAGTTGTTCATCACCCTGTGTATCGTAGTGAACACGCTGTTCATGGCGCTCGACCACCACGACATGGACAAAGATATGGAGAGA  2430
 F  V  E  L  F  I  T  L  C  I  V  V  N  T  L  F  M  A  L  D  H  H  D  M  D  K  D  M  E  R    810

GCGCTCAAAAGTGGCAACTATTTTTTCACTGCTACATTCGGAATAGAAGCCATGTCGAAATTAGTAGCTATGAGCCCTAAGTTTTACTTC  2520
 A  L  K  S  G  N  Y  F  F  T  A  T  F  G  I  E  A  M  S  K  L  V  A  M  S  P  K  F  Y  F    840

CAAGAGGGTTGGAATATATTTGACTTTATCATTGTGGCCTTATCACTGTTGGAGTTGGGATTGGAAGGAGTTCAGGGTTTGTCAGTGTTG  2610
 Q  E  G  W  N  I  F  D  F  I  I  V  A  L  S  L  L  E  L  G  L  E  G  V  Q  G  L  S  V  L    870

CGTTCCTTTCGTTTGCTTCGAGTATTCAAATTGGCAAAGTCATGGCCGACACTTAATTTACTCATCTCCATAATGGGTAGGACAATGGGT  2700
 R  S  F  R  L  L  R  V  F  K  L  A  K  S  W  P  T  L  N  L  L  I  S  I  M  G  R  T  M  G    900

GCCTTGGGCAACCTGACCTTCGTATTGTGCATCATTATTTTCATATTTGCGGTGATGGGTATGCAACTATTCGGGAAAAATTACGTGGAT  2790
 A  L  G  N  L  T  F  V  L  C  I  I  I  F  I  F  A  V  M  G  M  Q  L  F  G  K  N  Y  V  D    930

TACGTAGATCGATTCCCGGACGGGGACCTCCCGCGATGGAACTTCACGGATTTCATGCACAGCTTCATGATTGTGTTCCGAGTGCTCTGC  2880
 Y  V  D  R  F  P  D  G  D  L  P  R  W  N  F  T  D  F  M  H  S  F  M  I  V  F  R  V  L  C    960

GGAGAATGGATAGAAAGTATGTGGGACTGTATGTTGGTCGGAGATGTCTCTTGTATACCCTTCTTCTTGGCTACCGTCGTCATTGGCAAT  2970
 G  E  W  I  E  S  M  W  D  C  M  L  V  G  D  V  S  C  I  P  F  F  L  A  T  V  V  I  G  N    990

CTTGTGGTACTTAACCTTTTCTTGGCCCTGTTACTGTCAAATTTCGGCTCATCGAGTTTGTCGACACCGACTGCCGATCAGGATACCAAC  3060
 L  V  V  L  N  L  F  L  A  L  L  L  S  N  F  G  S  S  S  L  S  T  P  T  A  D  Q  D  T  N  1020

AAAATAGCAGAGGCCTTCAACAGAATATCAAGGTTTATTGATTGGGTGAAACGAAACGTAGCGGACGTTATGAAACTACTGAAGAACAAA  3150
 K  I  A  E  A  F  N  R  I  S  R  F  I  D  W  V  K  R  N  V  A  D  V  M  K  L  L  K  N  K  1050

CTGACCAATCAGATAGCGATCCACGCACCCGAGCGAGTCGACAACGAGCTGGAACTCGGCACAGACCTCGACGACGCCGTACTCTACAAA  3240
 L  T  N  Q  I  A  I  H  A  P  E  R  V  D  N  E  L  E  L  G  T  D  L  D  D  A  V  L  Y  K  1080

GACAAGAAACTCAAAGACCAAGTAGAAGTAGCTATAGGTGACGGTATGGAATTCACTATACCAGGCGACAATAAATACAAGAAAGGCAAA  3330
 D  K  K  L  K  D  Q  V  E  V  A  I  G  D  G  M  E  F  T  I  P  G  D  N  K  Y  K  K  G  K  1110

ATTTTGTTAAATAATATTAACGCAATAACAGATAATCATAGAGACAACCGTTTAGATTGTGAATTAAATCATCACGGATATCCTATACAG  3420
 I  L  L  N  N  I  N  A  I  T  D  N  H  R  D  N  R  L  D  C  E  L  N  H  H  G  Y  P  I  Q  1140

GATGACGATACAATTAGTCAAAAATCGTACGGCAGTCATAAAATCAGGTCGTTTAAAGATGAAAGTCATAAAGGTTCCGCAGACACGATA  3510
 D  D  D  T  I  S  Q  K  S  Y  G  S  H  K  I  R  S  F  K  D  E  S  H  K  G  S  A  D  T  I  1170

GATGGCGAAGAAAAGAAGGACGCTAGTAAAGAGGAATTGGGATTAGAAGAAGAACTGGTTGAGGAAGAGGAAGATGGGAAGTTAGACGGA  3600
 D  G  E  E  K  K  D  A  S  K  E  E  L  G  L  E  E  E  L  V  E  E  E  E  D  G  K  L  D  G  1200

GGTCTGGGTAAAACAGACATCATAGTAGCCGCAGACGAAGAAGTTGTTGACGACAGTCCTGCTGACTGCTGTCCTGTGCCATGTTACGCG  3690
 G  L  G  K  T  D  I  I  V  A  A  D  E  E  V  V  D  D  S  P  A  D  C  C  P  V  P  C  Y  A  1230

AAGTTTCCATTCCTTGTGGGTGATGACGAATCTCCCTTCTGGCAAGGCTGGGGCATGCTCCGGTTGAAAACCTTCAAACTCATTGAGAAC  3780
 K  F  P  F  L  V  G  D  D  E  S  P  F  W  Q  G  W  G  M  L  R  L  K  T  F  K  L  I  E  N  1260

ACATATTTCGAAACGGCTGTGATTACAATGATTTTGCTCAGTAGCTTGGCTTTGGCTTTAGAAGATGTAAATTTACCACATCGGCCGATT  3870
 T  Y  F  E  T  A  V  I  T  M  I  L  L  S  S  L  A  L  A  L  E  D  V  N  L  P  H  R  P  I  1290

CTTCAAGATATCTTGTATTATATGGATCGGATCTTCACCGTAATTTTCTTCATCGAGATGTTGATCAAATGGCTTGCCCTTGGCTTCCAG  3960
 L  Q  D  I  L  Y  Y  M  D  R  I  F  T  V  I  F  F  I  E  M  L  I  K  W  L  A  L  G  F  Q  1320
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Fig. 2.2 continued.

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AAATACTTCACAAATGCGTGGTGCTGGCTCGACTTCATCATTGTCATGGTCTCGCTTATAAACTTCGTAGCGGGGCTTTGTGGCGCCGGC  4050
 K  Y  F  T  N  A  W  C  W  L  D  F  I  I  V  M  V  S  L  I  N  F  V  A  G  L  C  G  A  G   1350

GGCATTCAGGCGTTCAAAACGATGCGAACGCTGCGCGCACTGCGCCCTCTCAGGGCCATGAGCCGCATGCAGGGCATGAGGGTGGTGGTA  4140
 G  I  Q  A  F  K  T  M  R  T  L  R  A  L  R  P  L  R  A  M  S  R  M  Q  G  M  R  V  V  V   1380

AACGCTCTCGTGCAAGCAATCCCGTCCATCTTCAACGTGTTGTTGGTGTGTCTTATCTTCTGGCTGATCTTCGCCATCATGGGAGTACAA  4230
 N  A  L  V  Q  A  I  P  S  I  F  N  V  L  L  V  C  L  I  F  W  L  I  F  A  I  M  G  V  Q   1410

CTGTTCGCTGGCAAATATTTCAAGTGCGTCGATCTAAACCACACGACGTTGAGCCACGAAATCATCCCGGACCGGAACGCGTGCATCTTA  4320
 L  F  A  G  K  Y  F  K  C  V  D  L  N  H  T  T  L  S  H  E  I  I  P  D  R  N  A  C  I  L   1440

GAGAACTACACCTGGGAGAACTCACCGATGAACTTCGACCACGTCGGCAAGGCGTGCCTCTGCCTGTTCCAAGTGGCCACCTTCAAGGGA  4410
 E  N  Y  T  W  E  N  S  P  M  N  F  D  H  V  G  K  A  C  L  C  L  F  Q  V  A  T  F  K  G   1470

TGGATACAGATCATGAACGACGCTATTGACTCGAGAGAAGTGGGCCGACAACCTATACGAGAGACGAACATCTACATGTACCTGTACTTC  4500
 W  I  Q  I  M  N  D  A  I  D  S  R  E  V  G  R  Q  P  I  R  E  T  N  I  Y  M  Y  L  Y  F   1500

GTGTTCTTCATTATATTTGGCTCATTCTTCACTCTCAACCTATTCATCGGTGTGATCATCGACAACTTTAACGAACAGAAGAAGAAAGCT  4590
 V  F  F  I  I  F  G  S  F  F  T  L  N  L  F  I  G  V  I  I  D  N  F  N  E  Q  K  K  K  A   1530

GGTGGCAGTCTCGAGATGTTCATGACTGAGGACCAGAAGAAATACTACAATGCCATGAAGAAAATGGGTTCCAAAAAACCATTAAAAGCT  4680
 G  G  S  L  E  M  F  M  T  E  D  Q  K  K  Y  Y  N  A  M  K  K  M  G  S  K  K  P  L  K  A   1560

ATTCCGAGACTGAAGTGGCGGCCACAAGCGATCGTGTTCGAGATAGTGACGGACAAGAAGTTCGACATGATCATCATGTTGTTCATCGGC  4770
 I  P  R  L  K  W  R  P  Q  A  I  V  F  E  I  V  T  D  K  K  F  D  M  I  I  M  L  F  I  G   1590

CTCAACATGTTGACGATGACGCTCGATCACTACCAGCAGTCGGAGACCTTCAGCACTGTCCTCGACTACCTCAACATGATATTCATCGTG  4860
 L  N  M  L  T  M  T  L  D  H  Y  Q  Q  S  E  T  F  S  T  V  L  D  Y  L  N  M  I  F  I  V   1620

ATATTCAGTTCAGAGTGCCTATTAAAAATGTTCGCCTTACGCTACCATTACTTTGTTGAGCCCGTGAGCTTGTTCGATTTCGTAGTAGTC  4950
 I  F  S  S  E  C  L  L  K  M  F  A  L  R  Y  H  Y  F  V  E  P  V  S  L  F  D  F  V  V  V   1650

AATTTCTCAATTCTTAGTTTGGTATTGAGTGATATTATAGAAAAATATTTTGTGTCGCCCACGTTACTGAGGGTAGTGAGAGTAGCGAAG  5040
 N  F  S  I  L  S  L  V  L  S  D  I  I  E  K  Y  F  V  S  P  T  L  L  R  V  V  R  V  A  K   1680

GTCGGTCGTGTGTTGCGTCTCGTGAAGGGCGCGAAGGGTATCCGGACGTTATTGTTCGCACTCGCCATGTCACTGCCAGCCTTGTTCAAC  5130
 V  G  R  V  L  R  L  V  K  G  A  K  G  I  R  T  L  L  F  A  L  A  M  S  L  P  A  L  F  N   1710

ATCTGTCTGCTGCTGTTCCTTGTGATGTTCATCTTCGCCATCTTCGGCATGTCGTTCTTCATGCACGTCAAAGACAAAGGTGGCCTCGAC  5220
 I  C  L  L  L  F  L  V  M  F  I  F  A  I  F  G  M  S  F  F  M  H  V  K  D  K  G  G  L  D   1740

GACGTGTATAACTTCAAGACTTTCGTGCAGAGTATGATCCTGCTATTTCAGATGTCGACGTCCGCCGGCTGGGACGGCGTGCTGGACGGC  5310
 D  V  Y  N  F  K  T  F  V  Q  S  M  I  L  L  F  Q  M  S  T  S  A  G  W  D  G  V  L  D  G   1770

ATCATCAACGAGGAGGAGTGCGACCTGCCGGACAACGAGCGCGGCTACCCCGGCAACTGCGGCTCAGCCACCATCGGCATCACCTACCTG  5400
 I  I  N  E  E  E  C  D  L  P  D  N  E  R  G  Y  P  G  N  C  G  S  A  T  I  G  I  T  Y  L   1800

CTGTCCTACCTCGTCATCTCCTTCCTCATCGTCATCAACATGTACATCGCCGTCATTCTCGAGAATTACTCGCAGGCGACAGAAGACGTG  5490
 L  S  Y  L  V  I  S  F  L  I  V  I  N  M  Y  I  A  V  I  L  E  N  Y  S  Q  A  T  E  D  V   1830
```

Fig. 2.2 continued.

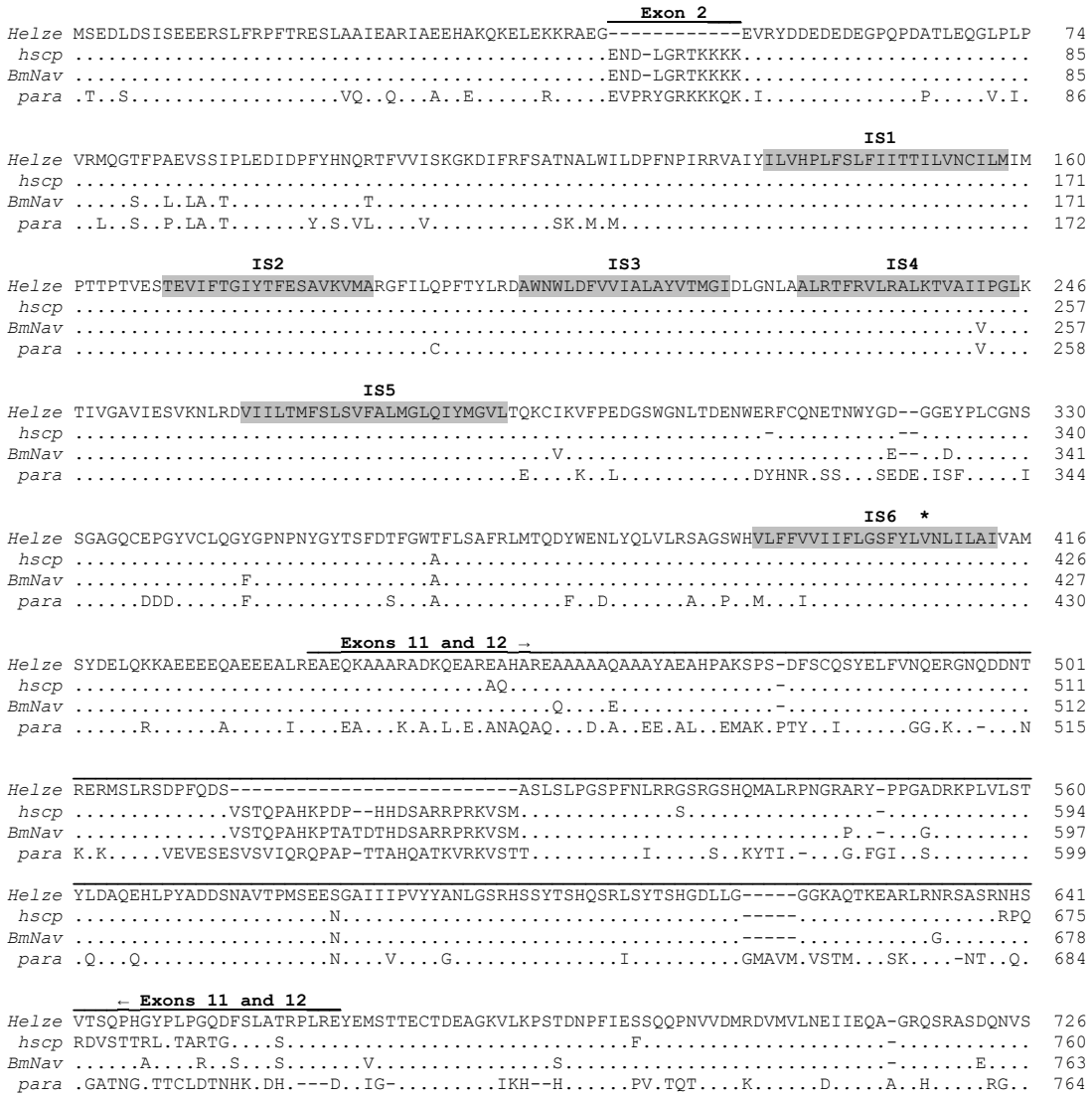


Fig. 2.3. The deduced translation of approximately 90% of the *Helicoverpa zea* voltage-gated sodium channel cDNA compared with *hscp* of *Heliothis virescens* (Park et al. 1999), *BmNa$_v$* of *Bombyx mori* (Shao et al. 2009) and *para* of *Drosophila melanogaster* (Loughney et al. 1989) (GenBank accession nos. GU574730, AF072493, EU822499 and M32078, respectively). The deduced sequence covers the open reading frame from the start codon to beyond the Domain IV S6 transmembrane region. Shaded residues indicate predicted transmembrane regions, periods indicate identity to *hscp*, *BmNa$_v$* and *para,* dashes indicate deletions and * indicate amino acid residue positions where polymorphisms associated with pyrethroid insecticide resistance have been identified (see also Fig. 2.5). Regions homologous to exon 2, exons 11 and 12, and exon 20 of *H. virescens* are indicated with a line above the sequence. ↓ indicates where a GLKAALCGRCVSSE sequence was identified in some clones. The sequence of exon d was shown in this sequence for residues 876 to 929 (see also Fig. 2.4).

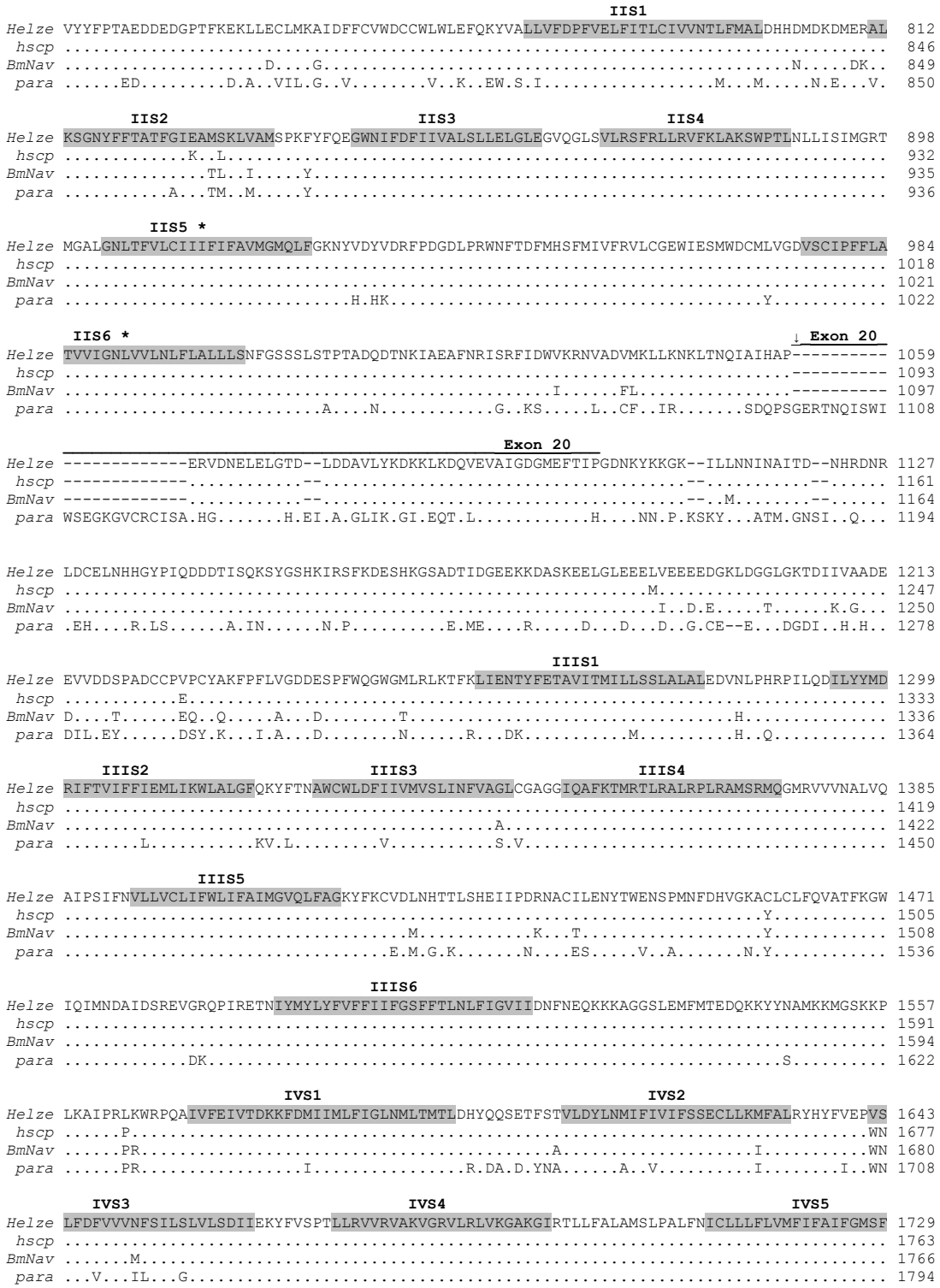


Fig. 2.3 continued.

```
                                                                                IVS6
Helze FMHVKDKGGLDDVYNFKTFVQSMILLFQMSTSAGWDGVLDGIINEEECDLPDNERGYPGNCGSATIGITYLLSYLVISFLIVINMY 1815
 hscp ..................................................................................... 1849
BmNav ..................................................S.............................. 1852
 para .....E.S.IN.......G...................A.....A..P...DK.........V...F.............. 1880

Helze IAVILENYSQATEDV 1830
 hscp ............... 1864
BmNav ............... 1867
 para ............... 1895
```

Fig. 2.3 continued.

The alternative exons homologous to *para* c and d, respectively, have been conserved across insect sodium channels and were present within *H. zea* (Fig. 2.4). Exon d was most prevalent from the *H. zea* cDNA clones sampled and is the exon represented in Fig. 2.3 (residues 876 to 929 in *Helze*).

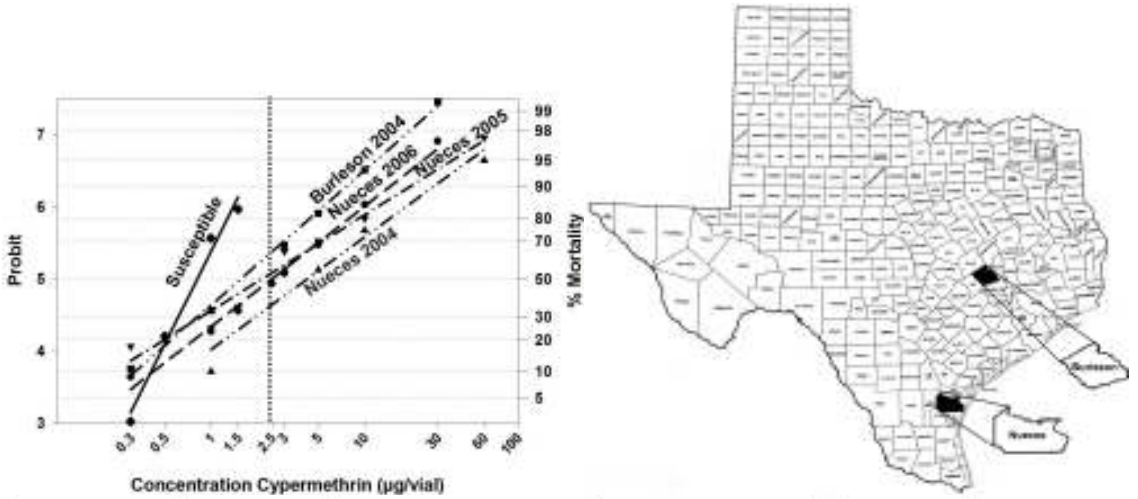
```
          IIS4                              IIS5    *
 Helze c LRVFKLAKSWPALNLIISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYT
 Helze d ...........T...L.......M.............................V
Helze R6 ...........T...L.......M.............V...............V
  hscp c ...................................................
  hscp d ...........T...L.......M.............................V
 BmNav c ...................................................
 BmNav d ...........T...L.......M.............................V
  para c ...........T...L.....................................
  para d ...........T...L.......M.............................H
```

Fig. 2.4. Alignment of mutually exclusive exons c and d from *Helicoverpa zea* (*Helze*) with those from *Heliothis virescens* (*hscp*), *Bombyx mori* (*BmNa*$_v$) and *Drosophila melanogaster* (*para*) (GenBank accession nos. GU574730, AF072493, EU822499 and M32078, respectively) (Park et al. 1999; Shao et al. 2009; Loughney et al. 1989). Shaded residues indicate transmembrane regions. *Helze* R6 represents a cDNA identified from a resistant specimen that contained a mutation homologous to I951V (*) of *para*, potentially an RNA-editing event associated with resistance to pyrethroid insecticides.

Resistance-associated mutations

The cDNA obtained encodes the majority of the sodium channel ORF, therefore allowing analysis of targeted sodium channel regions for possible resistance-conferring mutations from field-collected resistant males. Because the genome of *H. zea* has not yet been sequenced, there is no information on the total number of exons that may be present in this sodium channel gene. Therefore, when referring to resistance-associated mutations in the *H. zea* sodium channel, the residues involved are designated for the amino acid residue number described for *D. melanogaster* (Loughney et al. 1989) due to the similarity between the sodium channels of these two organisms.

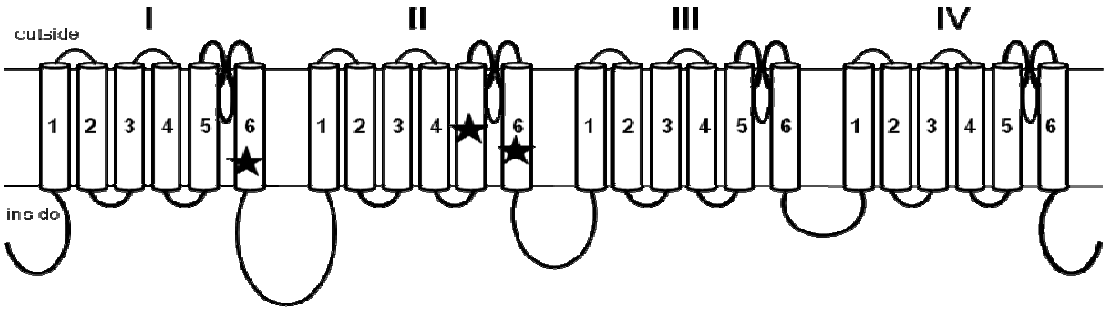
A total of eight individual *H. zea* adult males were identified with sodium channel mutations (Fig. 2.5), corresponding to amino acid residues V421, I951, and L1029 in the *Drosophila* sequence (Fig. 2.3; Fig. 2.6). None of the individual transcripts analyzed contained multiple mutations. At the V421 residue (residue 407 in the *Helze* sequence, Fig. 2.3), we identified three moths with the V421M mutation previously described in *H. virescens*, and also identified three moths with novel mutations at this residue: two with a V421A and one with a V421G (Fig. 2.5; Fig. 2.6). At the homologous site of the L1029F *kdr*-like mutation (residue 991 in the *Helze* sequence, Fig. 2.3), we identified one moth with the L to H mutation also previously described in *H. virescens* (Park and Taylor 1997). All specimens were heterozygous for resistance mutations at the V421 or L1029 residue based on genomic DNA sequencing (Fig. 2.5).



Individual Specimen	County	Concentration Survived (µg/vial)	Date Collected	Mutation	Genotype
R3	Nueces	30	July 5, 2006	V421A	SR
R6	Burleson	30	July 14, 2004	I951V	SR
R8	Nueces	60	June 22, 2004	L1029H	SR
R19	Nueces	10	June 19, 2006	V421M	SR
R22	Nueces	10	June 20, 2005	V421G	SR
R26	Nueces	10	July 4, 2005	V421M	SR
R37	Burleson	10	June 3, 2004	V421A	SR
R39	Burleson	10	July 22, 2004	V421M	SR

Fig. 2.5. Probit analysis, location, date, mutation and genotype associated with pyrethroid insecticide resistance in *Helicoverpa zea* moths surviving various dosages of cypermethrin in the adult vial test above the discriminating dosage of 2.5 µg per vial that kills all susceptible individuals. Under genotype, all individuals were heterozygotes, where S represents the susceptible allele for the respective resistant mutation R shown in each row (see text for specific nucleotide changes).

The homologous mutations at V421 and L1029 were confirmed from genomic DNA and cDNA for the seven *H. zea* resistant male specimens reported (Fig. 2.5). Interestingly, the *para*-homologous Ile 951 to Val mutation was identified in the cDNA sequence of one resistant moth (I913V in the *Helze* sequence, Fig. 2.3), but was not found in PCR amplifications of the corresponding genomic DNA fragment from the same animal despite having sequenced four cloned PCR products from each of five inde-



V421

```
                          IS6          *
 Helze SUSC  VLRSAGSWHVLFFVVIIFLGSFYLVNLILAIVAMSYDELQKKAEE
Helze V421M  ........................M.....................
Helze V421A  ........................A.....................
Helze V421G  ........................G.....................
  hscp SUSC  ............................................
 hscp V421M  ........................M.....................
      BmNav  ............................................
      Pluxy  ............................................
      Musdo  ..QA..P..M...I..............................
      Blage  ......P..M...I..............................
       para  ...A..P..M...I..............................
```

I951 and L1029

```
                *     IIS5                          IIS6         *
  Helze SUSC CIIIFIFAVMGMQLFGKNYVDYVDR//MLVGDVSCIPFFLATVVIGNLVVL
 Helze I951V ...V.....................//.........................
Helze L1029H .........................//.....................H...
   hscp SUSC ...................T.....//.........................
 hscp L1029H ...................T.....//.....................H...
      BmNav .........................//.........................
      Pluxy ...................T.H...//.........................
      Musdo ...................I.HK..//.Y.......................
      Blage ...................Y.N.E.//.....W...................
       para ...................H.HK..//.Y.......................
```

Fig. 2.6. Location (stars) and alignment of *para*-homologous sodium channel gene mutations (V421M, V421A, V421G, I951V, and L1029H) associated with pyrethroid insecticide resistance in *Helicoverpa zea* moths surviving high dosages of cypermethrin in the adult vial test (same specimens as in Fig. 2.5). Shaded residues indicate transmembrane regions and symbols // indicate the rest of the sequence, approximately 36 residues, that are not shown for brevity. The abbreviations for insect species and GenBank accession numbers for homologous sequences are as follows: *Helze* = *Helicoverpa zea*; *hscp* = *Heliothis virescens*, AF072493 (Park et al. 1999); *BmNa$_v$* = *Bombyx mori*, EU822499 (Shao et al. 2009); *Pluxy* = *Plutella xylostella*, AB265177 (Sonoda et al. 2006); *Musdo* = *Musca domestica*, X96668 (Williamson et al. 1996); *Blage* = *Blatella germanica*, U73584 (Dong 1997); *para* - *Drosophila melanogaster*, M32078 (Loughney et al. 1989).

pendent PCR reactions. This I to V mutation in specimen *Helze* R6 was present within the *para*-homologous exon d, which is mutually exclusive with exon c (Fig. 2.4). Econ c from specimen *Helze* R6 did not contain the I to V mutation.

Discussion

Gene structure

This *H. zea* sodium channel predicted protein sequence shared 96.2% identity with the published *H. virescens* sequence *hscp* (Park et al. 1999), 94.5% identity with the *B. mori* sequence $BmNa_v$ (Shao et al. 2009) and 78.6% identity with *para* (Loughney et al. 1989) (Fig. 2.3).

The majority of sequence analysis of the *H. zea* sodium channel was at the cDNA level, so little of the genomic organization is known. The lack of exon 2 in the *H. zea* cDNA (Fig. 2.3, *hscp* and $BmNa_v$ residues 49-59; *para* residues 49 to 60) could be due to a splice variant specific to adult male brain tissue.

In the *H. virescens* sodium channel gene, the deduced exons 11a-c homologous to the *para* exons 4-6 could not be amplified by RT-PCR from cDNA of 1-day old adult males and the corresponding sequences appear instead to be within an intronic region in the *H. virescens* sodium channel (Park et al. 1999). Therefore, it is unclear if these regions of 11a-c are actually expressed in the sodium channel protein in *H. virescens*. The same homologous region of the sodium channel cDNA in *B. mori* is comprised of exons 11-14 (Shao et al. 2009). When we compared the *H. zea* cDNA sequence to *B. mori* cDNA, *H. zea* contained almost the full homologous exon 11 (residues 438 to 515

of *Helze*) and both homologous exons 13 and 14 (residues 516 to 666 of *Helze*), but lacked the last 26 residues of exon 11 of *B. mori* (residues 527 to 552 of $BmNa_v$) (Fig. 2.3). Based on the findings of Shao et al. (2009) in *B. mori*, this is another region of the sodium channel that undergoes extensive stage-specific alternative splicing, which is likely the reason only some of these exons were identified from our adult male *H. zea* cDNAs and none were found by Park et al. (1999) from adult male *H. virescens* cDNAs.

There was another segment of the *H. zea* sequence just before the homologous exon 20 of *H. virescens* where both heliothines and *B. mori* lacked 23 residues of sequence when compared with the homologous *para* region (Fig. 2.3, residues 1099 to 1121 of *para*); however, the amino acid sequence of GLKAALCGRCVSSE was commonly found in this location among the clones analyzed from the different *H. zea* specimens analyzed (not shown). This 14 amino acid sequence is identical to the *B. mori* internally optional spliced exon 22 (Shao et al. 2009) and only the last residue E differs from the one present in the sodium channel of *Plutella xylostella* L. (Sonoda et al. 2008). It is possible that this splice variant may also occur in *H. virescens*, but just was not identified in the transcripts examined by Park et al. (1999).

In the *para* gene, splicing is such that exons c and d are mutually exclusive (Loughney et al. 1989). The *H. zea* exons were identical in sequence and length to those described in *H. virescens* (Park et al. 1999) and *B. mori* (Shao et al. 2009) (Fig. 2.4).

Resistance-associated mutations

We report coexistence of multiple mutations putatively conferring target site insensitivity to pyrethroids in male moths collected from geographically close areas. Most of the mutations that were identified are similar to those found previously in *H. virescens* (Park and Taylor 1997; Park et al. 1997), a moth that was previously classified in the same genus as *H. zea*. In both species, the homologous *kdr* mutation was a L1029H, caused by a single point mutation in the codon from CTT to CAT. This CTT nucleotide sequence would have to had been mutated in the first position to allow for the L1029F mutation (TTT) which has been identified in several pyrethroid-resistant insects (Davies et al. 2007b), but not found in *H. zea*. Due to a different codon usage in *H. zea*, a single point mutation of the CTT nucleotide sequence would not allow for the L1029S mutation, TTA (L) to TCA (S), which has been identified in some mosquitoes (Ranson et al. 2000; Davies et al. 2007b). The V421M mutation had previously only been identified from *H. virescens*, and the mutations found in *H. zea* are the first instances of other possible resistance-conferring mutation, Val 421 to Ala (GTG to GCG) or Val 421 to Gly (GTG to GGG), at this residue. Heterologous expression studies in *Xenopus* oocytes followed by electrophysiology experiments will be necessary to confirm that these novel mutations indeed reduce sensitivity to pyrethroids.

Interestingly, all of the specimens with mutations at the V421 and L1029 residues were heterozygous for these mutations, which has important implications for resistance management. Martin et al. (1999) set the discriminating dosage of cypermethrin required to kill all homozygous susceptible and heterozygous resistant *H.*

zea at 10 µg per vial based on previous work with *H. virescens* (Plapp et al. 1987). The assumption that only homozygous resistant moths survive 10 µg cypermethrin per vial has been used to estimate the frequencies of resistance alleles within populations of *H. zea* (Pietrantonio et al. 2007). The ability of these heterozygous resistant individuals to survive this 10 µg per vial dosage requires us to change the assumptions that have previously been made for resistance monitoring, at least for the target site insensitivity mechanism.

The finding of the *para*-homologous I951V mutation identified from one male moth is significant. Although there is always a possibility that this could arise from a sequencing error, this is unlikely. The cDNA sequence was confirmed from repeated PCR experiments using a high fidelity polymerase. In addition, the homologous housefly residue (I936) was predicted to be important for pyrethroid binding in the O'Reilly molecular docking model (O'Reilly et al. 2006), and the I to V mutation was further confirmed experimentally to cause pyrethroid resistance through mutant sodium channel expression in the *Xenopus* oocyte system and subsequent electrophysiological analysis (Usherwood et al. 2007). Davies et al. (2007a) did find a specimen from the "susceptible" *Culex pipiens quinquefasciatus* Whole Genome Shotgun (WGS) project with this mutation; however, the mutation in *H. zea* would be the first confirmed example of this I to V mutation in a field-collected insect that has been phenotypically confirmed as resistant by surviving 30 µg cypermethrin per vial, about one order of magnitude higher than the LC_{90} of the susceptible population (Fig. 2.6) (Kanga et al. 1996). Discovery of this predicted mutation in field-resistant insects substantiates the

validity and importance of computer molecular docking modeling systems as shown by O'Reilly et al. (2006).

Significantly, because of the inability to confirm the presence of this mutation in the genomic DNA from the same individual, we cautiously suggest that this I951V mutation may represent an RNA-editing event. Insect sodium channel transcripts have been shown to undergo extensive RNA-editing and can cause amino acid changes, resulting in functionally distinct channels with unique gating properties (Hanrahan et al. 2000; Palladino et al. 2000; Hoopengardner et al. 2003; Song et al. 2004; Dong et al. 2007). The I951V mutation would be a typical adenosine-to-inosine (A-to-I) modification by adenosine deaminases and was also observed within a highly conserved sequence region, which is characteristic for genes that undergo RNA-editing (Hoopengardner et al. 2003). In addition, this mutation was present within the mutually exclusive exon d (Fig. 2.2). Transcriptional expression patterns of mutually exclusive exons c and d (and their homologues) have been shown to be specific to different developmental stages (Sonoda et al. 2006; Shao et al. 2009), but their functional significance in sodium channel gating properties has yet to be examined. This I951V mutation within exon d would result in alternatively spliced channels with distinctly different properties and pyrethroid susceptibilities depending on if they contained exon c or d.

The literature on how alternative splicing and RNA-editing play integral functions in insect sodium channel pharmacology has greatly expanded in recent years (Liu et al. 2004; Song et al. 2004; Dong 2007); however, little is known about their role

in insecticide resistance. Alternative splicing was responsible for different sensitivities to pyrethroids in the diamondback moth, *P. xylostella* (Sonoda et al. 2006). Liu et al. (2004) identified an RNA-editing event that resulted in persistent tetrodotoxin-sensitive sodium channel currents, but this I951V mutation would be the first instance of RNA-editing at this residue and of pyrethroid resistance caused by RNA-editing (Dong 2007). Unfortunately, this mutation was only identified in one resistant specimen and this was the lone specimen preserved from this date and location (Fig. 2.5). If RNA-editing is responsible for pyrethroid resistance, this could make it much more difficult to quickly screen specimens for resistance because it would not be possible to identify this type of mutation using a genomic DNA template, but it would be necessary to synthesize cDNA, a process that is more costly and time consuming. Future studies to identify novel residues for target site resistance may need to analyze mutations in the cDNA and compare them to the corresponding genomic DNA of single individuals.

Fig. 2.5 show that the only the *kdr*-like mutation L1029H was identified from a male moth surviving 60 µg cypermethrin per vial. Although the moths that survived 10 and 30 µg cypermethrin per vial may also have been able to survive this 60 µg concentration, the mutations at the V421 and I951 residues were associated with only the lower concentrations and may be indicative of the level of resistance associated with these two mutations. In general, results from heterologous expression assays incorporating these homologous sodium channel mutations *in vitro* (Park et al. 2000; Lee and Soderlund 2001; Usherwood et al. 2007) corresponded to the fold increase in insecticide that male moths were able to survive in this study.

Significance

Identification of the sodium channel sequence from *H. zea* will allow for further insecticide modeling studies and eventual expression in the *Xenopus* oocyte system for hypothesis testing on target site resistance for insecticides that act on the voltage-gated sodium channel. Knowledge of the mutations associated with resistance will allow for the development of molecular diagnostic tools that could be used to determine if target site resistance is present in the field and at what frequency. RNA editing may be implicated in target site insensitivity to pyrethroids and this mechanism deserves further investigation in other resistant species. Understanding the molecular mechanisms responsible for resistance will greatly improve our ability to identify and predict resistance, preserve susceptibility to pyrethroid insecticides in pest populations, as well as design novel pyrethroids that could be toxic even for cypermethrin-resistant insects with mutated sodium channels.

CHAPTER III

METABOLIC RESISTANCE

Introduction

Metabolic resistance is the increased ability to degrade or sequester insecticidal compounds to nontoxic forms. Cytochrome P450-dependent monooxygenases (CYPs) are one of the main families of enzymes responsible for the metabolism of natural and synthetic xenobiotics (Feyereisen et al. 2005; Li et al. 2007). In addition to the development of target site resistance to pyrethroids, there was also the development of metabolic resistance, and CYPs have been the most common of these enzyme families associated with resistance to pyrethroids through enhanced gene expression. Significant synergism with piperonyl butoxide suggested that enhanced metabolism by could be a mechanism of resistance to cypermethrin (Kanga et al. 1996).

Regarding oxidative modes of insecticide resistance, the first cytochrome P450 cDNA cloned from heliothines was the pyrethroid-inducible *CYP6B2* from *H. armigera* (Xiao-Ping and Hobbs 1995). Pittendrigh et al. (1997) characterized eight genes from *H. armigera*, including *CYP4S1*, *CYP4S2*, *CYP4G8*, *CYP4G9*, *CYP4G10*, *CYP4M4*, *CYP4L3*, and *CYP9A3*, and Ranasinghe and Hobbs (1998) cloned cDNAs for *CYP6B6* and *CYP6B7*. *CYP4G8* and *CYP6B7* were overexpressed in pyrethroid resistant *H. armigera* individuals. *CYP9A12* and *CYP9A14* were isolated from *H. armigera* and were constitutively overexpressed with *CYP6B7* in pyrethroid resistant insects (Yang et al. 2006). *CYP6B10* has been cloned from *H. virescens* (Pimprale and Brown 1999b), and the overexpression of *CYP9A1* was associated with thiodicarb resistance (Rose et al.

1997). *CYP6B9* (Pimprale and Brown 1999a), *CYP9A12v3*, and *CYP9A14* (Chen and Li 2007) have been cloned from *H. zea*, and *CYP6B8* (Li et al. 2000a), *CYP6B27* and *CYP6B28* (Li et al. 2002a), and *CYP4M6, CYP4M7*, and *CYP321A1* (Sasabe et al. 2004) from *H. zea* express enzymes involved in xenobiotic metabolism.

Of the four genes identified from *H. zea* in the *CYP6* family, *CYP6B28* and *CYP6B27* are paralogous to *CYP6B8* and *CYP6B9*, respectively (Li et al. 2002a). All four genes seem to have evolved through gene duplication and conversion from a common ancestor and exhibit developmental and tissue-specific expression patterns (Li et al. 2002b). *CYP6B8* has 99.3% nucleotide and 99.8% amino acid identity to *CYP6B28*, and *CYP6B9* has 95.8% nucleotide and 97.4% amino acid identity to *CYP6B27* (Li et al. 2002a).

Our knowledge of the importance of *CYP* genes from *H. zea* has increased in recent years and there is much literature on the contribution of these genes to xenobiotic metabolism and the extent to which they are induced upon insect exposure to toxins and pesticides. Heterologous expression of recombinant enzymes has demonstrated that the different *CYP* genes are capable of metabolizing a wide range of xenobiotics. For example, *CYP6B8* metabolizes the insecticides α-cypermethrin, aldrin, and diazinon, as well as multiple allelochemicals such as flavones, rutin, xanthotoxin, angelicin, chlorogenic acid, indole-3-carbinol, and quercetin (Li et al. 2004; Rupasinghe et al. 2007).

In addition, exposure to xenobiotics can cause induction of *CYP* genes. Plant defense compounds such as jasmonate and salicylate were shown to induce expression

levels of *CYP6B8*, *CYP6B28*, *CYP6B9*, and *CYP6B27* as a sort of "eavesdropping" mechanism to protect *H. zea* from toxic plant compounds (Li et al. 2002c). Exposure of *H. zea* to α-cypermethrin resulted in only low levels of *CYP* induction (Li et al. 2000a; 2002b); however, exposure to plant compounds such as xanthotoxin or the general P450 inducer phenobarbital significantly induced *CYP* genes, thereby increasing tolerance levels to insecticides and mycotoxins (Li et al. 2000b; Wen et al. 2009).

Even as resistance to pyrethroids has been well documented in *H. zea* populations during the last two decades, the molecular mechanisms underlying this resistance have yet to be elucidated. The objective of this study was to compare the transcriptional expression levels of *CYP6B8* and *CYP6B9* from susceptible *H. zea* with field-collected specimens identified as resistant by survivorship to discriminating dosages of cypermethrin in the Texas monitoring program to determine if increased metabolism by these enzymes is a mechanism associated with moth resistance.

Materials and methods

Insects

Susceptible specimens were from a colony originally collected from Burleson Co., Texas, that was maintained insecticide-free in the laboratory for multiple generations at 27^{o}C with a photoperiod of 16:8 (L:D) h. For rearing, larvae were provided with a pre-mixed synthetic diet (Stonefly Heliothis Diet, Ward's Natural Science, Rochester, NY), and adults with 10% sucrose solution.

Vial assays

Vial assays were conducted with the pyrethroid cypermethrin as described by Pietrantonio et al. (2007). Resistant insects that survived discriminating dosages of cypermethrin (30 or 60 µg/vial) in the Texas pyrethroid resistance monitoring program were stored at -80^{o}C and subsequently used for analysis in this study. For the Uvalde 2009 assay, moths pretreated with PBO (Aldrich Chemical Company, Inc., Milwaukee, WI) were exposed to vials coated with 500 µg PBO/vial for 1 h prior to placement in cypermethrin vials (Kanga et al. 1996).

Total RNA isolation and cDNA synthesis

Adult male *H. zea* abdomens were individually dissected (mostly fat body and digestive system tissues) and placed immediately in RNAlater® (Ambion, Austin, TX, USA). This tissue was ground in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated as per the manufacturer's protocol with the additional optional isolation step. The RNA pellet was dissolved in 100 µL DEPC-water and 7 µL was used to synthesize cDNA using the SuperScript® III first strand cDNA synthesis kit (Invitrogen) as per the manufacturer's protocol.

Amplicon design for quantitative PCR

Primer Express® Software (Applied Biosystems, Foster City, CA, USA) was used to design the most optimal primers for three genes, *CYP6B8*, *CYP6B9*, and cytoplasmic actin (Table 3.1). Optimal primer concentrations (300 nM forward and 900

nM reverse) were determined for both sets of primers according to ABI directions (SYBR® Green PCR Master Mix Protocol) using template cDNA as described above. When designing primer amplicons, the *CYP* sequences were aligned using MEGALIGN (DNASTAR Inc., Madison, WI, USA) and CLUSTALW (DNASTAR), and primers were designed in the most dissimilar sequence regions of the genes in order to ensure that PCR products were specific to individual *CYP* genes. The *CYP6B8* amplicon may also include *CYP6B28*, as these two genes are extremely similar and were difficult to differentiate (Li et al. 2002a), but the *CYP6B9* primers amplified only this gene and not its paralog *CYP6B27*. The cytoplasmic actin amplicon comprises all three known genes (*HzA3a1* – AF286060, *HzA3a2* – AF286061, and *HzA3b* – AF286059) (Li et al. 2002a).

Table 3.1. Oligonucleotide primers designed for quantitative PCR.

Primer Name	Sequence (5'→3')
CYP6B8F	GGCTTCGCTCCCGTCAA
CYP6B8R	TCAGGAGAAGGTTCTTATGAACAAAA
CYP6B9F	GAACAGACGTGGTCATTGAGAAAG
CYP6B9R	GGGTCGTAGTGAATGCCTCTTG
ActinF	CGTTGCCCTGAGGCTCTCT
ActinR	GATGCCGTTGGCTTCCATAC

Quantitative PCR

A total of 17 moths (five susceptible and twelve resistant) were assayed individually using qPCR. Reactions using SYBR® Green PCR Master Mix (Applied Biosystems) were assembled as follows: to 1530 µl SYBR® Green reagent was added 642.6 µl water. This volume was divided into aliquots (127.8 µl) and 9 µl of ~12ng/µl cDNA template was added to each aliquot from individual *H. zea* cDNAs. This volume was equally divided in three tubes (45.6 µl each) and to each aliquot was added 3.6 µl of forward amplicon primer and 10.8 µl of corresponding reverse amplicon primer for estimation of the *CYP6B8*, *CYP6B9*, and actin transcripts, respectively, in a final volume of 60 µl each. On a 96-well MicroAmp® plate (Applied Biosystems), three wells (20 µl) were loaded for each template-primer combination. QPCR was performed for 45 cycles (95 °C 15 s, 60 °C 60 s) using the 7300 RT-PCR System (Applied Biosystems).

Statistical analysis

Relative abundance for each transcript was calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method and Sequence Detection Software v1.3.1 (Relative Quantification Study Application; Applied Biosystems) according to the manufacturer's directions, with statistical analysis using SPSS v12.0.1 for Windows (SPSS Inc, Chicago, IL, USA). Actin was used as the normalizer and the susceptible insects were used as the reference ratio (calibrator) for *CYP6B8* and *CYP6B9* quantification analysis.

Estimates of LC_{50} and LC_{90} values and 95% confidence intervals were determined by log-dose probit analysis using PoloPlus, Probit and Logit Analysis

software (LeOra Software, Petaluma, CA) (Robertson et al. 2007). The results of chi-square tests (χ^2) were used to estimate how well the data of each concentration-mortality curve fit the assumption of the probit model. Data were corrected for control mortality in the PoloPlus program. Resistance ratios (RRs) were determined at a given response level (50% or 90%) for the susceptible and resistant populations. In order to determine if there were statistically significant differences between the compared lethal concentrations, the 95% confidence intervals for the resistance ratios were calculated. In this pairwise comparison, lethal concentrations were considered significantly different if the value '1' did not fall within the confidence interval for the ratio (Robertson et al. 2007). The overlap of the confidence intervals for lethal concentrations was not used to determine significant differences between them because this method lacks statistical power (Robertson et al. 2007).

Results

The resistant specimens that were analyzed by quantitative PCR had survived concentrations of either 30 or 60 µg cypermethrin per vial in the adult vial test of the Texas pyrethroid resistance monitoring program (Table 3.2) (Pietrantonio et al. 2007). A concentration of 2.5 µg cypermethrin per vial is expected to kill all homozygous susceptible *H. zea* moths (Kanga et al. 1996), so these insects had survived dosages roughly 12 and 24 times greater than the LC_{90} of the susceptible population. Initially, seven different *CYP* genes were cloned for analysis; however, preliminary evidence of

overexpression was seen only for *CYP6B8* and *CYP6B9*, so these were the focus of this study.

Table 3.2. Adult vial test with cypermethrin for male bollworm, *Helicoverpa zea*, populations collected from pheromone traps. Results are for the overall population where resistant individuals used in qPCR were found (Table 3.3). Resistance ratios (RR) with 95% confidence intervals (CI) were calculated by the method of Robertson et al. (2007).

Population	n[a]	Slope ± SE	LC_{50}[b] (95% CI)	LC_{90}[b] (95% CI)	RR LC_{50} (95% CI)	RR LC_{90} (95% CI)	χ^2 (df)
Susceptible[c]	217	1.48 ± 0.36	0.33 (0.08-0.60)	2.44 (1.52-5.77)	1	1	0.46 (3)
R2	531	2.10 ± 0.26	2.93 (2.32-3.55)	11.94 (9.09-17.89)	8.85* (3.83-20.43)	4.89* (2.52-9.47)	5.06 (6)
R4 and R5	295	1.79 ± 0.22	2.41 (1.71-3.17)	12.47 (9.00-19.81)	7.28* (3.06-17.31)	5.10* (2.56-10.19)	1.86 (4)
R9	320	1.48 ± 0.22	1.96 (1.11-2.90)	14.35 (9.62-25.77)	5.93* (2.33-15.05)	5.88* (2.79-12.37)	4.95 (5)
R10	100	1.30 ± 0.27	2.12 (0.58-5.19)	20.40 (7.40-788.98)	6.40* (2.40-17.04)	8.35* (2.68-26.03)	11.54 (7)
R27	400	1.66 ± 0.28	2.56 (0.97-4.20)	15.17 (9.08-43.85)	7.75* (3.08-19.51)	6.21* (2.96-13.05)	9.56 (7)

Resistance ratios (RR) with 95% confidence intervals (CI) are calculated by the method of Robertson et al. (2007) where the resistant population LC is used as the numerator and the susceptible population LC as the denominator. RRs marked with *indicate that LCs are significantly different from the susceptible population ($P \leq 0.05$).

[a]Number of insects tested.

[b]Lethal concentration expressed in micrograms of insecticide per vial with 95% confidence intervals.

[c]Bioassay of Burleson County September 2005 susceptible field population.

Unfortunately, there were only a limited number of specimens that survived high dosages of cypermethrin available for comparison in this study. Repeated attempts to establish a resistant population in the laboratory were unsuccessful, so the only putatively resistant moths available were the ones saved from the resistance monitoring program (Pietrantonio et al. 2007). Only six of the twelve resistant moths tested showed levels of transcriptional expression apparently higher than that of the susceptible moths, so only these six moths were used to compare for statistical analysis (the other six moths were assumed to contain putatively different resistance mechanisms). For this reason, in order to determine if *CYP6B8* and *CYP6B9* transcripts are significantly overexpressed in field-resistant insects compared with that of the susceptible colony, qPCR results from six individual resistant and the five individual susceptible insects were pooled, respectively, to test the null hypothesis that there was no difference in the amount of CYP transcript between susceptible and resistant pools with observed overexpression. Upon statistical analysis, we rejected the null hypothesis and concluded that there was a significantly greater level of transcript of *CYP6B8* ($P = 0.008$) and *CYP6B9* ($P = 0.004$) in the resistant pool than the susceptible pool (Fig. 3.1).

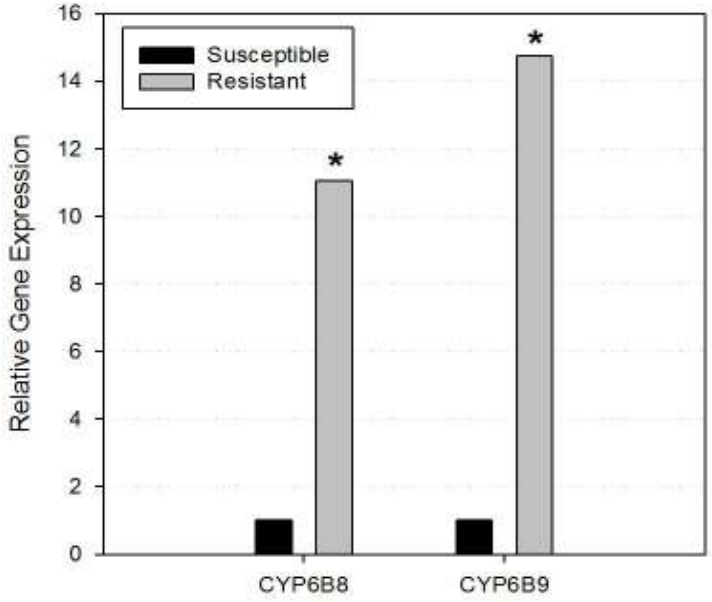


Fig. 3.1. Comparison of *CYP6B8* and *CYP6B9* relative transcriptional expression between pools of susceptible and resistant adult male *Helicoverpa zea* using quantitative PCR with actin as the normalizer. The null hypothesis that relative expression was the same between susceptible and resistant populations was rejected for *CYP6B8* (*P* = 0.008) and *CYP6B9* (*P* = 0.004) (One-Way ANOVA, SPSS).

The six resistant specimens were collected from years 2003 to 2009 from four different counties in Texas (Table 3.3). When individual resistant insects were compared to the susceptible pool, levels of transcriptional overexpression ranged from 3.7 to 33.3 for *CYP6B8* and from 5.6 to 39.6 for *CYP6B9* (Table 3.3).

Table 3.3. Relative expression of *CYP6B8* and *CYP6B9* in moths surviving discriminating dosages of 30 and 60 µg per vial cypermethrin that were tested for transcriptional overexpression using quantitative PCR.

Individual Specimen	County	Concentration Survived (µg/vial)	Date Collected	CYP6B8 Relative Expression	CYP6B9 Relative Expression
Susceptible	---	---	---	1.00	1.00
R2	Nueces	30	June 18, 2006	3.72	5.56
R4	Burleson	30	July 1, 2003	33.33	39.65
R5	Burleson	30	July 10, 2003	5.80	6.38
R9	Uvalde	30	July 13, 2007	12.38	23.64
R10	Williamson	60	July 8, 2008	6.68	7.78
R27	Uvalde	60	June 30, 2009	34.92	37.43

In addition, cypermethrin adult vial tests were performed on moths from the Uvalde Co. 2009 population (Table 3.2) with treatments of cypermethrin and of cypermethrin plus PBO to assess if the resistance levels were reduced due to PBO synergism with cypermethrin (Table 3.4). Results indicated that resistance was completely abolished with pretreatment of PBO.

Table 3.4. Uvalde Co., Texas, adult vial test with cypermethrin or cypermethrin + piperonyl butoxide (PBO) for male bollworm, *Helicoverpa zea*, collected from pheromone traps, 2009. Resistance ratios (RR) with 95% confidence intervals (CI) were calculated by the method of Robertson et al. (2007).

Population	n[a]	Slope ± SE	LC_{50}[b] (95% CI)	LC_{90}[b] (95% CI)	RR LC_{50} (95% CI)	RR LC_{90} (95% CI)	χ^2 (df)
Susceptible[c]	217	1.48 ± 0.36	0.33 (0.08-0.60)	2.44 (1.52-5.77)	1	1	0.46 (3)
Uvalde 2009	400	2.13 ± 0.30	3.01 (1.37-4.81)	12.05 (7.07-48.89)	9.12* (3.89-21.39)	4.93* (2.48-9.81)	18.41 (7)
Uvalde 2009 + PBO	210	2.92 ± 1.04	0.24 (0.05-0.37)	0.67 (0.45-2.12)	0.74 (0.27-2.02)	0.27 (0.13-0.58)	3.93 (4)

Resistance ratios (RR) with 95% confidence intervals (CI) are calculated by the method of Robertson et al. (2007) where the resistant population LC is used as the numerator and the susceptible population LC as the denominator. RRs marked with *indicate that LCs are significantly different from the susceptible population ($P \leq 0.05$).
[a]Number of insects tested.
[b]Lethal concentration expressed in micrograms of insecticide per vial with 95% confidence intervals.
[c]Bioassay of Burleson County September 2005 susceptible field population.

Discussion

In general, the constitutive expression levels of *H. zea CYP* genes has been shown to be relatively low, with low levels of induction occurring when exposed to α-cypermethrin (Li et al. 2000a; 2002b). Therefore, the higher levels of *CYP6B8* and *CYP6B9* seen in these insects were likely indicative of resistance and not just a response to pyrethroid exposure. These results are further supported by complete reduction in pyrethroid resistance with the addition of PBO, a cytochrome P450 inhibitor, to the cypermethrin treatment (Table 3.4). As all moths died that were treated with cypermethrin + PBO, there is also the possibility that other *CYP* enzymes could be contributing to the resistance.

The levels of transcriptional overexpression were similar for both *CYP6B8* and *CYP6B9* in each of the resistant insects, which supports the theory that resistance could likely be due to a mutations in a transcriptional regulatory mechanism common for both genes (Li et al. 2007). Chen and Li (2008) identified the first intact *Transib* transposon within the 5'UTR of the *CYP6B8* gene in a *H. zea* midgut cell line, and there is evidence that transposable elements can affect the transcriptional control or function of genes (ffrench-Constant et al. 2005).

Rupasinghe et al. (2007) compared insecticide metabolism for *CYP6B8* with *CYP321A1*, and while both were capable of effectively metabolizing cypermethrin, the predicted model of the *CYP321A1* catalytic site allowed for the metabolism of larger and more rigid molecules than that of *CYP6B8*. Further analysis of multiple insecticides in substrate modeling and heterologous expression systems may be key in determining effective compounds for overcoming *CYP6B8* and *CYP6B9* overexpression in *H. zea* populations by focusing on molecules that are not easily metabolized by these genes. Target site insensitivity has also been identified in resistant *H. zea* from Texas (see Chapter II), but of the specimens with significant metabolic resistance, none contained any of the sodium channel mutations that have thus far been identified.

While these results include only specimens from Texas, they may have far reaching implications on other production regions throughout the United States. *Helicoverpa zea* is a highly migratory insect and these resistant individuals have the capacity to migrate to other regions as shown in the model for Texas (Pietrantonio et al. 2007). Even though implementation of transgenic *Bt* (*Bacillus thuringiensis*) crops in

the US has reduced the amount of insecticides applied for controlling *H. zea*, pyrethroids are still recommended for high density populations of *H. zea* in *Bt* cotton (Seibert et al. 2008; Sivasupramaniam et al. 2008) and on average in 2008, three-quarters of the *Bt* cotton acres in the US were treated once for bollworms (Williams et al. 2009). The report of *Bt* resistance in *H. zea* field populations in Arkansas and Mississippi (Tabashnik et al. 2008) increases the likelihood that growers may continue to apply pyrethroids to control *H. zea* in *Bt* crops. Other insects such as stink bugs and *Lygus* spp. have emerged as primary cotton pests, resulting in increased non-targeted pyrethroid exposure of *H. zea* because pyrethroids are one of the main choices for their control, especially for stink bugs (Jackson et al. 2004; Snodgrass et al. 2008). In addition, pyrethroids are often applied as miticides in corn for feed in northern Texas. These pyrethroid applications may allow for further selection and/or maintenance of resistance genes throughout *H. zea* populations.

The characterization of metabolic resistance is important because of the potential for cross-resistance, where insects are not only resistant to pyrethroids, but may also be resistant to a broad range of other synthetic pesticides, greatly limiting the options with which they may be controlled. This mechanism of resistance can hinder the use of not only chemicals already on the market, but also may predispose *H. zea* to be resistant to novel pesticides that have yet to even come to market. A greater understanding of the molecular mechanisms responsible for pyrethroid resistance will help us to make better informed decisions for *H. zea* management and improve our ability to monitor temporal and spatial fluctuations in resistance. As overexpression of *CYP6B8* and *CYP6B9* is the

first described mechanism of metabolic resistance for *H. zea* in the field, it provides an important first step for developing high-throughput protein-based assays that can quantify resistance frequency among field populations, such as an enzyme-linked immunosorbancy assay (ELISA) that may be used in concert with current monitoring programs.

CHAPTER IV

RESISTANCE MONITORING*

Introduction

Pyrethroids are widely used for control of *H. zea* because they are extremely effective and relatively inexpensive. Pyrethroid molecules are typically esters of an acid and alcohol that contain some of the esteric properties of the pyrethrins, which are esters of a cyclopropanecarboxylic acid and a cyclopentenolone alcohol (Soderlund et al. 2002). A standard type II pyrethroid such as cypermethrin has an aliphatic cyclopropylalkene acid moiety and an α-cyano-3-phenoxybenzyl alcohol moiety (Fig. 4.1). Structural variations in either the acid or alcohol moiety can affect their potency, metabolism and environmental stability (Fig. 4.1) (Soderlund et al. 2002; Forrester et al. 1993; Khambay and Jewess 2005; Gunning et al. 2007; Tan and McCaffery 2007). For example, esfenvalerate shares the α-cyano-3-phenoxybenzyl alcohol moiety with cypermethrin, but has an aromatic, non-cyclopropane ring, benzene acid moiety (Fig. 4.1). Bifenthrin has an aliphatic cyclopropylalkene acid moiety as cypermethrin, but has a non-cyano biphenyl alcohol moiety (Fig. 4.1).

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*Much of this chapter is reprinted with permission from "Differential efficacy of three commonly used pyrethroids against laboratory and field-collected larvae and adults of *Helicoverpa zea* (Lepidoptera: Noctuidae) and significance for pyrethroid resistance management" by Hopkins, B.W. and Pietrantonio, P.V., 2010. *Pest Management Science*, Volume 66, p. 147-154, Copyright 2010 by John Wiley & Sons, Inc.

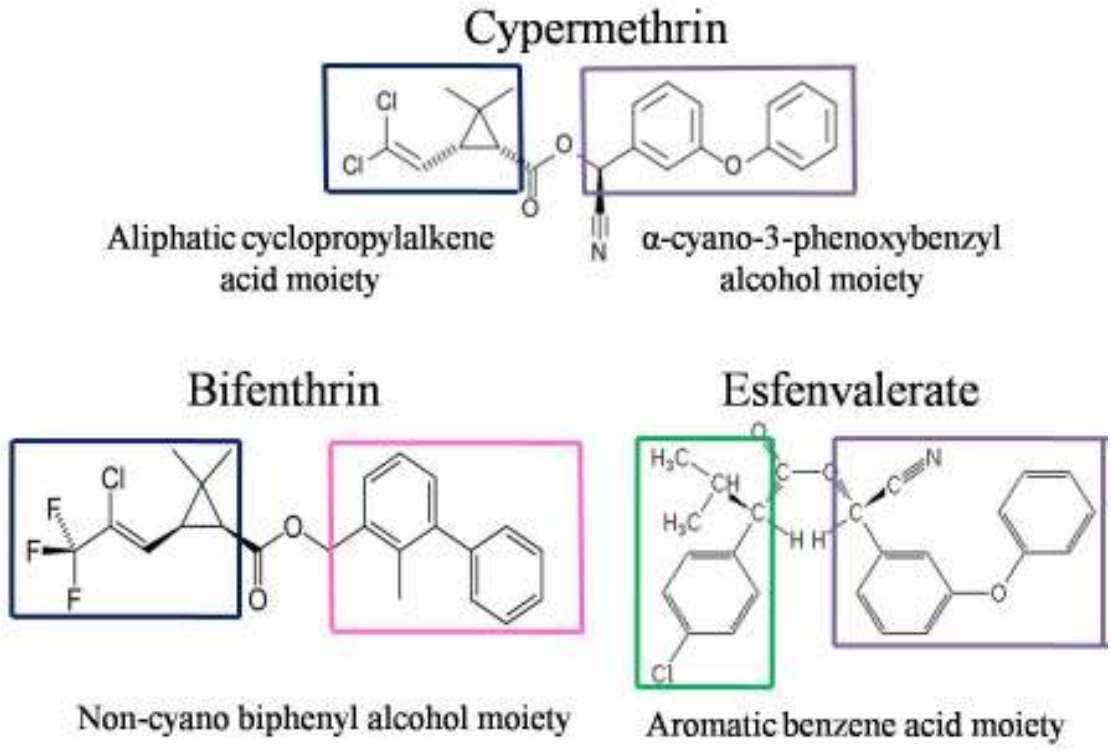

Fig. 4.1. Structural comparison of the acid and alcohol moieties of three pyrethroids: cypermethrin, bifenthrin and esfenvalerate. Cypermethrin and bifenthrin share aliphatic cyclopropylalkene acid moieties (blue boxes) while cypermethrin and esfenvalerate share α-cyano-3-phenoxybenzyl alcohol moieties (violet boxes). Bifenthrin has a distinctive non-cyano biphenyl alcohol moiety (pink box) and esfenvalerate has a distinctive aromatic benzene acid moiety (green box).

Resistance to pyrethroids was identified in *H. zea* in the early 1990's when widespread use of pyrethroids eventually led to resistance in some populations. Stadelbacher et al. (1990) first observed low levels of moth survivorship at a discriminating dose of 10 µg/vial cypermethrin in Mississippi. Hsu and Yu (1991) identified a population of larvae in Florida that exhibited a 3-fold resistance to permethrin. Abd-Elghafar et al. (1993) established colonies of field collected larvae from Illinois and Arkansas and reported LC_{50} resistance ratios of 2.2 to 18 for

permethrin, cypermethrin, and fenvalerate. Kanga et al. (1996) indicated that populations from Texas in 1988 and 1989 showed LC_{50} resistance ratios of 3.4 to 8.8 to cypermethrin. In 1996, LC_{50} resistance ratios of 4.0 to 34.9 to permethrin, cypermethrin, and cyhalothrin were identified in South Carolina *H. zea* populations (Brown et al. 1998). As these cases of pyrethroid resistance were identified, multiple mechanisms of resistance were characterized. Abd-Elghafar and Knowles (1996) identified decreased penetration as a likely mechanism of resistance to fenvalerate. Low frequencies of target site insensitivity to allethrin were discovered in field insects in a neurophysiological assay (Ottea and Holloway 1998). Significant synergism with piperonyl butoxide suggested that enhanced metabolism by cytochrome P450-dependent monooxygenases could be a mechanism of resistance to cypermethrin (Kanga et al. 1996).

Different methods have been used to characterize resistance in lepidopteran field populations. The most commonly used methods for large-scale monitoring of lepidopterans are the vial assay that exposes insects to a residual concentration of insecticide (Plapp et al. 1997) and the topical assay where the pesticide is applied directly to the insect cuticle (Anon. 1980; Mullins and Pieters 1982). In addition to different assays, different growth stages have been tested in these methods as well (e.g., neonates, larvae, and adults). Regardless, the most important element in a monitoring program is consistency to allow the comparison of data through the years (Mullins and Staetz 1994). A multi-state monitoring program was funded by the Insecticide Resistance Action Committee (IRAC) in 1998 and 1999 due to a growing concern about the increase in pyrethroid resistance in *H. zea* (Martin et al. 1999; Martin et al. 2000).

Survivorship in the adult vial test was used to assess resistance of adult male moths collected from traps baited with artificial sex pheromone from populations in 11 states. The program used two discriminating dosages of the pyrethroid cypermethrin: one that should kill all susceptible adults (i.e., 5 μg/vial cypermethrin), and one that should allow only resistant individuals to survive (i.e., 10 μg/vial cypermethrin) (Kanga et al. 1996; Bagwell et al. 1998). Cypermethrin solutions were prepared in acetone and coated on the inside of glass vials to provide residual exposure to moths (Plapp et al. 1987). Resistance monitoring using these discriminating dosages of cypermethrin in the adult vial test has been continued throughout many regions of the US (Luttrell et al. 2006; Payne et al. 2006; Fleischer et al. 2007; Hutchison et al. 2007; Temple et al. 2008). In Texas a range of dosages has been used to gauge resistance at the population level through probit analyses and to allow analyses of the evolution of resistance (Pietrantonio et al. 2007a; Pietrantonio et al. 2007b; Pietrantonio et al. 2009).

Dr. Pietrantonio's laboratory has monitored *H. zea* resistance to cypermethrin across the state of Texas since 2003 (and since 1997 in the Brazos Valley) using the Adult Vial Test (Plapp 1987) that exposes pheromone trap-collected adult male moths to residues of different concentrations of the pyrethroid cypermethrin in 20 ml glass vials (Pietrantonio *et al*. 2007).

However, the main targets for pyrethroid applications in the field are eggs and early instar larvae, and many pyrethroids structurally different from cypermethrin are used in production agriculture to control *H. zea*. Critically, the relationship between susceptibility to cypermethrin in adults versus larvae has only been preliminarily

explored (Schreiber and Knowles 1991). It is not known to what extent the results obtained with cypermethrin relate to the results that may be obtained with other frequently used pyrethroids, even in susceptible populations. Similarly, using cypermethrin in the adult vial assay may not be appropriate for predicting pyrethroid resistance in larvae, females or even in males expressing different mechanisms of resistance that may render other pyrethroids ineffective. A comparison of the vial assay using larvae and adults and structurally distinct pyrethroids should provide greater knowledge to assess the reliability of cypermethrin in the adult vial assay for resistance monitoring and detection of resistance in field populations. The objectives of this study were to first, establish baseline susceptibility data for third instars and male moths from the same generation of *H. zea* in the vial assay using the pyrethroids cypermethrin, esfenvalerate, and bifenthrin. Second, use these data from susceptible *H. zea* established herein to determine resistance ratios of a field population of *H. zea* and compare concentration-mortality probit lines of larvae and adults to determine to what extent results obtained with cypermethrin relate to the responses to esfenvalerate and bifenthrin in resistant populations. Third, determine if resistance diagnosed through the vial test with male moths is diagnostic of (or equivalent to) larval resistance.

Materials and methods

Insects

Eggs from a susceptible colony of *H. zea* were generously provided by Nancy Adams of Monsanto; this colony has been maintained insecticide-free in the laboratory

for multiple generations at 27°C with a photoperiod of 16:8 (L:D) h. For rearing, larvae were provided with a pre-mixed synthetic diet (Stonefly Heliothis Diet, Ward's Natural Science, Rochester, NY), and adults with 10% sucrose solution.

Approximately 500 late-instar *H. zea* larvae were collected from field corn in Uvalde County, Texas, on 30 Oct 2008 and reared individually in plastic cups with synthetic diet under the same conditions as the susceptible colony. Upon eclosion adults were mass-reared (ten males and ten females per each gallon jar) to produce an F1 generation that was tested in vial assays.

Vial assays

The inner surface of 20 ml glass scintillation vials was coated evenly with 0.5 ml acetone solutions of one of three pyrethroids: cypermethrin, esfenvalerate, or bifenthrin (Plapp et al. 1987). A wide range of preliminary concentrations were tested to determine the range of dosages to use for experiments (data not shown). Control vials were coated with dehydrated acetone (48 h on 4Å molecular sieves, EM Science, Gibbstown, NJ) and stock solutions of 1000 µg/ml were prepared with technical grade cypermethrin (95.2%; 40:60 *cis:trans*) and bifenthrin (95.9%) (both from FMC Corporation, Princeton, NJ), and esfenvalerate (99.09%) (DuPont, Wilmington, DE) in dehydrated acetone. The baseline susceptibility for the three pyrethroids was estimated using three replicates from the susceptible laboratory colony. One bioassay was possible from the Uvalde field population due to a limitation in the number of insects available. For both laboratory insects and field collected insects, in each replicate the full range of treatments was run

on one single, continuous generation (i.e., the larvae and adults were all from the same egglay).

Dosages for testing the three pyrethroids were selected in logarithmic increments. For the susceptible laboratory colony experiments, the larval vial assay treatments ranged from 0.1 to 2.0 µg cypermethrin/vial with 862 larvae, 0.1 to 2.0 µg esfenvalerate/vial with 861 larvae, and 0.05 to 1.0 µg bifenthrin/vial with 854 larvae. Adult vial assay treatments ranged from 0.2 to 2.0 µg cypermethrin/vial with 693 moths, 0.1 to 1.0 µg esfenvalerate/vial with 598 moths, and 0.02 to 0.5 µg bifenthrin/vial with 778 moths. For the Uvalde Co. field population experiments, the larval vial assay treatments ranged from 0.5 to 20.0 µg cypermethrin/vial with 221 larvae, 0.1 to 10.0 µg esfenvalerate/vial with 190 larvae, and 0.2 to 5.0 µg bifenthrin/vial with 165 larvae. Adult vial assay treatments ranged from 0.5 to 10.0 µg cypermethrin/vial with 155 moths, 0.5 to 5.0 µg esfenvalerate/vial with 141 moths, and 0.2 to 5.0 µg bifenthrin/vial with 156 moths.

Larvae were reared on artificial diet 5-7d to the third instar (20 ± 3 mg), placed individually in vials, and evaluated at 48 h for mortality (Campanhola and Plapp 1989). Larvae were categorized as alive or dead based on the ability to right themselves and crawl when probed. Adult male moths were fed 10% sucrose solution for 1-2 d, placed individually in vials, and evaluated at 24 h for mortality (Plapp et al. 1987). Upon removal from the vials, moths were categorized as alive or dead based on their ability to fly a distance of 2 m.

Statistical analyses

Estimates of LC_{50} and LC_{90} values and 95% confidence intervals were determined by log-dose probit analysis using PoloPlus, Probit and Logit Analysis software (LeOra Software, Petaluma, CA) (Robertson et al. 2007). The results of chi-square tests (χ^2) were used to estimate how well the data of each concentration-mortality curve fit the assumption of the probit model. Concentration-mortality regressions were plotted using SigmaPlot software. Data were corrected for control mortality in the PoloPlus program. Probit lines were compared for parallelism and equality using likelihood ratio tests in PoloPlus.

Lethal concentration ratios (LCRs; tolerance ratios) and resistance ratios (RRs) were determined at a given response level (50% or 90%) to test the relative efficacy of the three pyrethroids for larvae and male moths in the susceptible and resistant populations. In order to determine if there were statistically significant differences between the compared lethal concentrations, the 95% confidence intervals for either lethal concentration ratios or resistance ratios were calculated. In this pairwise comparison, lethal concentrations were considered significantly different if the value '1' did not fall within the confidence interval for the ratio (Robertson et al. 2007). The overlap of the confidence intervals for lethal concentrations was not used to determine significant differences between them because this method lacks statistical power (Robertson et al. 2007).

Hypothesis testing to compare concentration-mortality probit lines was evaluated by likelihood ratio tests for equality and parallelism (chi-square tests and *P* values for

comparing the slope and intercept). The three possible outcomes were that the lines were parallel and equal, parallel but not equal, or not parallel and not equal. When the slopes and intercepts of the concentration-mortality lines were not significantly different, the treatments were considered equivalently toxic. When only the slopes of the concentration-mortality lines were not significantly different, lines were considered parallel, indicating that the two treatments had the same relative potency (i.e., the same variability in response) (Robertson et al. 2007).

Results

Susceptible laboratory colony

Estimates of the LC_{50} and LC_{90} and 95% confidence intervals for the pyrethroids cypermethrin, esfenvalerate, and bifenthrin were calculated for larvae and adults (Table 4.1). The predicted values of the probit model did not differ significantly from the observed values in the vial assays ($P \leq 0.05$), indicating that the probit model was suitable for the concentration-mortality analyses (Table 4.1).

Table 4.1. Toxicity of three pyrethroids in vial assays towards third instars and male moths of *Helicoverpa zea* from the same generation of a susceptible laboratory colony.

Insecticide	Growth Stage	n[a]	LC_{50}[b] (95% CI)	LC_{90}[b] (95% CI)	Slope ± SE	χ^2 (df)
Cypermethrin	Third instar[c]	862	0.37 (0.30-0.44)	1.22 (0.97-1.67)	2.45 ± 0.17	25.03 (13)
	Adult[d]	693	0.70 (0.60-0.81)	1.86 (1.50-2.54)	2.99 ± 0.21	17.84 (10)
Esfenvalerate	Third instar	861	0.40 (0.29-0.54)	1.45 (1.02-2.55)	2.31± 0.16	55.28 (13)
	Adult	598	0.49 (0.39-0.65)	1.35 (0.95-2.47)	2.93 ± 0.23	29.58 (9)
Bifenthrin	Third instar	854	0.13 (0.10-0.16)	0.38 (0.29-0.57)	2.80 ± 0.20	38.02 (13)
	Adult	778	0.17 (0.11-0.26)	0.51 (0.31-1.62)	2.62 ± 0.18	118.44 (13)

[a]Number of insects tested.
[b]Lethal concentration expressed in micrograms of insecticide per vial with 95% confidence intervals (CI).
[c]Evaluations made 48 h after treatment for the three pyrethroids.
[d]Evaluations made 24 h after treatment for the three pyrethroids.

In order to compare the relative efficacy of the three pyrethroids for the larval and adult growth stages separately, probit lines of the three pyrethroids were compared simultaneously for equality and parallelism (Table 4.2; Fig. 4.2). The hypothesis of equality of susceptibility to the three pyrethroids was rejected for both larvae and adults (Table 4.2; Fig. 4.2). The hypothesis of parallelism between all three pyrethroids was not rejected for either growth stage, indicating that cypermethrin, esfenvalerate, and bifenthrin have the same relative potency for both larvae and adults, respectively (Table 4.2; Fig. 4.2). As the hypotheses of equality of the three probit lines were rejected for both larvae and adults, further pairwise comparisons were subsequently made to identify differences between individual pyrethroids within each growth stage (Table 4.2; Fig. 4.2). The pyrethroid with the lowest LC estimate was used as the denominator to calculate the lethal concentration ratio between pyrethroids. Pairwise comparisons of equality between bifenthrin and cypermethrin and bifenthrin and esfenvalerate were rejected for larvae and pairwise comparisons between each of the three pyrethroids were rejected for adults (Table 4.2; Fig. 4.2). Larvae were about three times more sensitive to bifenthrin than to cypermethrin and esfenvalerate, for which they were equally susceptible (Table 4.2; Fig. 4.2). Adults were also more sensitive to bifenthrin than to esfenvalerate and cypermethrin, about three to four times, but were slightly more sensitive to esfenvalerate than cypermethrin (Table 4.2; Fig. 4.2).

Table 4.2. Relative efficacy of three pyrethroids towards third instars and male moths of *Helicoverpa zea* from the same generation of a susceptible laboratory colony.

Insecticide	n[a]	LCR LC_{50} (95% CI)	LCR LC_{90} (95% CI)	Equal	χ^2 (df), tail prob.	Parallel	χ^2 (df), tail prob.
Third Instar All pyrethroids[b]	2577	---	---	Reject#	215. (4), 0.000	Do Not Reject	4.22 (2), 0.121
Third Instar Esfenvalerate/ Cypermethrin	1723	1.10 (0.91-1.32)	1.18 (0.91-1.54)	Do Not Reject	1.98 (2), 0.372	---	---
Third Instar Cypermethrin/ Bifenthrin	1716	2.76* (2.32-3.29)	3.20* (2.51-4.09)	Reject#	151. (2), 0.000	---	---
Third Instar Esfenvalerate/ Bifenthrin	1715	3.03* (2.54-3.62)	3.80* (2.94-4.90)	Reject#	175. (2), 0.000	---	---
Adult All pyrethroids[b]	2069	---	---	Reject#	265. (4), 0.000	Do Not Reject	2.02 (2), 0.363
Adult Cypermethrin/ Esfenvalerate	1291	1.41* (1.21-1.65)	1.38* (1.05-1.81)	Reject#	18.96 (2), 0.000	---	---
Adult Cypermethrin/ Bifenthrin	1471	4.19* (3.60-4.88)	3.65* (2.79-4.78)	Reject#	210. (2), 0.000	---	---
Adult Esfenvalerate/ Bifenthrin	1376	2.97* (2.52-3.50)	2.64* (1.96-3.57)	Reject#	149. (2), 0.000	---	---

Lethal concentration ratios (LCR) with 95% confidence intervals (CI) calculated by the method of Robertson et al.[44] where the pyrethroid with the lowest LC estimate (Table 4.1) was used as the denominator to calculate LCRs between pyrethroids. LCRs marked with * indicate that LCs are significantly different between pyrethroids ($P \leq 0.05$).

Likelihood ratio tests for equality and parallelism calculated by the method of Robertson et al. (2007). The # indicate that the hypothesis of equality was rejected ($P \leq 0.05$).

[a]Number of insects for each category is the sum obtained from Table 4.1.

[b]For either larvae or adults separately, the probit lines for all three pyrethroids were tested simultaneously for equality and parallelism. As the hypothesis of equality was rejected, pairwise comparison analyses followed.

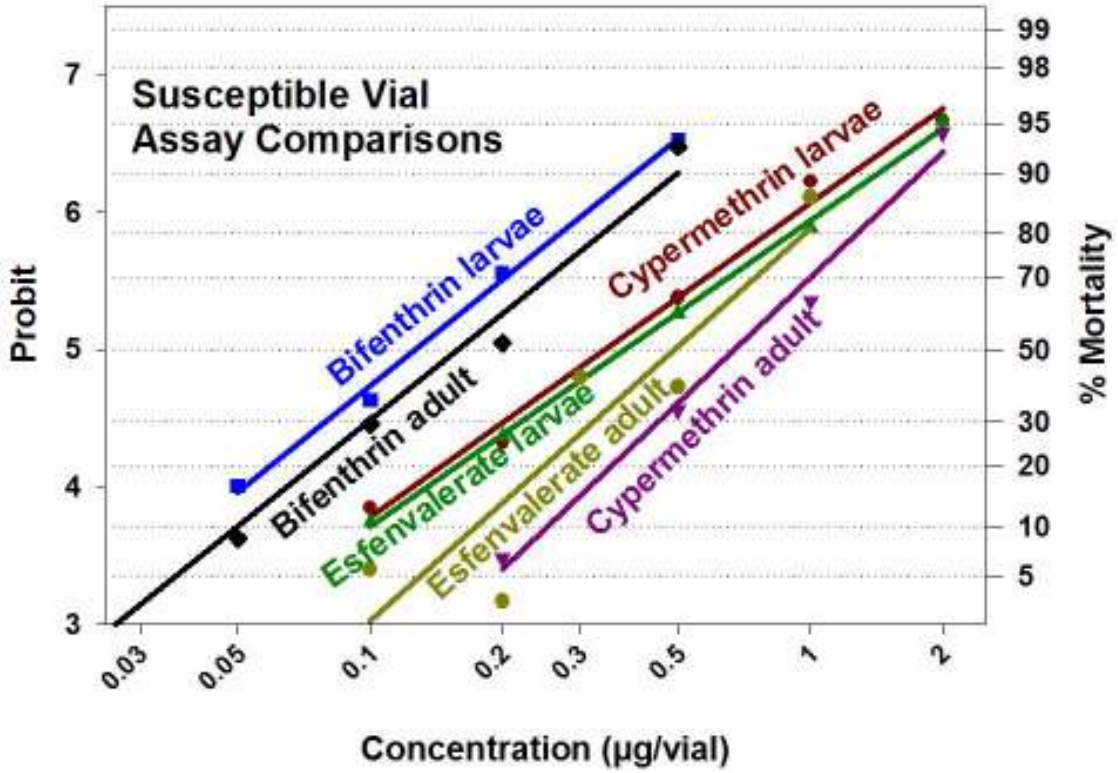

Fig. 4.2. Probit analysis for *Helicoverpa zea* third instars and male moths from a susceptible laboratory colony exposed for 48 h and 24 h in the vial assay, respectively, to cypermethrin, esfenvalerate, and bifenthrin. The hypothesis of equality between all three pyrethroids was rejected for both growth stages but the hypothesis of parallelism was not rejected ($P \leq 0.05$) (Table 4.2). Pairwise comparisons of equality between bifenthrin and cypermethrin and bifenthrin and esfenvalerate were rejected for larvae and pairwise comparisons of equality between all three pyrethroids were rejected for adults ($P \leq 0.05$) (Table 4.2).

Uvalde Co. field population

In order to determine the response of larvae and adults of a resistant population to the three pyrethroids, collections of larvae were made in Uvalde Co., Texas, where resistance had been previously detected through monitoring of male moths (Pietrantonio et al. 2007b; Pietrantonio et al. 2009). Estimates of the LC_{50} and LC_{90} values and 95%

confidence intervals were calculated for the F1 generation of this Uvalde population (Table 4.3). These LC estimates were used to calculate resistance ratios and the 95% confidence intervals of the ratios with respect to the susceptible laboratory colony (Table 4.4). Third instars and male moths of the F1 Uvalde Co. population were significantly more resistant than the susceptible laboratory colony to the three pyrethroids based upon LC_{50} and LC_{90} resistance ratios (Table 4.4). The highest LC_{50} resistance ratios were for bifenthrin and were 9.41 for larvae and 9.28 for adults (Table 4.4). These were followed by LC_{50} resistance ratios of 4.68 for larvae with cypermethrin, 4.67 for adults with esfenvalerate, 4.16 for adults with cypermethrin, and 3.75 for larvae with esfenvalerate (Table 4.4). Pairwise comparisons of equality between probit lines of the Uvalde and susceptible populations of larvae (Fig. 4.3) and adults (Fig. 4.4) were rejected for all pyrethroid treatments, indicating that the Uvalde population was resistant compared to the susceptible one (Table 4.4). Pairwise comparisons of parallelism were rejected only for bifenthrin in the adult vial assay (Table 4.4; Fig. 4.4), meaning that the relative potency of all the other treatments was the same between the susceptible and resistant field population (Table 4.4; Figs. 4.3 and 4.4).

Table 4.3. Toxicity of three pyrethroids in vial assays towards third instars and male moths of *Helicoverpa zea* from the same F1 generation of a Uvalde Co. field population collected from field corn on 30 Oct 2008.

Insecticide	Growth Stage	n[a]	LC_{50}[b] (95% CI)	LC_{90}[b] (95% CI)	slope ± SE	χ^2 (df)
Cypermethrin	Third instar[c]	221	1.71 (1.23-2.27)	7.68 (5.34-13.53)	1.97 ± 0.28	1.40 (4)
	Adult[d]	155	2.89 (2.23-3.64)	7.12 (5.42-10.92)	3.27 ± 0.51	2.44 (3)
Esfenvalerate	Third instar	190	1.51 (1.20-1.90)	4.59 (3.35-7.69)	2.65 ± 0.39	2.99 (4)
	Adult	141	2.30 (1.68-3.02)	5.90 (4.19-12.28)	3.13 ± 0.69	0.23 (2)
Bifenthrin	Third instar	165	1.25 (0.92-1.85)	5.80 (3.29-19.93)	1.92 ± 0.39	3.06 (4)
	Adult	156	1.54 (1.20-1.89)	3.01 (2.36-4.94)	4.40 ± 0.96	1.34 (3)

[a]Number of insects tested.
[b]Lethal concentration expressed in micrograms of insecticide per vial with 95% confidence intervals (CI).
[c]Evaluations made 48 h after treatment for all three pyrethroids.
[d]Evaluations made 24 h after treatment for all three pyrethroids.

Table 4.4. Determination of pyrethroid resistance in field collected *Helicoverpa zea*: Comparison of third instars and male moths from the same F1 generation of a Uvalde Co. population collected from field corn with respect to the susceptible laboratory colony using three pyrethroid insecticides in the vial assay.

Insecticide Growth Stage	n[a]	RR LC_{50} (95% CI)	RR LC_{90} (95% CI)	Equal	χ^2 (df), tail prob.	Parallel	χ^2 (df), tail prob.
Cypermethrin Third instar	1083	4.68* (3.37-6.48)	6.28* (3.89-10.14)	Reject#	117. (2), 0.000	Do Not Reject	2.26 (1), 0.133
Cypermethrin Adult	848	4.16* (3.21-5.38)	3.82* (2.63-5.55)	Reject#	104. (2), 0.000	Do Not Reject	0.28 (1), 0.597
Esfenvalerate Third Instar	1051	3.75* (2.89-4.87)	3.17* (2.05-4.92)	Reject#	86.04 (2), 0.000	Do Not Reject	0.73 (1), 0.394
Esfenvalerate Adult	739	4.67* (3.48-6.27)	4.37* (2.63-7.26)	Reject#	97.90 (2), 0.000	Do Not Reject	0.11 (1), 0.744
Bifenthrin Third Instar	1019	9.41* (6.65-13.31)	15.22* (6.83-33.90)	Reject#	215. (2), 0.000	Do Not Reject	3.82 (1), 0.051
Bifenthrin Adult	934	9.28* (7.32-11.76)	5.90* (4.02-8.64)	Reject#	169. (2), 0.000	Reject#	5.91 (1), 0.015

Resistance ratios (RR) with 95% confidence intervals (CI) calculated by the method of Robertson et al. (2007) where the resistant population LC is used as the numerator and the susceptible population LC as the denominator. RRs marked with * indicate that LCs (Table 4.3) are significantly different from the susceptible population ($P \leq 0.05$).

Likelihood ratio tests for equality and parallelism calculated by the method of Robertson et al. (2007) The # indicate that hypotheses of equality or parallelism were rejected when susceptible and resistant population probit lines were compared ($P \leq 0.05$).

[a]Number of insects for each insecticide and growth stage category is the sum of the susceptible and resistant insects for each category (Tables 4.1 and 4.3).

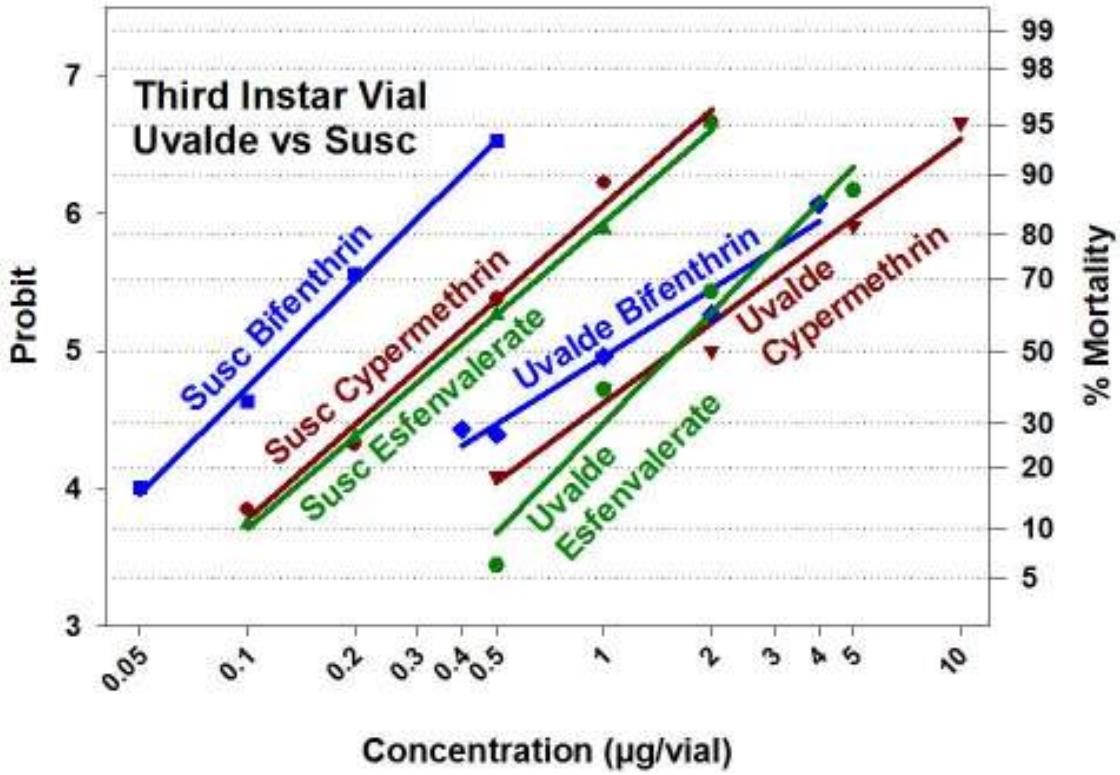

Fig. 4.3. Probit analysis for *Helicoverpa zea* third instars from a susceptible laboratory colony and the F1 generation of a Uvalde Co., Texas, resistant field population exposed for 48 h in the vial assay to cypermethrin, esfenvalerate, and bifenthrin. Pairwise comparisons of equality between susceptible and resistant probit lines were rejected for each of the three pyrethroids but pairwise comparisons of parallelism between susceptible and resistant probit lines were not rejected for any of the three pyrethroids ($P \leq 0.05$) (Table 4.4).

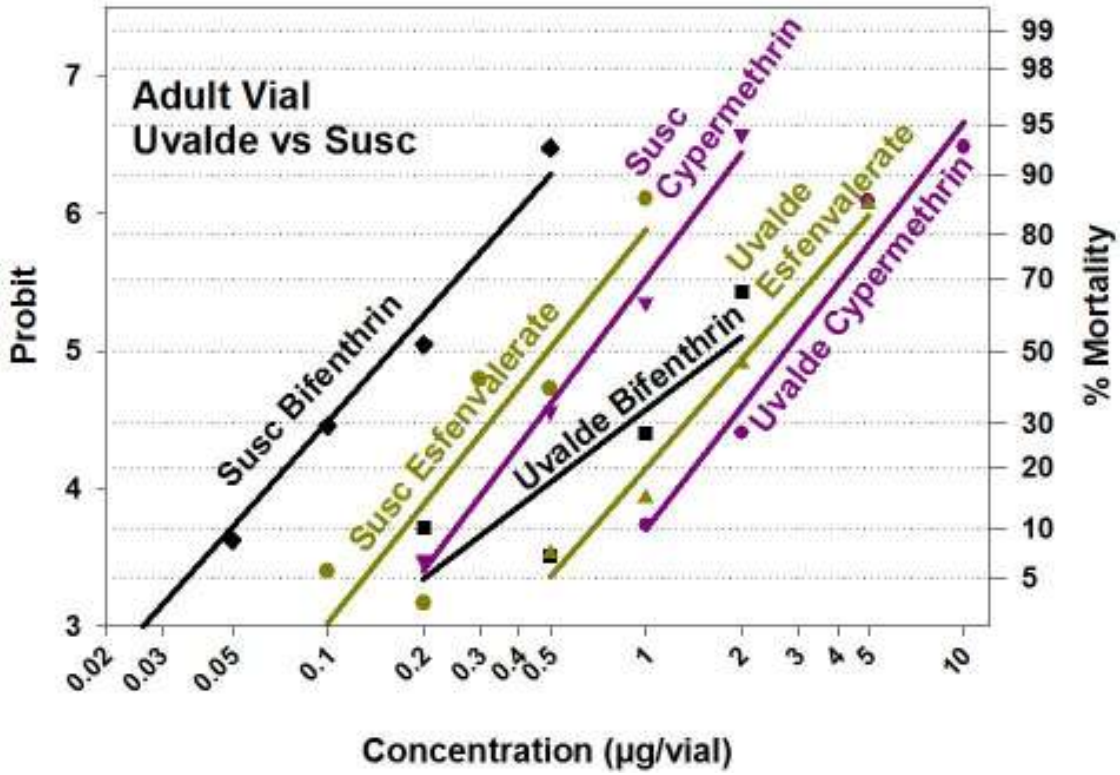

Fig. 4.4. Probit analysis for *Helicoverpa zea* male moths from a susceptible laboratory colony and the F1 generation of a Uvalde Co., Texas, resistant field population exposed for 24 h in the vial assay to cypermethrin, esfenvalerate, and bifenthrin. Pairwise comparisons of equality between susceptible and resistant probit lines were rejected for each of the three pyrethroids but pairwise comparisons of parallelism between susceptible and resistant probit lines were rejected only for bifenthrin ($P \leq 0.05$) (Table 4.4).

Discussion

The main stages of *H. zea* targeted for control with pyrethroid insecticides are eggs and early instar larvae. An emphasis for any monitoring program should be to use a standardized method that closely relates methodology used in the laboratory to field exposure and can be applied to a large enough sample to provide an accurate portrayal of the population (Mullins and Staetz 1994). This allows for increased accuracy in

detection of resistance and enhances relationships between monitoring results and the prediction of field failures due to resistance (ffrench-Constant and Roush 1990). Ideally, monitoring would be conducted on early instar larvae to best represent the situation occurring in the field. However, it is much more difficult to collect large numbers of larvae from the field to use for monitoring. For practicality, male moths have been utilized in resistance monitoring programs due to the ease of collection from pheromone traps (Mullins and Staetz 1994). Using cypermethrin in the adult vial test to monitor for resistance to pyrethroids has been quite successful across many regions of the US (Luttrell et al. 2006; Payne et al. 2001; Fleischer et al. 2007; Hutchison et al. 2007; Pietrantonio et al. 2007a; Pietrantonio et al. 2007b; Temple et al. 2008) and changes in susceptibility levels are quickly evident. However, there are currently shortcomings in using this method for *H. zea*. First, it is not known how susceptibility of larvae and adults is related, and second, if different mechanisms of resistant are present in different growth stages, this method may not be a good predictor of resistance. Therefore, the baseline susceptibility of a susceptible laboratory colony of *H. zea* was evaluated using third instars and male moths with three structurally distinct pyrethroids to see how they compared.

When comparing the differences in efficacy between the three pyrethroids, both growth stages were most sensitive to bifenthrin and adults were more sensitive to esfenvalerate than cypermethrin (Table 4.2; Fig. 4.2). For both growth stages compared independently, when all three pyrethroids were evaluated simultaneously (Table 4.2; Fig. 4.2), the hypothesis of parallelism was not rejected, suggesting that the relative potency

was the same among cypermethrin, esfenvalerate, and bifenthrin. While this was the case for the susceptible laboratory colony, the question that comes into play in a resistance monitoring program is how these results might differ when resistance is present. For example, elevated levels of metabolism might only be present in the larval stage and could be specific to only one type of pyrethroid (Forrester et al. 1993). In this situation, the resistance ratio seen for one of the pyrethroids in a particular growth stage (larvae) would greatly increase, but might not be detectable when testing with the other pyrethroids or in the adult stage.

To address this question, it was important to run the same set of vial assays on a pyrethroid-resistant population. The pyrethroid resistance monitoring program for *H. zea* in Texas has revealed that Uvalde Co. has had some of the most resistant populations of bollworm during the last few cotton growing seasons (Pietrantonio et al. 2007a; 2007b; 2009). Preliminary results indicate that increased transcriptional expression of cytochrome P450 genes (*CYP6B8* and *CYP6B9*) were associated with pyrethroid resistance in Uvalde Co. populations in 2007 and 2009 (see Chapter III). In June 2008, the LC_{50} resistance ratio for male moths for cypermethrin was more than 8, indicating that the population was resistant (Pietrantonio et al. 2009). To have available a resistant field population to compare with our susceptible laboratory colony data, *H. zea* were collected as larvae from field corn in Uvalde Co., Texas, at the end of the season when resistant insects should be present, and reared to the F1 generation in the laboratory to test for resistance (compare Tables 4.1 and 4.3). Upon testing, both the LC_{50} and LC_{90} resistance ratios for all treatments showed that the Uvalde population was indeed more

resistant than the susceptible laboratory colony. This F1 population showed an LC_{50} resistance ratio for cypermethrin in the adult vial assay of almost 5, a value high enough to consider this population resistant in the Texas resistance monitoring program (Table 4.4) (Pietrantonio et al. 2007b). Results from the adult vial assay for esfenvalerate and bifenthrin also indicated a resistant population, thus resistance was detectable in the adult stage with all three pyrethroids tested. Resistance ratios for the LC_{50} similar to that of the adults were observed for the larvae for all three pyrethroids, respectively (Table 4.4). Schreiber and Knowles (Schreiber and Knowles 1989) found similar results for *Heliothis virescens* (Fabricius) when comparing resistance ratios in third instar and adult vial assays using cypermethrin and the pyrethroid-resistant PEG-87 tobacco budworm strain.

However, in our study the LC_{50} resistance ratios for bifenthrin were approximately twice of those of cypermethrin and esfenvalerate (Table 4.4). Some field corn in the same area where this larval population was collected had been sprayed earlier in the season with bifenthrin for control of fall armyworms and spider mites (Troxclair N, 2009, pers. comm.). One possibility for the increased resistance ratio for bifenthrin could be due to selection pressure on an earlier generation of *H. zea* in the Uvalde area. Intrinsic properties of the insecticides may also affect persistence in the field and therefore, resistance. Photolysis and degradation rates differ among pyrethroids, which affect their persistence, and Laskowski (2002) showed that bifenthrin had a considerably longer half-life than cypermethrin or esfenvalerate. The increased time to break down bifenthrin may increase the time for potential exposure for *H. zea* and may also expose

insects to sub-lethal dosages of this insecticide, thus increasing the potential of survival of resistant heterozygotes that may greatly influence resistance levels in subsequent generations. Additionally, analyses of wind trajectories by Pietrantonio et al. (2007b) indicated that migration of resistant populations from other locations may be an important factor in local developments of resistance and perhaps moths migrating into Uvalde could have received bifenthrin pressure elsewhere.

Variations in the pyrethroid structure (see Fig. 4.1; cyclopropane vs. isobutyl and aliphatic vs. aromatic acid; alpha-cyano vs. non-cyano and phenoxybenzyl vs. biphenyl alcohol) along with different combinations of optical and geometric isomers can greatly affect the binding and metabolism of the compound in general (Forrester et al. 1993; Khambay and Jewess 2005; Gunning et al. 2007; Tan and McCaffery 2007). Inherent differences in metabolism and binding properties between different pyrethroid molecule structures could explain the differences in resistance ratios among cypermethrin, esfenvalerate, and bifenthrin described for the Uvalde population. The greater levels of resistance seen to bifenthrin herein were most likely due to its non-cyano, biphenyl alcohol moiety (Fig. 4.1) that makes it structurally divergent from cypermethrin and esfenvalerate.

The same resistance ratios were observed for both larvae and adults for each of the three pyrethroids tested, which indicates that using adults to monitor pyrethroid resistance is an acceptable methodology to estimate larval resistance for a specific pyrethroid. However, as the magnitude of resistance almost doubled when using bifenthrin compared to cypermethrin or esfenvalerate, the choice of pyrethroid used for

monitoring is of great importance. In the case of the Uvalde Co. field population, the current resistance monitoring program with cypermethrin (or potentially one with esfenvalerate) may have underestimated the magnitude of the resistance ratios for bifenthrin, or conversely, evaluations with bifenthrin may have overestimated the magnitude of the resistance ratios for the population if cypermethrin or esfenvalerate were to be used for control. Thus, monitoring results with cypermethrin appear to be clearly applicable to what would be expected with esfenvalerate, but not with bifenthrin. Until we gain a better understanding of the underlying molecular mechanisms responsible for resistance in *H. zea*, it may be necessary to use an additional pyrethroid such as bifenthrin when monitoring for resistance to ensure that resistance does not go undetected. Determining the molecular mechanisms of resistance will allow for the development of diagnostic tools that can be used to identify what mechanisms are present and increase the efficiency of resistance management programs by establishing diagnostic pyrethroids specific for those mechanisms.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The overall goal of this project was to determine the molecular mechanisms responsible for pyrethroid resistance in *H. zea*, and to determine if results from the current resistance monitoring technique, the adult vial test using cypermethrin, correlate with results of bioassays with larvae, the primary targeted developmental stage for field pesticide applications.

To evaluate target site resistance, almost the entire *H. zea* sodium channel was cloned from susceptible males and sequenced. The analysis of the sodium channel sequence in moths selected because of their resistance to high concentrations of pyrethroids in the vial assay revealed a diversity of mutations in field-collected insects. Single point mutations associated with resistance were identified that included the V421M and L1014H mutations previously found in *H. virescens* (Park et al. 1999), as well as two novel mutations at the V421 residue: V421A and V421G. The first two mutations confer reduced sensitivity to pyrethroids in recombinant channels analyzed by electrophysiology (Zhao et al. 2000; Lee and Soderlund 2001) while the novel mutations have yet to be confirmed to cause insensitivity to pyrethroids through similar studies. All of the *H. zea* specimens examined with mutations at these two residues were heterozygous for the mutation. In addition, an I951V mutation was identified. This mutation was predicted to be important for pyrethroid binding (O'Reilly et al. 2006) and was further confirmed to cause insensitivity to pyrethroids in *Drosophila* sodium

channels analyzed using the *Xenopus* oocyte expression system (Usherwood et al. 2007). However, this is the first time a field-collected resistant insect has been characterized with this mutation. Interestingly, the I951V mutation was found only from cDNA and not genomic DNA, which indicates that this may also be the first case of RNA-editing associated with resistance to pyrethroid insecticides.

Metabolic resistance due to transcriptional overexpression of cytochrome P450s was investigated by comparing the relative expression of *CYP6B8* and *CYP6B9* transcript from individual resistant *H. zea* male moths with susceptible moths. A pool of resistant moths had significantly higher levels of transcriptional expression compared to that of the susceptible pool, and the range of overexpression varied from a factor of ~3.7 to 39.6 greater than that of the susceptible. Levels of overexpression were similar for both *CYP* genes, indicating that resistance may be due to interference with a common transcriptional regulatory mechanism.

Although many instances of resistance to pyrethroids have been documented for *H. zea* in the US, this research is the first to describe the molecular mechanisms associated with resistance. Both target site and metabolic resistance mechanisms were identified in male moths from multiple locations across the state of Texas from 2003-2009 (Table 5.1). While both mechanisms were identified, even from insects within the same populations (see Nueces 2006, Table 5.1), there were never any instances identified where one individual insect had both mechanisms of resistance present. Metabolic resistance was the first mechanism indentified (2003), followed by predominantly target site resistance (2004-2005), a mixture of the two mechanisms

(2006), and then only metabolic resistance was identified in the last three growing seasons (2007-2009). Due to the small number of insects analyzed it is difficult to extract conclusions about a succession of specific mechanisms of resistance mechanisms evolving through time.

Table 5.1. Temporal occurrence of pyrethroid resistance mechanisms in male moths of *Helicoverpa zea*.

Date Collected	County	Concentration Survived (μg/vial)	Resistance Mechanism
July 1, 2003	Burleson	30	Metabolic
July 10, 2003	Burleson	30	Metabolic
June 3, 2004	Burleson	10	Target Site
June 22, 2004	Nueces	60	Target Site
July 14, 2004	Burleson	30	Target Site
July 22, 2004	Burleson	10	Target Site
June 20, 2005	Nueces	10	Target Site
July 4, 2005	Nueces	10	Target Site
June 18, 2006	Nueces	30	Metabolic
June 19, 2006	Nueces	10	Target Site
July 5, 2006	Nueces	30	Target Site
July 13, 2007	Uvalde	30	Metabolic
July 8, 2008	Williamson	60	Metabolic
June 30, 2009	Uvalde	60	Metabolic

For the most part, the population levels of pyrethroid resistance as measured by resistance ratios have decreased over the past few seasons, likely due to a combination of different factors. Dr. Pietrantonio's laboratory has recommended that low rates of pyrethroids no longer be used to control sorghum pests such as sorghum midge, *Contarinia sorghicola* (Coquillett), and rice stink bug, *Oebalus pugnax* (Fabricius), as this was likely contributing to resistance by applying early selection pressure to generations of *H. zea* before they moved into cotton, likely allowing survivorship of heterozygotes (Pietrantonio et al. 2007b); a greater adherence to this policy has likely reduced levels of pyrethroid resistance. In addition, the general weather patterns for 2008 and 2009 likely had great influence on *H. zea* resistance, as during both years there were extensive droughts for much of Texas, especially in the southern region including Nueces Co., resulting in lower overall crop acreages, fewer pesticide applications, and in general, lower numbers of *H. zea* moth catches for many areas within the Texas resistance monitoring program (Pietrantonio et al. 2009; Hopkins et al. 2010). With this combination of factors, it is difficult to determine if the prevalence of metabolic resistance in insects analyzed from these last few seasons was reflecting a true shift and increase of its frequency or if it was just a circumstantially biased result due to the smaller sample size of resistant insects available for testing.

Most current pyrethroid resistance monitoring programs across the US incorporate the adult vial test with cypermethrin. The majority utilize bioassays using discriminating dosages of cypermethrin, typically 5 and 10 µg per vial (Luttrell et al. 2006; Payne et al. 2001; Fleischer et al. 2007; Hutchison et al. 2007; Temple et al.

2008), while the Texas program uses a full range of ten dosages (Pietrantonio et al. 2007b). In both instances, these programs measure changes in frequencies of susceptibility to cypermethrin, whether it is at a few specific dosages or for an entire concentration range. However, there are two problems with these current monitoring strategies. First, adult male moths are used in monitoring as they are easier to collect than larvae due to the commercial availability of a sex pheromone to lure the moths into a wire-cone trap. While this is much easier than attempting to collect larvae from the field, pyrethroid applications are typically applied for control of small larvae, typically in the first or second instar. It has not been verified that using adults in the monitoring program accurately represents larval resistance levels; therefore, this was investigated with a resistant population collected in Uvalde, Texas. Second, to lend continuity to previous data, the pyrethroid cypermethrin is used almost exclusively for resistance monitoring. As many other pyrethroids are commonly applied for insect management, it was also important to determine if using this one pyrethroid for resistance monitoring was predictive of other pyrethroids as well.

In order to address these questions, first, vial assays were conducted on third instar larvae and adult *H. zea* from a susceptible and field-resistant population from Uvalde using three structurally different pyrethroids: cypermethrin, esfenvalerate, and bifenthrin. I found that resistance ratios were similar between larvae and adults for all three pyrethroids, respectively, indicating that the adults do accurately predict resistance in larvae for a particular pyrethroid. However, resistance ratios for bifenthrin were double those of cypermethrin and esfenvalerate, indicating that resistance ratios obtained

with one pyrethroid may not be indicative of those for other compounds. Understanding the underlying mechanisms of pyrethroid resistance at the molecular level will help us better understand these differences seen among the different pyrethroids in the vial test. For example, as resistance ratios for bifenthrin were twice as high as those seen for cypermethrin and esfenvalerate, and individuals from that Uvalde resistant field population exhibited transcriptional overexpression of *CYP6B8* and *CYP6B9*, it is logical to hypothesize that perhaps oxidative metabolism of bifenthrin in these populations is responsible for the differential resistance ratios observed. Hypotheses such as this may be tested by heterologously expressing those *CYP* genes and comparing the rates at which they are able to metabolize each of the three pyrethroids. These new questions and the greater ability to address them are important aspects for making the best decisions on controlling populations of *H. zea* and the knowledge obtained therein will greatly improve current monitoring programs by paving the way for the development of specific assays that can rapidly assess if resistance is present in a population, to what degree it is present, and what the mechanism is.

For instance, single nucleotide polymorphisms associated with resistance can quickly be identified in field populations with the development of assays using quantitative real time PCR. Allele-specific primers can be designed to use in high-throughput single nucleotide polymorphism (SNP) genotyping by single tube PCR with T_m-shift that can identify resistance mutations, whether individuals with these mutations are homozygous or heterozygous, and what the frequency is within the population tested (Wang et al. 2005). An enzyme-linked immunosorbent assay (ELISA) can be developed

to test for the overexpression of specific metabolic enzymes such as cytochrome P450s and can show the frequency of resistant individuals and to what extent the overexpression occurs compared to susceptible insects.

Both of these types of assay provide many benefits compared to the traditional resistance monitoring methods because they are extremely specific, can be used on all insect growth stages, and lend themselves to high-throughput resistance assessment. Quickly understanding what mechanisms of resistance is present, and to what extent, will greatly affect the best management practices for controlling *H. zea*. For example, if resistance levels are high, it may be best to switch to a different pyrethroid or a different mode of action altogether depending on what mechanism is present. However, although fast, molecular assays alone may not provide complete assessment in a resistance monitoring program. The inherent specificity of these assays can cause problems when the mechanism responsible for resistance has not yet been identified. In order to design molecular-based assays to screen for resistance, the mechanism must have already been identified, so if a novel sodium channel mutation or a different cytochrome P450 gene or other enzyme were responsible for resistance, these mechanisms would not be identified.

Thus, the best results may be obtained with complementary approaches using traditional bioassays and molecular resistance monitoring in tandem. The traditional vial assays will show when resistance levels are present or changing, and molecular assays can be used to identify which mechanisms are present and to quantify how prevalent the resistant genotype is within the population. If resistance has increased but is not

detectable using the current spectrum of molecular assays, it may be necessary to search for novel resistance mechanisms responsible for the resistance.

While this research was a good initial exploration of resistance mechanisms in *H. zea*, there is still much left to explore. Repeated attempts to select and maintain a resistant *H. zea* colony in the laboratory were unsuccessful and thus prevented much research on population genetics for this dissertation. For this, field insects were brought to the laboratory from regions where pyrethroid resistance was identified in the resistance monitoring program. Many different attempts were made to select for resistance in the laboratory through multiple methods such as exposure to pyrethroids in vials, by topical application, by leaf dip, and by diet incorporation, and breeding selected insects through mass crosses and later incorporating selecting specific more prolific families through single pair matings. Every time measurable resistance began to develop, there would be a problem with bottlenecks and crashing of the populations. To our best knowledge, there are no other successful instances of selecting for a pyrethroid resistant colony of *H. zea* either, and this suggests that there is a high fitness cost associated with resistance in this pest. Perhaps selection could be improved by introducing field-collected insects after each selection to prevent loss of fitness, which was not done for the attempts described above.

There must be a further focus on the population genetics of pyrethroid resistance based on our finding that all of the moths with sodium channel mutations at the V421 and L1029 residues were heterozygous for the mutation. This has serious implications on resistance management strategies because it is imperative to know the genotype of

resistant moths in order to predict how quickly resistant alleles may spread within a population. The previous assumption that 3 µg cypermethrin per vial would kill all homozygous susceptible moths and that 10 µg cypermethrin per vial would kill all homozygous susceptible and heterozygous resistant moths (Martin et al. 1999) has been used to assess genotypic frequencies of resistant populations (Pietrantonio et al. 2007b). There is likely a combination of factors that describe why we found no specimens homozygous resistant for target site resistance. *Helicoverpa zea* is highly polyphagous compared to *H. virescens* and has a much broader natural refuge where it is not subject to pyrethroid insecticide treatment. Thus, there should be a high number of susceptible moths available for mating and a greater chance to dilute resistance alleles from a population that is under selection by outcrossing with this susceptible population. In addition, if there is a higher fitness cost associated with the resistance allele, there is the possibility that homozygous resistant insects are not fit enough to survive under field conditions. With either scenario, the lower levels of resistant alleles found from *H. zea* have likely contributed to the low numbers of field control failures observed in this species compared to *H. virescens* and why we have not seen such a steady increase in resistance since its original discovery in 1990.

The Texas resistance monitoring program has many years of data and samples of moths across different locations (http://insecticideresistance.tamu.edu). This spatial and temporal data can be further analyzed to test hypotheses on migration and the spread of pyrethroid resistance alleles, not only throughout Texas, but also to other diverse cropping regions across the United States. There are many theories of how resistance

moths travel with wind currents, possibly spreading resistance problems to other locations and for further generations (Pietrantonio et al. 2007b). These hypotheses should be tested using molecular markers to assess if the development of resistance is due to local selection pressure, migration of other resistant moths, or a combination of both. This analysis at the population level could effectively incorporate the resistance mechanisms described in this paper to further validate if these mechanisms manifest themselves within populations and spread through migration.

It would be very interesting to conduct a similar experiment to that of Shao et al. (2009) where the sodium channel transcriptome was sequenced in order to determine the frequency of differentially spliced sodium channel transcripts among different growth stages. To follow this study, it would be important to also examine the proteome to determine how many of these transcripts form functional proteins. This information can be used with *in vitro* expression assays to learn much more about how the diversity of sodium channels affect function within insects. Further experiments may be able to better identify the residues responsible for insecticide binding and identify other residues that may be important for resistance.

This research only focused on the effects of *CYP6B8* and *CYP6B9* in metabolic resistance. In addition to exploring the other known *CYP* genes, knowledge of the *H. zea* genome would allow for identification of all *CYP* genes and further analysis of their association with pyrethroid metabolism. While this dissertation described transcriptional overexpression of *CYP6B8* and *CYP6B9* associated with pyrethroid resistance, it did not describe the underlying causes for overexpression, so it would be interesting to analyze

the upstream sequence for any evidence of interference with transcription. In addition, it would be important to explore if esterases or glutathione-S-transferases have any role in pyrethroid resistance in *H. zea*.

A total of 47 moths that had survived 10, 30, or 60 µg cypermethrin in the adult vial test were screened for sodium channel mutations at the V421 and L1029 amino acid residues, and 12 moths were screened for transcriptional overexpression of *CYP6B8* and *CYP6B9* (only the 30 and 60 µg cypermethrin survivors). A putative mechanism of resistance was described for nine of the twelve moths examined for both of these mechanisms (Fig. 5.1); however, evidence for an additional mechanism exists because some of the moths that survived high concentrations in the vial assay did not exhibit sodium channel mutations nor high overexpression of the two *CYP* genes analyzed here.

Determination of the molecular mechanisms responsible for pyrethroid resistance in *H. zea* will be informative when making resistance management decisions to optimize control of this pest. The results of this research are important for crop production across the United States and the world. The bollworm is a pest of multiple crops, and pyrethroids are often the most cost effective measure for control. Understanding the mechanisms of resistance will assist in the development of effective resistance management strategies to allow producers to make the most cost-effective, environmentally sound decisions for controlling bollworm as well as help preserve the susceptibility of bollworm to pyrethroids. Additionally, the discovery of overexpression of oxidative enzymes in *H. zea* may jeopardize the success of pesticides other than pyrethroids that act through a different molecular target. Therefore, continued

investigation of metabolic mechanisms present may impact the success of other novel and more expensive chemistries. The development of *in vitro* and *in silico* models incorporating resistance research such as described throughout this dissertation should improve our understanding of insecticide pharmacology, insecticide resistance, and can be integral for future development of novel insecticides.

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