CYTOCHROME P450 MONOOXYGENASE-MEDIATED RESISTANCE IN THE SOUTHERN HOUSE MOSQUITO: CHARACTERIZATION, GENETICS AND FITNESS

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Mosquitoes vector a multitude of deadly and debilitating pathogens that present serious threats to the health of humans and animals. Insecticides are often the only feasible method of controlling disease vectoring mosquitoes in endemic areas and during epidemic outbreaks. Pyrethroids are the primary insecticide class used on bed nets and for indoor residual spraying. Worldwide, permethrin resistant populations of *Culex pipiens quinquefasciatus* have been recorded.

The two major mechanisms of resistance to permethrin are cytochrome P450 monooxygenase-mediated detoxification and a mutation (*kdr*) which renders the targeted voltage-sensitive sodium channel protein insensitive. Despite the importance of P450 detoxification, little is known. This study has characterized the P450 mechanism, investigated interactions with *kdr*, examined fitness associated with P450-mediated resistance and confirmed the probable P450 gene responsible for resistance. Understanding the complexity of P450 resistance is essential for determining how control programs will affect mosquito populations.

The ISOP450 strain of *Cx. p. quinquefasciatus* was isolated through backcrossing permethrin-resistant JPAL into an SLAB (susceptible) genetic background. Resistance in ISOP450 was conferred exclusively by P450

detoxification in a larval-specific manner (1,300-fold relative resistance). P450 detoxification in ISOP450 was only able to readily detoxify permethrin and bioactivated temephos. Analysis of the inheritance pattern revealed that P450 detoxification was incompletely dominant and monofactorial. Interactions of P450 detoxification with *kdr* were determined by deducing the permethrin resistance contribution for each mechanism in various genotypic states. The interactions among all genotype combinations were multiplicative.

In *Culex* colonies segregating for P450 resistance and maintained in an insecticide-free environment, the P450 resistance allele decreased slightly but not significantly over time. A heterozygote advantage was observed in the cold temperature environment and the resistance allele significantly decreased in the temephos exposed environment. Biological estimates of fitness showed no parameter to be associated with P450 detoxification. Therefore, a large fitness cost is not associated with the detoxification mechanism under insecticide-free laboratory conditions.

CYP9M10 was determined to be over-expressed by 2,200-fold and 12-fold in ISOP450 larvae and adults, respectively, compared to SLAB. Polymorphic sites which differentiated the resistant and susceptible *CYP9M10* alleles were determined, providing a possible molecular monitoring tool for assessing P450-mediated resistance.

BIOGRAPHICAL SKETCH

Melissa was born on November 16, 1981 in San Francisco, California to Paul and Antoinette (Toni) Hardstone. Her interest in science started at an early age with trips to Golden Gate Park and other neighborhood parks where she would watch with wonder at the wildlife and clumsily run around trying to catch grasshoppers. Melissa's family moved to Pleasant Hill, California in 1989 where she attended Valhalla Elementary School for 3rd grade and then transferred to Sequoia Elementary School for 4th and 5th grades. She began to develop her interest in science more fully during her time at Sequoia Middle School where she was an active participant in biology class and competed in science fairs throughout the region. Melissa graduated from College Park High School in 1999 having taken many honors level courses.

Throughout her life, Melissa's parents nurtured, supported and embraced her hobbies, interests, and extracurricular activities. These have included dance (ballet and jazz), girl scouts, music (piano, viola, drums, and choir), painting, 4-H (raising swine and leadership), sports (basketball and wrestling), science clubs (Odyssey of the Mind, Focus for Teens, and summer courses at Diablo Valley Community College), cooking and baking, traveling around the world, and gardening.

After high school, Melissa enrolled at the University of California at Davis as a general biology major with a pre-medicine emphasis. Although she had been interested in science her whole life, it was in college that Melissa's interest in insect biology began. The spring quarter of her sophomore year a fellow dorm mate mentioned that she knew of a lab in need of an undergraduate research assistant, so Melissa applied for the job and was hooked. She began to incorporate entomology classes into her coursework,

and by winter quarter of junior year was officially an entomology major. It was when she took the medical entomology class from Dr. Thomas Scott that she realized her two interests, medicine and insects, could be combined. It was during these years that she worked in a multitude of research laboratories and developed her passion for research. She remained in the lab of Dr. Walter Leal for 3 years as the primary researcher examining the proteomics of olfaction in *Drosophila* as well as conducting experiments using electroantennography to study mosquito and moth olfaction.

Melissa began her graduate student career at Cornell University in the fall of 2003 working under Dr. Jeffrey Scott and continued her interest in research in medical entomology by studying insecticide resistance in mosquitoes. Melissa was scared to move across the country and away from her family, but the 5 day drive with her father eased her nerves. Since her time at Cornell, Melissa has embraced every experience possible, including enjoying Ithaca summers (and snowy winters), serving in many capacities of Jugatae as well as perfecting and honing her grant writing and research skills. She has had the opportunity to be a teaching assistant for a variety of courses and was awarded the Environmental Protection Agency Science to Achieve Results fellowship which supported her research for 3 years.

To my parents for their unconditional love and support.

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Much of what I have been able to accomplish could not have been possible without funding opportunities. Thank you to the Entomology Griswold and Rawlins Fund committees, CALS Mellon Grant committee and notably the EPA STAR Fellowship selection committee who has allowed me to focus my attention on the research that I have so enjoyed for the last 3 years.

I want to thank my family and most importantly, my parents who have given me unconditional support and love. They have provided me with all the skills which have made me who I am today. They taught me the value of education, hard work, kindness and humility. They have embraced my education as their own and make me want to strive to be a better scientist.

Lastly, thanks to Nori for being a wonderful partner and great companion. I treasure every moment we have had together and look forward to the many more adventures we will have in the future. You have been my family in Ithaca and I can not imagine my life without you.

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2-DIGE	two dimensional fluorescence difference gel electrophoresis
Ace	*Altered aceylcholine esterase*
AChE	acetylcholine esterase
ACh	acetylcholine
APN	aminopeptidase N
BC	backcross generation
bp	base pair
BSA	bovine serum albumin
Bti	*Bacillus thuringiensis israelensis*
Buffer A	100 mM Tris-HCl, pH 7.5; 100 mM EDTA; 100 mM NaCl; 0.5% SDS
cDNA	complimentary deoxyribonucleic acid
cm	centimeters
DALYS	disability adjusted life years
ddH_2O	double distilled water
DDT	dichlorodiphenyltrichloroethane
DTT	dithiothreitol
dyo	days old
EDTA	ethylenediaminetetracetic acid
ESTs	expressed sequence tags
F_1	first filial generation, offspring of a genetic cross
GABA	gamma-aminobutyric acid
gDNA	genomic deoxyribonucleic acid
Gfi-1	transcriptional repressor
GST	glutathione S-transferase

hrs	hours
HWE	Hardy-Weinburg Equilibrium
IGR	insect growth regulator
ISOJPAL	permethrin resistant strain of *Cx. p. quinquefasciatus* which is isogenic to SLAB containing *kdr* and P450 detoxification
ISOP450	permethrin resistant strain of *Cx. p. quinquefasciatus* which is isogenic to SLAB containing P450 detoxification and lacking *kdr*
ITN	insecticide treated bed net
JH	juvenile hormone
JPAL	permethrin resistant strain of *Cx. p. quinquefasciatus* containing *kdr* and P450 detoxification
kb	kilobases
kDa	kiloDaltons
kdr	*knockdown resistance*, L1014F allele
LC_{25}	lethal concentration killing 25% of the population
LC_{50}	lethal concentration killing 50% of the population
LPR	Learn Pyrethroid Resistant, house fly strain
M	molar
μg	micrograms
μl	microliters
mg	milligrams
min	minutes
ml	milliliters
mM	millimolar
mRNA	messenger ribonucleic acid
mV	millivolts

NADPH	reducing agent, electron donator in biochemical reactions
nmol	nanomolar
ng	nanograms
OP	organophosphate insecticides
ORF	open reading frame
oz	ounces
P450	cytochrome P450 monoxygenase-mediated resistance
PB	phenolbarbital, a P450 inducer
PBO	piperonyl butoxide, a P450 inhibitor
PCR	polymerase chain reaction
pen	*reduced cuticular penetration*
PMSF	phenylmethylsulfonyl fluoride
PTPE	2-propynyl 2,3,6-trichlorophenyl ether, a P450 inhibitor
PTU	pheylthiourea
qRT-PCR	quantitative real-time polymerase chain reaction
Rdl	*Resistance to dieldrin*
rDNA	ribosomal deoxyribonucleic acid
RHS	residual house spraying
RNA	ribonucleic acid
RNAi	ribonucleic acid interference, a gene silencing pathway
R	resistant allele
R/R	homozygous resistant genotype
RR	resistance ratio, LC_{50} resistant strain/LC_{50} susceptible strain
R/S	heterozygous resistant and susceptible genotype
RT-PCR	reverse transcription-polymerase chain reaction
S	susceptible allele

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
SKA	dieldrin resistant house fly strain
SLAB	susceptible laboratory strain of *Cx. p. quinquefasciatus*
SNP	single nucleotide polymorphism
S/S	homozygous susceptible genotype
T	teneral females
TTX	tetrodotoxin
VSSC	voltage-sensitive sodium channel
WNV	West Nile virus, an arbovirus in the family Flaviviridae
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The following chapter will provide a detailed and extensive review of literature associated with insecticide resistance in mosquitoes. Understanding the biology, molecular basis and implications of insecticide resistance within a vector management system require knowing the various classes of insecticides available, what resistance mechanisms can evolve and potential ways in which the system can be manipulated. Although my dissertation research specifically focuses on the cytochrome P450-mediated permethrin detoxification in *Culex pipiens quinquefasciatus*, in the following chapter I have reviewed the suite of insecticides used in vector control campaigns and the correlated resistance mechanisms which have evolved from their use.

1.2 Mosquitoes: Impacts on human and animal health

Mosquitoes have plagued humans and animals as disease carriers and nuisance pests throughout history [1]. Mosquitoes are capable of vectoring a multitude of deadly and debilitating pathogens that present serious threats to the health of both humans and other animals. These include filarial nematodes, protozoans, and more than 20 viral pathogens [2–4]. For many pathogens, the mosquito serves as a required host in which they complete development and/or replicate. Mosquitoes can also mechanically transfer some pathogens on their mouthparts [5].

In order to transmit pathogens, a female mosquito must take a blood meal from at least two hosts. Female mosquitoes can be attracted to a host over a long distance by carbon dioxide plumes and body odor. These cues stimulate olfactory sense receptors on the antennae and the female then undergoes a

behavioral alteration and begins to fly upwind towards the source. As the female approaches the host, visual stimuli and heat plumes become the primary cues [6,7]. Blood is essential as part of the pathway for disease transmission between hosts, and also provides obligatory proteins for egg production [4–6].

Integrated control of mosquito populations includes traditional chemical control techniques along with the use of biological agents and environmental manipulation [4,8]. Many successful and unsuccessful attempts have been made in controlling vectors of disease. One example is *Anopheles* mosquitoes which transmit *Plasmodium falciparum*, the parasite that causes malaria. Worldwide, malaria causes up to 2.7 million deaths annually, with an additional 400-900 million clinical cases per year [9,10]. As such, in 1956, the World Health Organization (WHO) started a worldwide Malaria Eradication Programme [11]. The program combined drug therapy with wide-scale insecticide spraying of houses (using DDT). Initally, the campaign was successful, but eventually insecticide resistance, drug resistance and economic pressures became insurmountable problems and eradication was no longer a feasible option [9]. Malaria remains eradicated from the United States, much of Europe [12], Israel, Lebanon, Syria, Jordan [12], Cyprus [11], Egypt [13], Japan [14] and Taiwan [15], but currently exists in 107 countries in Africa, Central and South America, Asia and the Pacific [10].

1.3 Southern house mosquito, *Culex pipiens quinquefasciatus*

1.3.1 Biology

Among the most important mosquito vectors is *Culex pipiens quinquefasciatus*, commonly called the southern house mosquito. This species is the primary vector of the filarial nematode, *Wuchereria bancrofti* [4]. Human

infection by these nematodes can block the lymphatic system and lead to extreme swelling of the limbs (elephantiasis) and scrotum (hydrocoele). Lymphatic filariasis is present in 83 countries, infects 120 million people [16], and accounts for the loss of 4.6 million DALYS (disability adjusted life years) [17] with India accounting for 40% of the prevalence globally [18]. The extreme disfigurement and physical incapacitation resulting from swelled extremities prevents people from functioning normally. This can create economic struggles in endemic countries where it is estimated that on average a total of $811 million are lost annually [18]. In 1995, the WHO classified human lymphatic filariasis as the second leading cause of permanent and long-term disability [16].

Adult *Cx. p. quinquefasciatus* are able to transmit a multitude of viruses, including West Nile virus (WNV) [19,20]. This virus is usually maintained in a mosquito-bird cycle, where *Cx. p. quinquefasciatus* serves as an amplification vector [21]. Often dead-end hosts, such as humans or horses, can be infected and die. Since its discovery in New York City in 1999, WNV in the United States has caused over 10,000 clinical cases and 400 human deaths, along with many fatal infections in horses [21]. *Cx. p. quinquefasciatus* mosquitoes are also involved in the transmission of avian malaria pathogens, avian pox virus, dog heartworm (*Dirofilaria immitis*), and urban outbreaks of St. Louis encephalitis virus in eastern United States [5]. This species has experimentally been shown to be capable of transmitting Japanese encephalitis virus as well. Other arboviruses have been isolated from *Cx. p. quinquefasciatus* populations throughout the world, but subsequent laboratory studies do not implicate it as an efficient vector of these disease pathogens (for example: Oropouche virus) [4].

1.3.2 Life stages

Adult female *Cx. p. quinquefasciatus* lay a few hundred eggs aligned in a floating raft structure. Each egg is oviposited separately and the vertical eggs adhere to one another by surface forces [1]. The time interval between oviposition and hatching is temperature dependant. Eggs will hatch in 26-36 hrs when subject to the most favorable temperatures (21°C-30.5°C) [2]. The larvae filter-feed during all four instars. The duration of each instar is approximately 24 hrs, except the fourth instar which is about 3 days and is accompanied by a large increase in body mass [1]. The mosquito will remain a pupa for approximately 2-4 days before it emerges as an adult [1]. Upon eclosion, adult males and females will seek out a sugar meal such as nectar from flowers, and honey dew. Both sexes use sugars, primarily *D*-fructose and *D*-glucose [6], as a metabolic source of energy throughout their adult lives. At the optimum developmental temperature (25°C) female and male longevity averages 43 and 19 days, respectively, in field conditions [4]. Adults will mate by swarming around a visual cue and males will become attracted to a female by the sound of her wing-beats (500-600 Hz) [4]. Females must be at least 18-24 hrs old to be successfully inseminated and use only the sperm from one male to fertilize a raft of eggs. *Cx. p. quinquefasciatus* females are anautogenous (*i.e.*, require a blood meal for egg maturation) and primarily ornithophilic (*i.e.*, prefer to feed on birds) [4]. Once a bloodmeal has been ingested, the female has the metabolic energy necessary to utilize stored sperm from the spermatheca to fertilize the next raft of eggs to be laid. Oviposition sites include a wide range of organically rich water sources, such as brackish ponds, sewer drains, pit latrines and ditches [4,22]. Each stage of the mosquito life cycle and many of the behavioral aspects are dependant on and can be altered

by temperature, humidity, diet and crowding.

1.3.3 *Culex pipiens* complex

Since mosquitoes play such an important role in disease transmission around the world, it is important to be able to classify them correctly. Using morphology exclusively is very difficult since some species/subspecies have intermediate characteristics which in the past has created controversy. Currently, the morphological characters used are the shape and distance of the dorsal and ventral phallosome arms in males (DV/D ratio) and the pattern of larval siphon structure. Biological features of females, such as autogeny versus anautogeny, host feeding preferences and the ability to diapause, are also used to determine taxonomy [4]. In China, the complex is thought to be only two subspecies (*Cx. p. pallens* and *Cx. p. quinquefasciatus*) based on hybridization studies, morphology, cuticular hydrocarbon analysis and allopatry [4]. In Australia, the complex includes four separate species, *Cx. p. australicus*, *Cx. p. quinquefasciatus*, *Cx. p. pipiens molestus*, and *Cx. globocoxitus*.

Using electrophoretic assays and molecular studies of rDNA sequence divergences along with morphological DV/D ratios [4], the complex includes: *Cx. p. quinquefasciatus* (anautogenous, occurring south of 36°N), *Cx. p. pipiens* (anautogenous, occurring north of 39°N in North America), *Cx. p. pipiens molestus* (autogenous, geographically occurring with *Cx. p. pipiens* but are exclusively associated with closed, underground breeding sites), *Cx. p. pallens* (anautogenous, occurring north of 32°N in China and Japan), *Cx. p. australicus* (anautogenous, occurs in Australia, Tasmania, New Caledonia and the New Hebrides), *Cx. torrentium* (anautogenous, occurring in the west Palaearctic), and *Cx. vegans* (anautogenous, occurring in China, India, Korea, Japan and southeastern Russia). The distribution of *Cx. p. pipiens* and *Cx. p.*

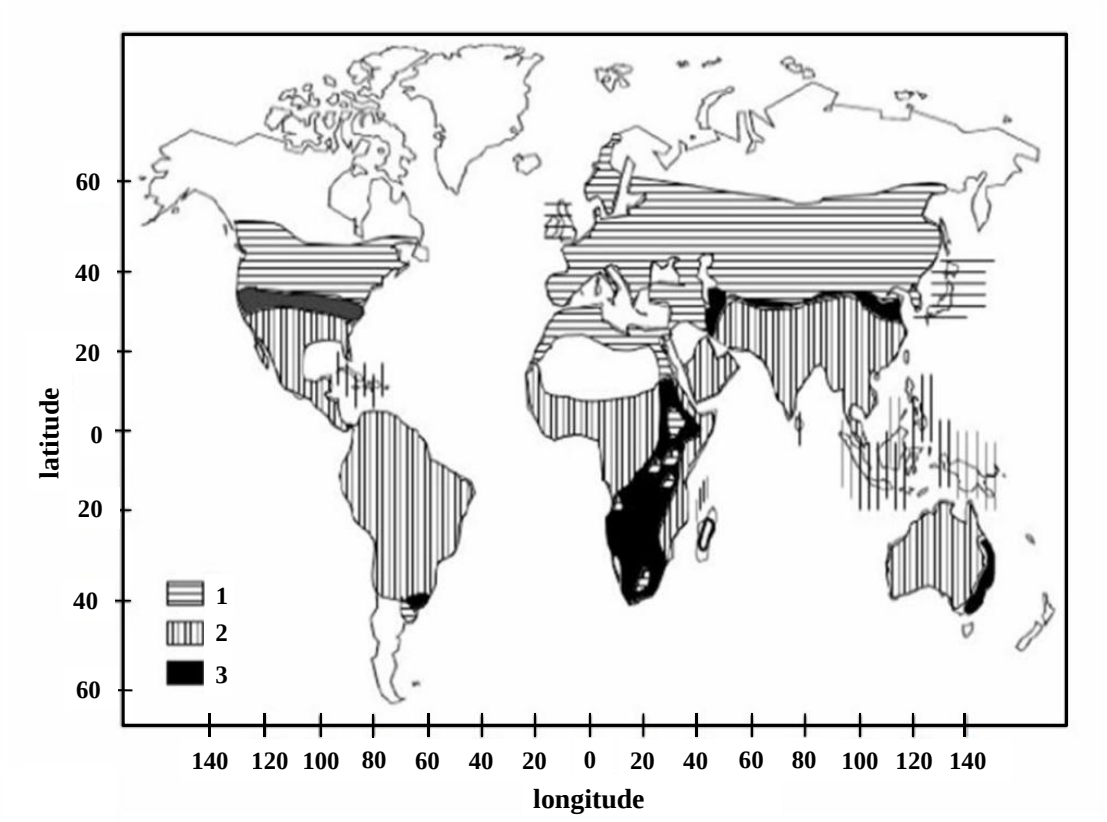


Figure 1.1: Distribution of *Cx. p. pipiens* (1), *Cx. p. quinquefasciatus* (2) and both species (3) within a hybrid region (modified from [4]).

quinquefasciatus are shown in Figure 1.1.

Cx. p. quinquefasciatus occurs in the southern hemisphere worldwide (south of 36°N) while populations north of latitude 39°N are *Cx. p. pipiens* [4,6]. A hybrid distribution area of these two species occurs between these two latitudes in the Americas, the Middle and Far East, Australia and Africa. Within this region, *Cx. p. pipiens* and *Cx. p. quinquefasciatus* are readily able to hybridize [4]. The ability to hybridize is necessary for gene flow between populations, especially related to insecticide resistance.

1.3.4 Sex determination

In mosquitoes, the homologous chromosomes are somatically paired so that they are tightly synapsed along the entire length of the chromosome, not just at the centromere. *Culex* mosquitoes have three pairs of homomorphic chromosomes, with chromosome 1 being the shortest, chromosome 2 being the longest, and chromosome 3 being an intermediate length [6]. Sex is determined by a single pair of alleles or chromosome segments, *M* and *m*, located on chromosome 1 [23]. Maleness is dominant over femaleness, therefore, males are of the genotype *Mm* and females are *mm* [6]. The *M* factor is visually determined by condensed chromatin in the zone 10C3 of arm 1L, whereas the *m* factor will contain puffs at this location [6].

The expected 1:1 sex ratio can be altered by a single recessive factor *d* (distorter factor) located on chromosome 1. The distorter factor operates only when the father is heterozygous (*i.e.*, with the genotype $M^D m^d$, yielding approximately 90% males in the offspring. The result of this sex ratio distortion is that the frequency of the male-determining chromosome increases over the female-determining chromosome [24], due to breakage of the female-determining chromosome.

1.3.5 Control of *Cx. p. quinquefasciatus*

Many studies have been conducted in attempts to find control measures that do not involve (or minimize) the use of chemical insecticides. One approach includes the use of polystyrene beads. These non-toxic floating beads inhibit the development of mosquito larvae and pupae by suffocation, and decrease the availability of oviposition sites by blocking access to water surfaces [25]. In Tanzania and India, not only did polystyrene bead treatment reduce vector populations, it also decreased the annual man-biting rate from

approximately 50,000 bites per villager pre-treatment to almost 1,000 within the first year of treatment [25].

While many integrative techniques can be used, often the only way to control vector densities in endemic areas, control an epidemic outbreak, and decrease populations of nuisance mosquitoes, is to use traditional chemical insecticide applications. A list of insecticides used for the control of *Cx. p. quinquefasciatus* larvae and adults is shown in Table 1.1. Populations of *Cx. p. quinquefasciatus* have been found to be resistant to many of the major classes of insecticides (OPs, carbamates and pyrethroids).

Application procedures which target *Cx. p. quinquefasciatus* vary depending on the condition of the water source. For example, with sewage drains a drip technique can be used, for large ponds floating booms can be placed along the periphery, and in some breeding sites granular or compression sprays may be most effective. Whatever the application method, currently the most commonly applied insecticides are OPs, which can remain effective for 2-8 weeks [22].

The insecticides listed in Table 1.1 are registered for mosquito control. Larviciding is primarily conducted using OPs, but applications of biopesticides and insect growth regulators have been used in polluted waters. Since *Cx. p. quinquefasciatus* is a domestic mosquito, it prefers to rest indoors on material that usually cannot be sprayed (curtains, furniture, etc.). As such, residual indoor spraying is not as effective as other personal protection options. Insecticide treated bed nets (ITNs) do provide some protection, since low doses of pyrethroids have a rapid effect on mosquitoes and the risk of toxicity to humans is minor [31]. Commonly used pyrethroids include: α-cypermethrin, cyfluthrin, deltamethrin, etofenprox, λ-cyhalothrin, and permethrin [22,32].

Table 1.1: Insecticides used to control *Cx. p. quinquefasciatus*.

Insecticide	Chemical Class	Reference
oils		[22]
B. sphaericus	biopesticide	[26]
B. thurigiensis israelensis	biopesticide	[22]
bendiocarb	carbamate	[22]
carbosulfan	carbamate	[27]
propoxur	carbamate	[22]
diflubenzuron	insect growth regulator	[22]
fenoxycarb	insect growth regulator	[28]
methoprene	insect growth regulator	[22]
pyriproxyfen	insect growth regulator	[22]
triflumuron	insect growth regulator	[29]
chlorpyrifos	organophosphate	[22]
chlorpyrifos-methyl	organophosphate	[30]
fenthion	organophosphate	[22]
fenitrothion	organophosphate	[29]
malathion	organophosphate	[30]
phoxim	organophosphate	[29]
pirimphos-methyl	organophosphate	[22]
temephos	organophosphate	[22]
bifenthrin	pyrethroid	[30]
cyfluthrin	pyrethroid	[22]
λ-cyhalothrin	pyrethroid	[22]
α-cypermethrin	pyrethroid	[22]
deltamethrin	pyrethroid	[22]
etofenprox	pyrethroid	[22]
permethrin	pyrethroid	[22]

1.4 Insecticides used for mosquito control

While integrative techniques can be more environmentally friendly, chemical insecticides are still the primary method for keeping mosquito populations under control, particularly under conditions of disease outbreaks.

1.4.1 Early inorganics

The earliest forms of chemical insecticides were inorganic stomach poisons. These environmentally harsh compounds were originally used to control agricultural pests. White arsenic (or arsenic trioxide) was used as early as 900 A.D. by the Chinese [33] to protect their gardens. The most widely used inorganic insecticide was Paris green (copper acetoarsenite). In 1942 and 1943, mosquito control agencies in the United States used over 200,000 pounds of Paris green and Puerto Rico used another 120,000 pounds [33].

1.4.2 Botanical insecticides

The use of naturally derived compounds from plants or flower extracts came about shortly after early inorganics. Of the botanically derived compounds, pyrethrum is the most commercially important. Use began in Persia (now Iran) and Dalmatia (now Yugoslavia) and became widespread in the mid-1800s [34]. Commercial production started in Dalmatia, but switched to Japan during World War I. During World War II, Japan was not able to maintain pyrethrum production and Kenya, then a British colony, took control of the international market. Since then, Kenya has remained the world leader in pyrethrum production, supplying about two-thirds of the world's demand [34,35].

Crude pyrethrum extract is made from the dried flower heads of *Crysanthemum cinerariaefolium* and *C. coccineum*. Pyrethrins, a general term for

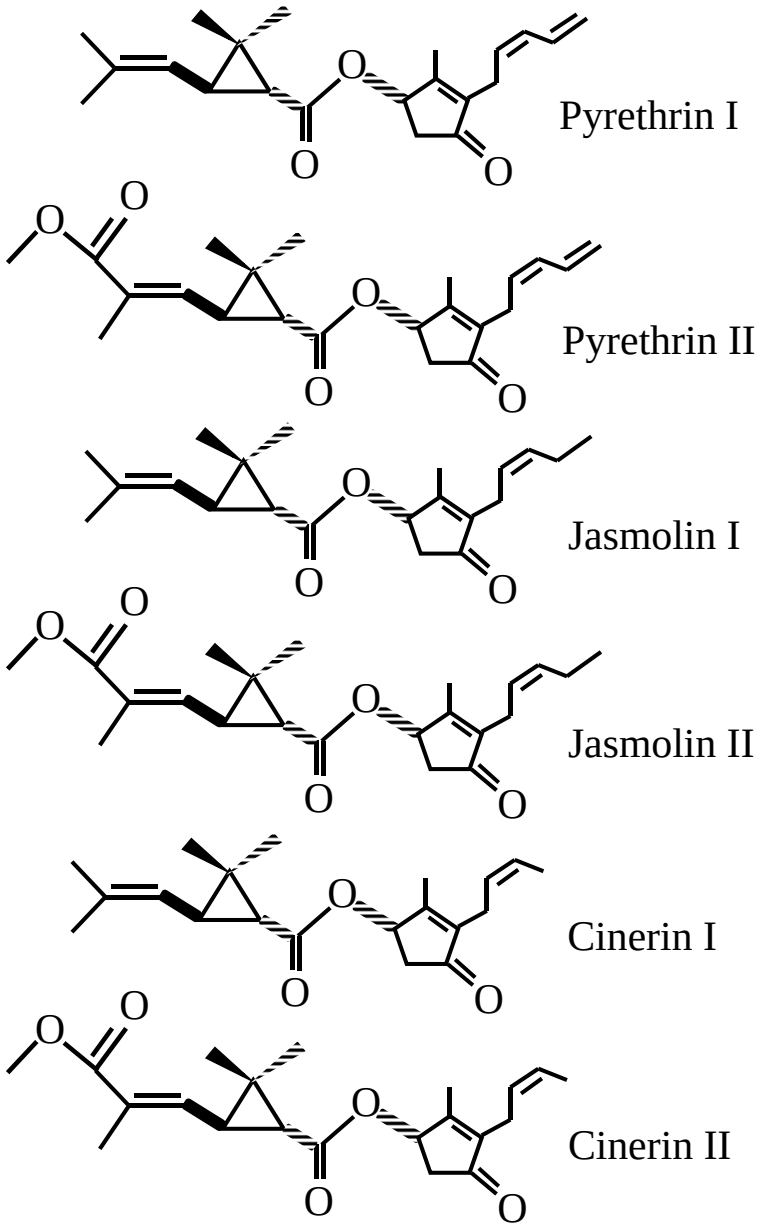


Figure 1.2: Structures of pyrethrins (modified from [36]).

the mixture of active compounds found in the flowers, make up about 3 percent of the flower mass [34]. Pyrethrum extract contains "a mixture of three naturally occurring, closely related insecticidal esters of chrysanthemic acid (pyrethrins I) and three closely related esters of pyrethric acid (pyrethrins II)" (Figure 1.2) [37]. The dominant derivatives are pyrethrin I (38%) and pyrethrin II (35%), followed by cinerin II (11.7%), cinerin I (7.3%), jasmolin I (4%) and jasmolin II (4%) [36].

Early insecticidal experiments on the two dominant derivatives suggested that pyrethrin I exposure caused more rapid kill, but pyrethrin II exposure was capable of rapid knockdown and slow death (Staudinger and Ruzicka 1924 as cited by [33,38]). Pyrethrins were incorporated into a powder form, which became useful for mosquito control in the late 1880s. Then in the early 1900s, it was observed that when the powder was burned it would repel adult mosquitoes in homes [39]. Most importantly, the eventual commercial synthesis of compounds based on the structures of pyrethrins I and II, lead to the creation of one of the most effective classes of insecticides to control mosquitoes, the pyrethroids.

1.4.3 Bacteria derived insecticides

Bacillus thuringiensis israelensis

Bacillus thuringiensis israelensis (*Bti*) was discovered in the mid 1970s in Israel (Negev desert) and was determined to have mosquitocidal properties [40] due to Cry and Cyt proteins [41]. The *Bti* crystals active against mosquito larvae contain three types of polypeptides: (1) 130 kDa Cry4A, Cry4B or Cry4C, (2) 72 kDa protein Cry4D and (3) 28 kDa cytolytic factor CytA [42]. Domain 1 of the toxins are involved in membrane ion channel formation, while domains 2 and 3 are involved in selectivity through receptor binding.

The crystals are ingested and dissolved in the alkaline midgut of the insect [43]. The toxin proteins are then proteolytically activated when the C- and N- termini are cleaved. The active forms of the toxins then bind to specific receptors (cadherin and aminopeptidase-N) on the brush border membrane of the midgut epithelium. The membrane-bound toxins then induce pore formation by inserting into the epithelial cells. These pores are either non-specific or K^+-specific causing problems in osmotic balancing resulting in cell lysis [43–45].

Mosquitoes are susceptible to *Bti* particularly *Culex* spp. and *Aedes* spp., while *Bti* has much lower toxicity to *Anopheles* spp. [44]. *Cx. p. quinquefasciatus* strains resistant to Cry4A, Cry4B or Cry4D became sensitive when tested in the presence of a CytA toxin. It was concluded from these results that the Cyt toxins synergize the toxic effects of the Cry toxins. This was most evident, though present in all of the Cry4 resistant strains, in the Cry4D selected strain where resistance changed from >1,000-fold to 8-fold when combined with CytA [46]. Synergism was also observed in *Cx. p. quinquefasciatus* selected with Cyt1Aa, Cry11Aa or a 1:3 mixture of the two toxins for 30 generations. The resulting resistance ratios were 1237, 242 and 8, respectively. Not only did the Cyt toxin decrease the toxic effects of the Cry toxin, but the Cyt toxin also delayed the onset of resistance. After 48 generations, the resistance of the 1:3 mixture strain to Cry11Aa was 9.3-fold [47].

Some insect pest species, *Plutella xylostella* [48], *Plodia interpunctella* [49], *Cadra cautella* [50], *Pectinophora gossypiella* [51] and *Trichoplusia ni* [52], have developed resistance to Cry toxins expressed in transgenic field crops. While laboratory selection has produced resistant strains in many insect pest species [53–59], there are only two reported cases of resistance in mosquitoes,

one in field caught *Culex* [60] and one in laboratory selected *Aedes aegypti* [54]. The rare occurrence of field resistance, high toxicity, and high selectivity has increased the use of this bacterium for control programs worldwide. For example, the German Mosquito Control Association has used *Bti* exclusively and decreased *Culex* spp. and *Aedes* spp. densities by >90% [44,61].

Bacillus sphaericus

Bacillus sphaericus was originally discovered before *Bti* [62], but did not become a widespread option for some time because it took longer to find highly active isolates (serotypes). The only way to determine if an isolate is active is to conduct time-consuming bioassays on mosquito larvae. Currently, nine active serotypes have been identified [26]. These serotypes are highly toxic to *Culex* spp. and *Anopheles* spp., but ineffective against *Aedes* spp. [63].

Mosquitocidal activity of *B. sphaericus* results from two crystal toxins, Bin and Mtx, produced during sporulation [64]. Very little is known about Mtx toxins (Mtx and Mtx2) and their functions except that they are produced during the vegetative growth phase [64] and can synergize other mosquitocidal toxins [65]. In *Cx. p. quinquefasciatus*, only BinA (41.9 kDa) has been shown to be toxic, while BinB (51.4 kDa) is inactive but is involved in receptor binding. The mode of action of this bacterium is quite different than *Bti*. In *Culex* spp., after ingestion of the spore/crystal complex, the BinA toxin is released when the complex is dissolved by proteases and high gut pH. The toxin binds to the specific midgut receptor, Cpm1, and then creates large vacuoles in the cell. These vacuoles induce mitochondrial swelling which results in decreased oxygen uptake [26].

While this bacterium has been a successful component in mosquito control programs, there are some reported cases of resistant laboratory

selected [66] and field populations [67–69]. A field population of *Cx. p. quinquefasciatus* from California under laboratory selection obtained resistance in 12 generations, such that fourth instar larvae could survive a concentration of *B. sphaericus* that was 7,000 times higher than the LC_{50} of the susceptible laboratory strain [70]. Inheritance of *B. sphaericus* was determined by standard backcrosses and binding assays revealing that it is a recessive trait associated with a single locus [70]. In France, a similar situation occurred along the Mediterranean coast where intensive control with *B. sphaericus* had been conducted since the late 1960s. This population developed a >10,000-fold resistance after a few generations of selection [71]. A resistant strain of *Cx. pipiens*, called GEO, contained undetectable amounts of Cpm1 protein despite normal transcription of the gene. The sequence of *Cpm1* from the resistant strain differed from the susceptible strain by seven mutations: six missense and one premature stop codon. The truncation possibly prevents the protein from anchoring to the membrane properly which conferred the resistance since the Bin receptor remained unaltered [72]. Another possible resistance mechanism found in field populations of *Cx. pipiens* could be the insertion of a transposable element into the coding sequence of *Cpm1* creating a new intron and altering the membrane protein. This type of alteration could prevent the Mtx toxin from interacting and binding to the receptor [73].

Since *B. sphaericus* produces only one toxin and binds to one receptor on the insect midgut, it is more biologically constrained than *Bti*. *Bti* produces four major toxins, Cry4A, Cry4B, Cry11A and Cyt1Aa, and binds to multiple receptors in the gut of insects. To defeat the restrictions of using *B. sphaericus*, recent attempts have been made to create recombinant bacteria that contain the endotoxins of both bacteria [74]. Binding studies revealed no competition

between labeled *B. sphaericus* toxin and non-labeled *Bti* toxins, indicating that different receptors along the brush border membrane bind each biopesticide [75].

Interestingly, the interactions of the endotoxins from the two bacteria are synergistic and effective against *B. sphaericus* resistant mosquitoes (BS-R strain) [76]. When *B. sphaericus* was combined with the Cyt1Aa toxin of *Bti*, resistance in the BS-R strain was reduced by >30,000-fold [77]. The mechanism of synergism conferred by *Bti* Cyt toxins is still unknown. Some theories are based on the idea that Cyt toxins can augment any of the steps leading up to cell lysis, such as proteolytic activation, receptor binding or toxin insertion. Another thought is that Cyt toxins bind to the brush border membrane and then serve as a receptor for the Cry toxins, such that a binding site is present even in resistant populations [44].

1.4.4 Synthetic insecticides

The era of synthetic insecticides began in the 1930s with the discovery of DDT. A shift from using inorganic toxins and pyrethrum extract went to DDT and subsequently OPs, carbamates and pyrethroids (described in detail below). Many of these synthetic compounds are able to maintain insecticidal activity while having additional properties that include: fast action, increased selectivity reducing toxicity to non-target organisms, increased toxicity to the target pest, decreased persistence in the environment, and cheaper methods for manufacture.

DDT

DDT, dichlorodiphenyltrichloroethane, $C_{14}H_9Cl_5$, was first synthesized in 1874 by a German chemist Othmar Ziedler, but went unnoticed for almost 60

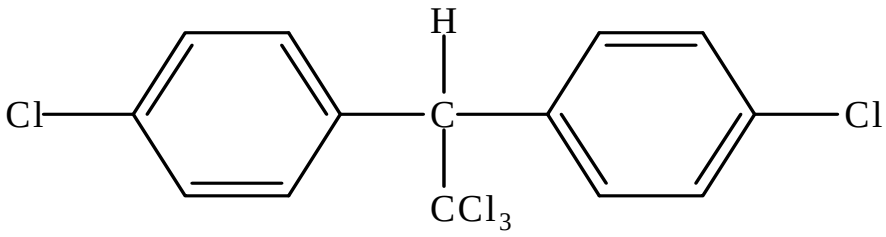


Figure 1.3: Structure of DDT.

years. It was not until 1939 when Paul Muller of J. R. Geigy (Basle, Switzerland) synthesized and observed the insecticidal properties of DDT, that this compound became popular worldwide [78]. The synthesis of DDT is inexpensive due to its simple chemical structure (Figure 1.3). This particular feature allowed for mass synthesis resulting in the entire production output until 1945 to be used by the military. One famous example of DDT use by the American military occurred in 1944 when typhus broke out in Naples, Italy. Over 1 million civilians were dusted with DDT, and within a month the outbreak was controlled. It was the first time in history that a typhus outbreak was halted in the middle of winter [33,78].

It is estimated that between 1942 and 1953, DDT use to control vector-borne diseases saved over 5 million lives and prevented over 100 million illnesses [79]. During World War II, DDT was used to control mosquitoes transmitting malaria and lice transmitting typhus. Troops in both the German and Allied militaries had significant decreases in mortality rates from these vector-borne diseases. To control body lice, the United States Army used "Louse Powder" which was made of 10% DDT and 90% pyrophyllite (aluminum silicate hydroxide) and was effective for over 30 days on unwashed clothing [80]. Throughout World War II, there were only 64 cases of typhus in the entire US Army due to the use of the louse powder [78]. In 1945 alone, 33

million pounds of DDT were produced in the United States and even more was produced in post-war times for agriculture and household uses [81]. Following these events, DDT won the title of the "Insecticide that Won the War" and earned Muller the Nobel Prize in Medicine in 1948.

At the end of World War II, focus on the development and potential application of DDT was replaced with experiments to determine the toxins' mode of action. Kennedy [82] observed the symptoms of mosquitoes after tarsal contact to a surface treated with DDT. Mosquitoes became excited, ataxic (uncoordinated), followed by visual convulsions, paralysis ("knock-down") and finally death [82]. The initial excitatory response caused adult mosquitoes that usually preferred dark environments to be attracted to light. This "repellent" effect of DDT may have increased the potential for a given mosquito to exit a building without getting a lethal dose. It was also noted that DDT could be used successfully as a residual larvicide [12], and that using sub-lethal doses would elicit the excitation and ataxia symptoms in larvae causing them to break from the water surface and drown [33,82].

Symptoms of DDT intoxication are due to the prolonged opening of the voltage sensitive sodium channels in the central nervous system of insects [83,84]. DDT acts by extending the depolarization phase of the action potential. When the after-potential exceeds the membrane potential, repetitive firing of the action potential results [83]. The prolonged tail currents result from normal sodium channel activation followed by a second open state. When the channel is in the second open state it is either not subject to inactivation or is inactivated slowly. Therefore the amplitude of the tail conductance is directly correlated with the number of open sodium channels [85,86].

Despite the overall consumer satisfaction with the insecticidal properties and widespread application, the use of DDT decreased. The decline was due to the discovery of other active compounds, research into environmental effects, and the emergence of resistance in the field. In 1972, DDT was banned from agricultural, medical and household uses in the United States.

Organophosphates

Organophosphorus chemistry has been studied since the late 1800s, but in 1936 organophosphate (OP) insecticides were discovered. OPs originally did not get as much attention as DDT for their insecticidal properties because OP compounds were being synthesized primarily for use as nerve gases. In World War II there was a large campaign in chemistry to create fast acting and effective nerve gases for use on the battlefield. Then, in the mid-1900s, attention was placed on their use as compounds for controlling insects [87]. These compounds became and are still some of the most predominant in agriculture and vector control programs worldwide. Much of the success as an insecticide class comes from the compounds being stable, long lasting and having a broad-spectrum of activity. In insects symptoms include: restlessness, hyperexcitability, tremors and convulsions and paralysis [88].

OPs can undergo oxidative activation or detoxification in a target insect. For example, in the case of oxidative activation the phosphoric acid of the parent compound parathion is metabolized by a complete cytochrome P450-monooxygenase (P450) enzyme system. The resulting active compounds are paraoxon, diethyl phosphorothioic acid and diethyl phosphoric acid [89] (Figure 1.4).

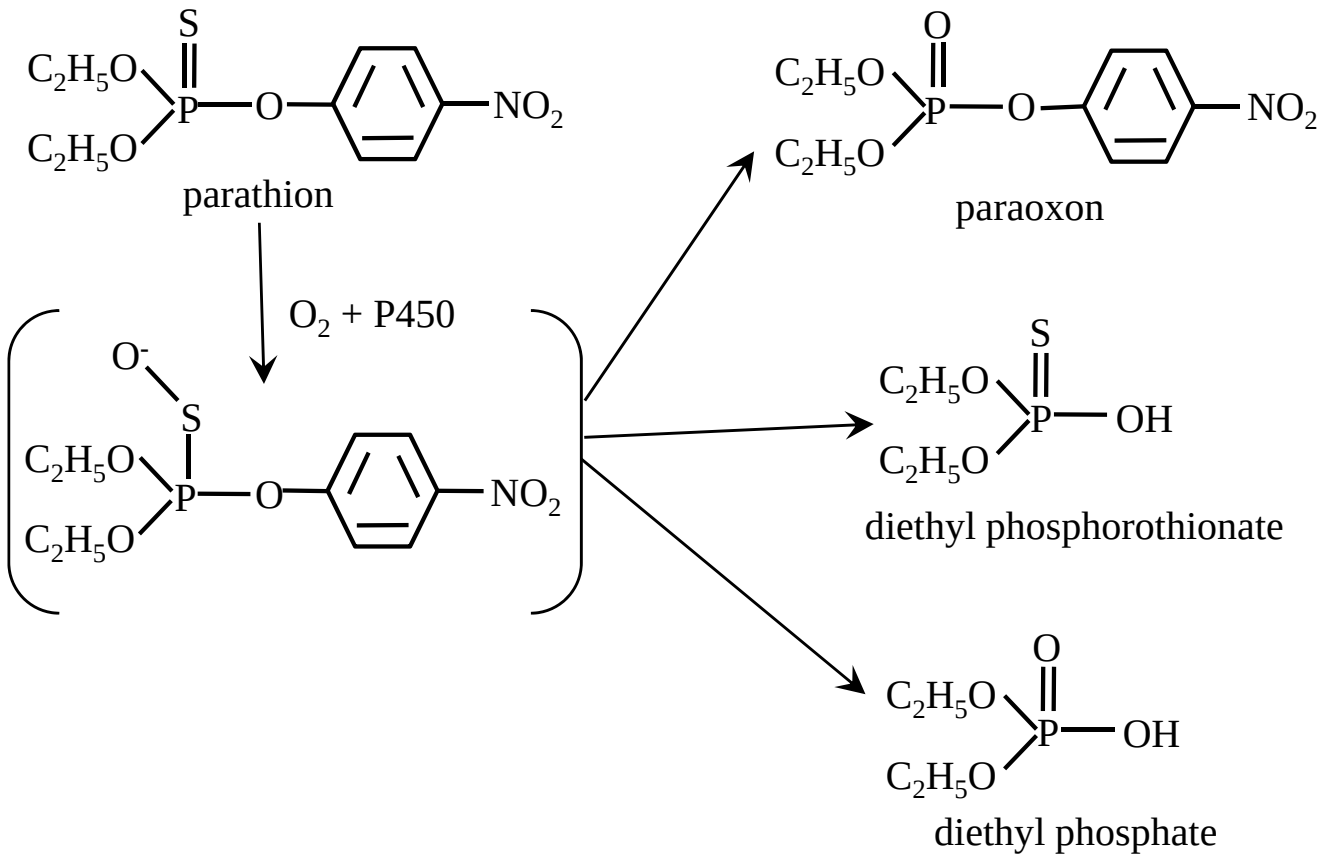


Figure 1.4: Simplified mechanism for the activation of the OP parathion (modified from [89]) to paraoxon.

The mechanism of action of this insecticide class is the inhibition of acetylcholinesterase (AChE). AChE is an enzyme that breaks down the excitatory neurotransmitter acetylcholine (ACh) in the synapses of the nervous system. OP insecticide compounds mimic the general structure of ACh because they contain both an esteratic site and an anionic site. The rate of AChE inhibition is highly dependent on the initial affinity of the phosphate for the enzyme, which takes place in the first part of the reaction (Figure 1.5).

In the last step of the reaction (k_4) under OP intoxication (Figure 1.5), a highly stable phospho-intermediate is produced and the phosphoryl moiety is cleaved at an extremely slow rate. This makes the OPs effectively "irreversible" AChE inhibitors [88].

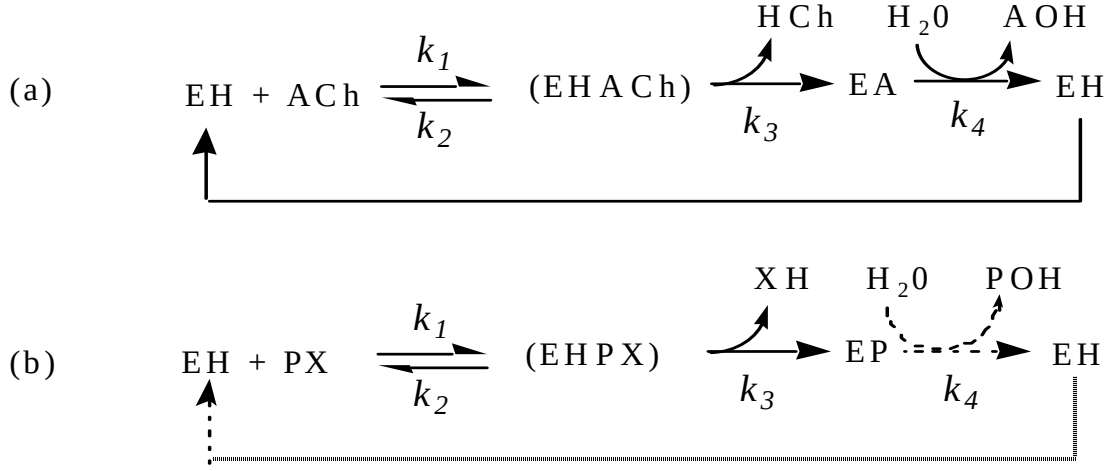


Figure 1.5: Depiction of the steps involved in (a) AChE (EH) degradation of ACh in a normal nervous system and (b) inhibition of AChE in a nervous system exposed to OPs (modified from [88]).

Insects poisoned by OPs accumulate ACh in the synapses resulting in the constant stimulation of the postsynaptic nerve. This causes over-stimulation of the nervous system, leading to massive universal bursts of

neural activity and death. The following OPs, also listed in Table 1.1, are used as larvicides in mosquito control throughout the world: chlorpyrifos, fenthion, pirimphos-methyl and temephos (Figure 1.6).

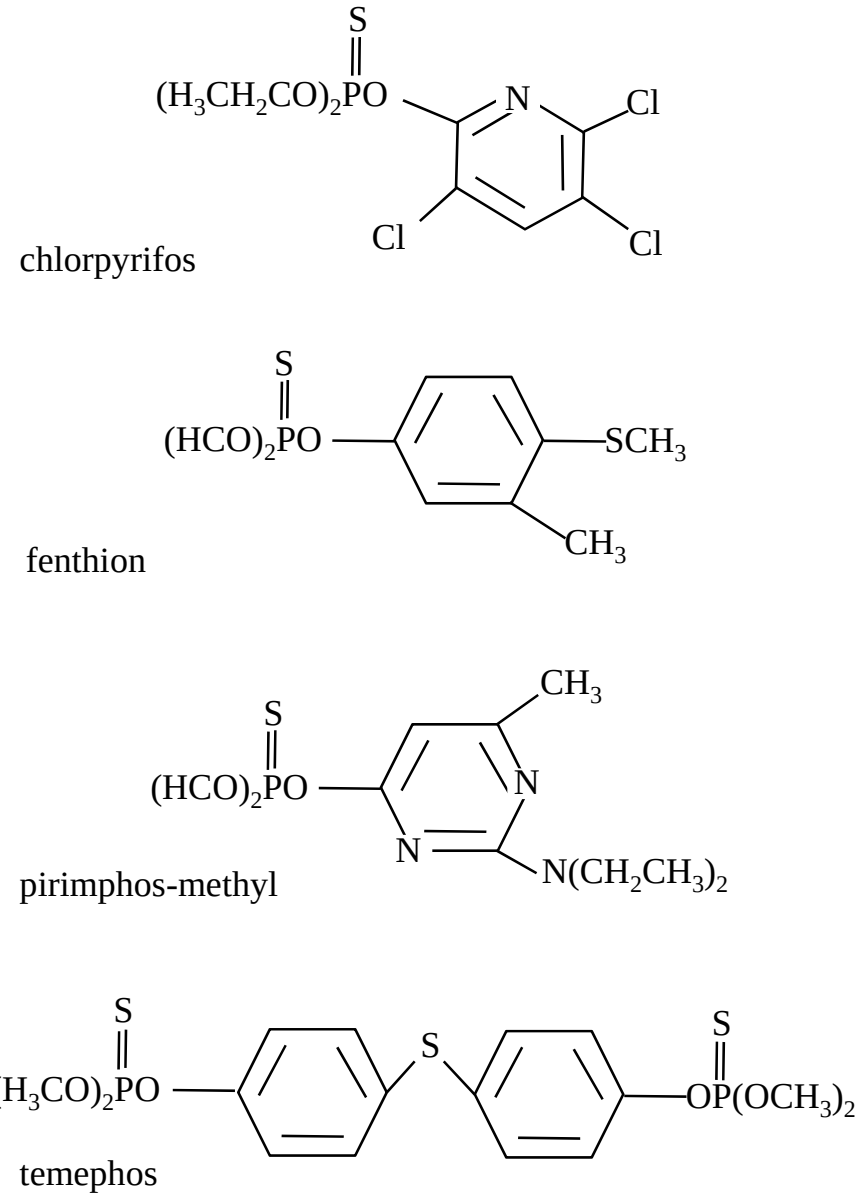


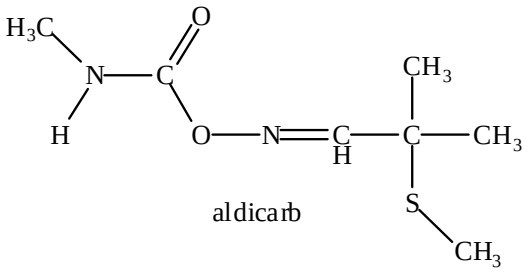
Figure 1.6: Organophosphate insecticides referred to in the text and used as larvicides.

Carbamates

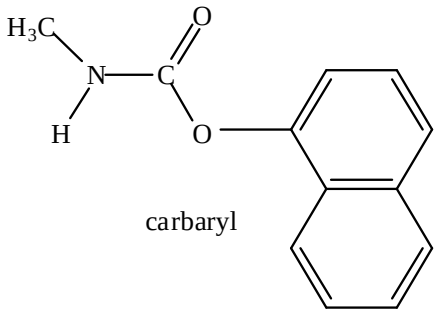
Carbamates were originally discovered in the 17th century when Europeans explored and colonized West Africa. The Egbo Society served as a government over the indigenous people of the area that is now Calabar Province in Nigeria. It was illegal to practice witchcraft, and if found guilty the Egbo Society would make the accused drink a poisonous milky substance made out of Calabar beans [90].

Europeans were so intrigued by this substance that they brought the beans back to Scotland and began to study the components of the toxin. In 1925, the molecular configuration was determined, and in 1935 approval for synthesis was obtained. Geigy Chemical Company, E. I. du Pont de Nemours Company, and Union Carbide Corporation were all key players in the development of new active carbamates. Most of the synthesis occurred in the 1960s and used the general formula $R_1R_2NC(O)OX$. In the formula, X is a substituted phenol if R_1 is methyl and R_2 is hydrogen, or X is a N-heterocyclic or hydroaromatic enol when both R_1 and R_2 are methyl groups [91]. It was Union Carbide who made the first registered oxime carbamate, aldicarb, and also made carbaryl (Figure 1.7) [90].

The mode of action of this class of insecticides is very similar to that of OPs, inhibition of AChE. Carbamates are also known to inhibit pseudocholinesterases and aliesterases, but not arylesterases [91]. When the enzyme binds with the insecticide a "reversible" complex is formed. The first step of the reaction is reversible by the addition of high AChE concentration and the final step of decarbamylation (k_4=4x10^{-1}min^{-1}) is rapid allowing for the original AChE to be reactivated at a steady rate (Figure 1.8) [88,91].



aldicarb



carbaryl

Figure 1.7: Structures of the carbamate insecticides aldicarb and carbaryl.

The insecticide merely competes with ACh for the active site on the enzyme, as it has been determined that AChE is not destroyed during the reaction. The most important step of the reaction is the first step and the electrophilic substitutions on the carbamate compound serve as the limiting factor [88,90].

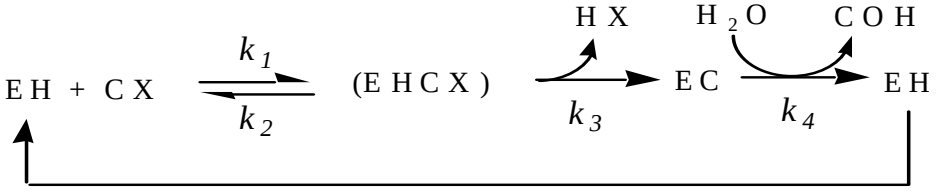
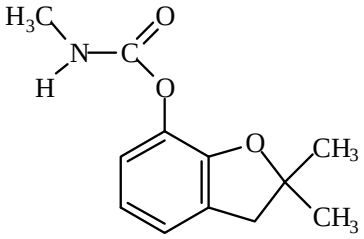
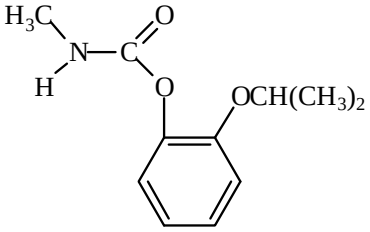


Figure 1.8: Depiction of steps involved in the inhibition of AChE in a nervous system exposed to carbamate insecticides (modified from [88]).

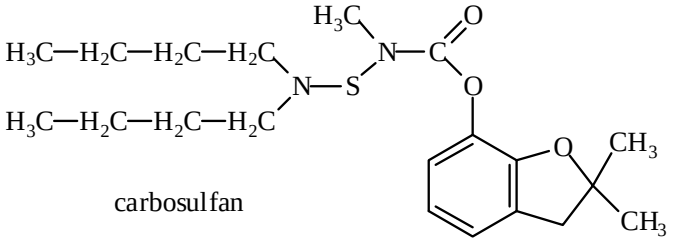
For mosquito control, bendiocarb and propoxur [22] are carbamates used for indoor residual treatments of dwellings, while carbosulfan [27] is used on bed nets (Figure 1.9). Carbosulfan applied to polyester bed nets decreased the rate of *Cx. p. quinquefasciatus* bloodfeeding on experimental hut occupants in Africa. Human bloodfeeding rates in the control huts were 17%, in huts with pyrethroid only treated nets it was 12-14%, and in huts with carbosulfan treated nets the rate was only 3% [92].



bendiocarb



propoxur



carbosulfan

Figure 1.9: Structures of carbamates listed for use as mosquito control compounds: bendiocarb, propoxur and carbosulfan.

Pyrethroids

Commercial pyrethroids were discovered at a time when resistance and environmental impacts were hindering DDT use, and when OP resistance was becoming a growing problem. Synthetic pyrethroids are still one of the most important classes of insecticides used against mosquitoes (especially for treated bed nets). This class of compounds makes up approximately 20% of the global insecticide market share and global sales are estimated at $1,280 million [93].

Chemical manipulations of pyrethrin I began once it was determined that pyrethrin I had enhanced insecticidal activity over pyrethrin II. Allethrin, the first commercially available synthetic pyrethroid, was discovered when the side chain of the alcohol moiety of pyrethrin I was unsaturated [93, 94]. One of the major hurdles in the rapid development of pyrethroids was that many of the early compounds were not photostable, which impaired the ability to keep up with the widespread demand. In response, Sumitomo Chemical Company in Japan developed fenvalerate and Rothamsted Experimental Station in the United Kingdom developed permethrin [93]. Permethrin was then manipulated by adding a cyano group to the α-carbon of the phenoxybenzyl moiety to make cypermethrin [94]. By synthesizing an alcohol containing an α-cyano group with a dibromo-crysanthemic acid, deltamethrin was created (Figure 1.10). While many of the early synthetic pyrethroids contained a mixture of isomers, deltamethrin was composed of a single isomer. At its introduction, deltamethrin was more toxic than any other insecticide ever made [95].

Pyrethroids disrupt electrical impulses along nerves by acting on the voltage-gated sodium channels. In a normal nervous system, an action potential results from an increase in membrane sodium conductance (g_{Na})

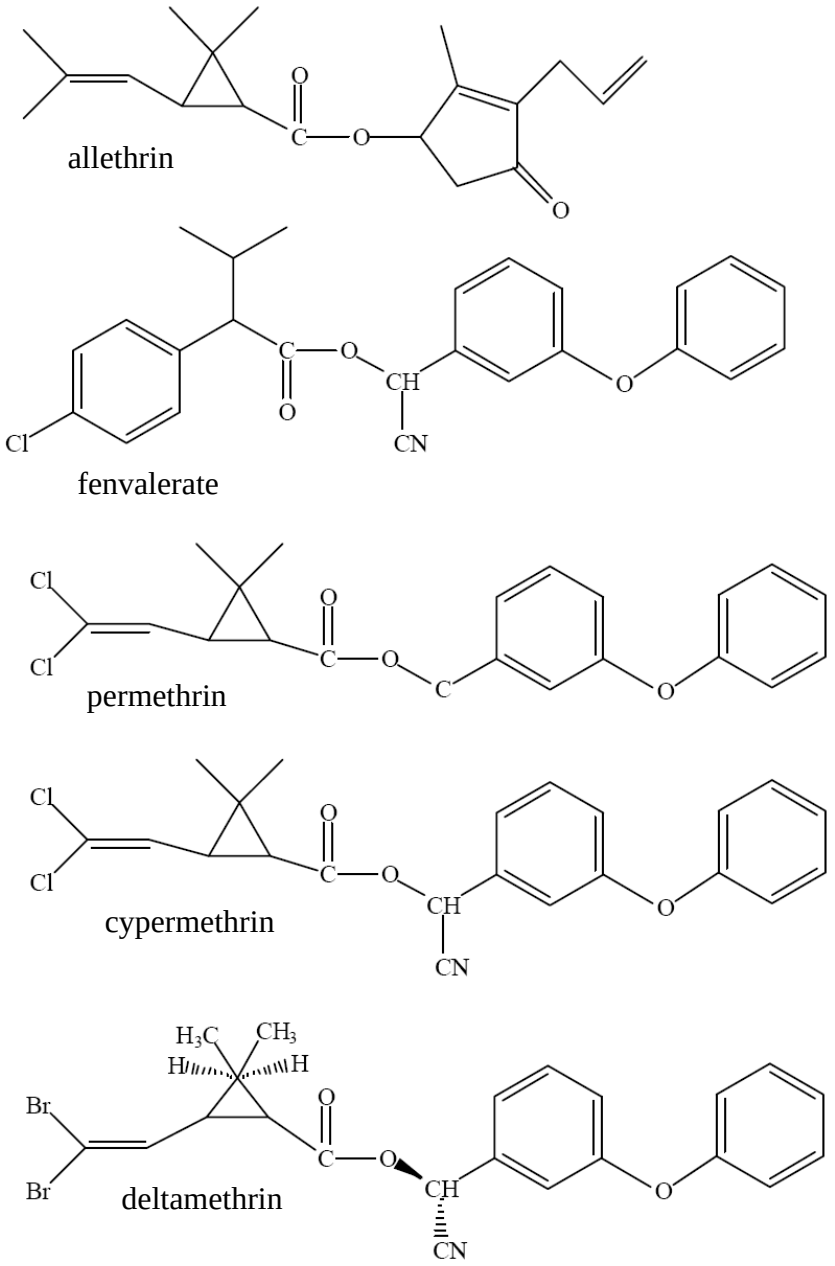


Figure 1.10: Synthetic pyrethroid insecticide structures in order of appearance in the text.

causing the rising phase followed by a falling phase that results from a decrease in g_{Na} along with an increase in membrane potassium conductance (g_K). In an insect treated with pyrethroids, the poisoning symptoms are very similar to those exhibited by insects treated with DDT [86]. Overall symptoms include hyperactivity, tremors, lack of coordination, convulsions, paralysis and finally death. Pyrethroid poisoning symptoms are further classified as either Type I or Type II [96,97]. These two classifications represent a spectrum of responses as some pyrethroids have characteristics of both groups [98].

Type I pyrethroids, such as allethrin and permethrin, cause a rapid onset of the symptoms listed above. Electrophysiological experiments determined that these compounds bind to closed (resting or inactivated) sodium channels causing the rapid decay of tail currents. By using TTX, a puffer fish toxin that inhibits the sodium current without acting on the potassium current, the action potential of a poisioned nervous system could be analyzed. Type I pyrethroid insecticides suppress the amplitude of g_{Na}, which decreases the amplitude of the action potential. Type I pyrethroids extend the depolarization time of the action potential such that the tail current exceeds the membrane potential, generating repetitive firing in the axons [99,100]. The time constant of the sodium current decay for Type I pyrethroids fits a single exponential function [101,102]. Pyrethroid alterations of sodium channel kinetics (resulting in repetitive discharges) may be due to the modification of less than 1 percent of all sodium channels [101,103].

Type II pyrethroids, such as cypermethrin and deltamethrin, have a slower onset of symptoms and exert such stress on the sodium channels that recovery is improbable. Poisoned insects become ataxic and uncoordinated with interspersed periods of hyperexcitation and convulsions [97]. Type II

pyrethroids bind to activated channels in a use-dependent manner. Type II pyrethroids, like Type I pyrethroids, prolong the depolarization phase of the action potential, but the decay of the tail current is extremely slow [104]. Type II pyrethroids cause the immediate and complete depolarization of the membrane resulting in a blockage of conduction at the synapses [93].

Blum and Kearns [105] observed that like DDT, pyrethrum had increased toxicity to *Periplaneta americana* at lower temperatures (called negative temperature coefficient). There are many factors, such as life stage, gender, and other environmental features, that can influence insecticide toxicity-temperature relationships. While there was a positive correlation associated with temperature and penetration of the insecticide, it seems this factor is of minor importance [106]. Munson, et al. [107] believed that at high temperatures DDT was highly soluble in the lipids and that when the temperature was decreased, the DDT would be released. No such experimental observations were made to support this hypothesis. Since only small differences were found at high and low temperatures it is not likely that a decrease in metabolism of the insecticide compound contributes to the negative temperature coefficient. Altered distribution does not appear to be a factor either. The increased pyrethroid toxicity at lower temperatures appears primarily to be due to increased interactions at the target site [106].

It is therefore possible that the kinetics of sodium channels differ under different temperatures. This hypothesis was observed at low temperature conditions where sodium tail currents were significantly extended [108]. It was observed that at 33°C when bathed in 10^{-8}M allethrin cockroach giant axons experienced repetitive discharge and a decrease in temperature enhanced conduction block [109]. Pyrethroids that produce bursts have an upper and

lower temperature limit at which only a single action potential results from a single stimulus, called the inversion temperature. In general, for pyrethroids the lower inversion temperature is near 19°C. While it is clear that bursts occur at lower temperatures, it has also been shown that conduction blocks occur more rapidly at lower temperatures [96].

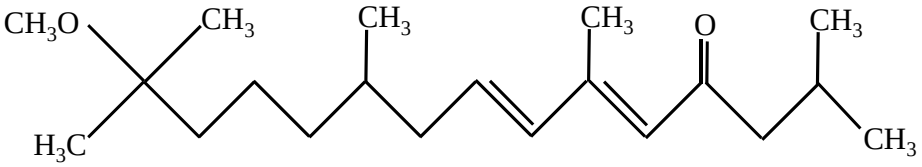
Insect growth regulators

Insect growth regulators (IGRs) are insecticides which regulate metamorphosis, development and reproduction. Juvenile hormone (JH) controls many developmental processes in insects (*i.e.*, embryogenesis, diapause, molting and metamorphosis, reproduction, etc.) [110]. As such, the types of IGRs include chitin synthesis inhibitors, JH mimics, JH analogs, molting hormone agonists, and molting inhibitors.

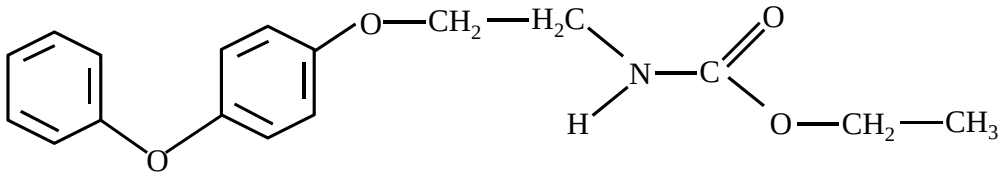
The first use of an IGR occurred in 1956 when JH was isolated from male Cecropia (*Hyalophora cecropia*) moths and was topically applied to other moths preventing metamorphosis and halting normal developmental processes. IGRs did not become widely used until 1965 when the "paper factor" incident occurred. Harvard researchers experienced a decrease in egg hatch rates and supernumerary larvae of the European linden bug (*Pyrrhocoris apterus*) kept in colony. Upon investigation of this initially empirical observation, they found that it was the paper towels used during rearing that caused the developmental abnormalities. The paper contained a compound called juvabione, which is a JH mimic of the family Pyrrhocoridae (Hemiptera) [110].

IGRs are incorporated into integrated management programs because they are insect-specific [28]. Chitin synthesis inhibitors are generally used as larvicides, exerting their effects at the time of molt. Larvae develop normally until they are to molt, at which time they are unable to ecdyse since new cuticle

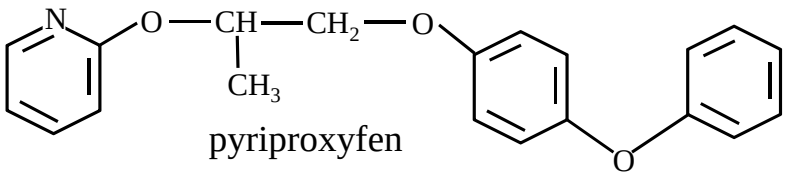
synthesis is inhibited. JH mimics and agonists compete with JH in binding to receptors and success of this group of IGRs is highly dependant on application timing [110]. Although IGRs are slow acting they can have LC_{50}s that are 1-2 orders of magnitude less than neurotoxic insecticides [111].



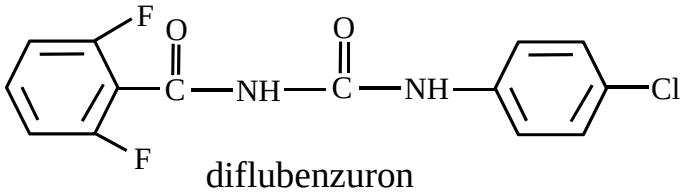
methoprene



fenoxycarb



pyriproxyfen



diflubenzuron

Figure 1.11: Examples of insect growth regulators used in mosquito control.

Mosquito control programs use IGRs (Figure 1.11) mostly because they prevent the harmful adults from developing and also keep the ecologically important larvae in the environment. Special formulations and control-released applications will often need to be implemented in order to target the larvae which hatch asynchronously [28]. Methoprene has been used effectively against *Aedes* spp. larvae, but this JH analog was later formulated as a slow-release compound in order to be effective against *Culex* spp. and *Anopheles* spp. Other notably used IGRs in mosquito control include the juvenoids fenoxycarb, pyriproxyfen and the chitin synthesis inhibitor diflubenzuron (Figure 1.11) [28].

Novel insecticides

Since resistance is one of the major obstacles facing mosquito control efforts around the world, there is pressure to design new synthetic insecticides. Despite the inherent need for novel compounds that exploit new target sites, very few are developed and commercialized specifically for vector control. This is attributed to the downsizing of agrochemical companies, the high cost of discovery, and low profitability in the vector control market since the countries that need these resources have limited monetary resources [112]. Some of the novel chemicals currently in trial stages for vector control include a multitude of phytochemicals [113] as well as the insecticides listed in Table 1.2. Neonicotinoids are currently the fastest growing new insecticide class with a market value of over 600 million euros per year [114] and increased promise for use in vector control.

Table 1.2: List of novel insecticides evaluated for mosquito control.

Insecticide	Class	Mode of Action	Reference
halofenozide	IGR (non-steroidal ecdysone agonist)	Binds to ecdysone receptor, which activates gene transcription and starts the molting cycle	[115]
novaluron	IGR	Chitin synthesis inhibitor	[116]
fipronil	phenyl pyrazole	Impedes the passage of Cl^- ions through the GABA-gated Cl^- channels [103]	[30,117]
dinotefuran	neonicotinoid	Agonist of nicotinic acetylcholine receptor	[118]
chlorfenapyr	protoxin	Activated by cytochrome P450 monooxygenases. Uncoupler of oxidative phosphorylation.	[111]
hydramethylnon	amidinohydrazone	Inhibits mitochondrial electron transport and blocks production of ATP	[111]
indoxacarb	oxadiazine	Decarbomethoxylated to active compound DCJW which blocks Na^+ channels [103]	[111]
imidacloprid	neonicotinoid	Agonist of nicotinic acetylcholine receptor	[111]
diafenthiuron	IGR	Chitin sythesis inhibitor	[111]
pyriproxyfen	IGR	JH mimic	[111,119,120]
Beauveria bassiana	entomopathic fungi	Vegetative growth in haemocoel	[121,122]
dsRNA *AaeIAP1*	molecular	RNAi gene silencing of an apoptosis inhibitor	[123]

1.5 Insecticide resistance

Resistance to insecticides is defined as "the inherited ability of a strain of some organism to survive doses of a toxicant that would prove lethal to the majority of individuals in a normal population of the same species" [124]. The first description of resistance occurred in 1914 on San Jose scale (*Quadraspidiotus perniciosus*) which were surviving different mixtures of lime-sulfur that had been effective two years earlier [125]. The first occurrence of resistance to a synthetic insecticide was observed in DDT-resistant house flies collected in Stockholm, Sweden in 1946, only two years after the insecticide was introduced (Wiesmann 1947 as cited in [126]). In 1947, DDT resistance was first observed in the mosquito vector *Cx. molestus* in Italy [126]. Resistance was subsequently found in *Ae. taeniorhynchus* [127] and *Ae. sollicitans* in Florida [127]. Shortly after, DDT-resistance was recorded in *An. sacharovi* from Greece in 1951 and dieldrin resistance was recorded in *An. gambiae* from northern Nigeria in 1955 [126]. Since those preliminary reports, over 500 insect (over 200 of which are of medical or veterinary importance) and mite species have become resistant to a multitude of insecticidal compounds [128]. By the mid-1980s, 109 mosquito species had developed resistance to organochlorines (DDT and/or dieldrin), and 59 species were resistant to OPs of which 17 showed adult resistance to carbamates and 10 showed cross-resistance to pyrethroids [129].

Resistance is extremely detrimental in the on-going struggle to control or eradicate vectors of disease [129]. Worldwide 2.5 tons of pesticides are used annually costing around $20 billion [130]. For vector control, residual house spraying was found to be 7.2 times more expensive than insecticide treated bed nets in South America [131], while the opposite was true in South Africa [132].

Overall, the cost of DDT is still the least expensive at $3.20/year/household, while pyrethroids are undergoing a decline in cost ($10-$12/year/household) and propoxur is overwhelmingly the most expensive compound at $74.4/year/household [133]. The total cost of resistance is estimated to be approximately 10-25% of current treatment costs, which equals around $30 million annually in the USA [130]. Maintaining sufficient levels of control of pest populations in the face of resistance creates additional costs (such as, increased application of insecticides, reduced crop yield, medical care) beyond the expense of using insecticides on non-resistant populations. As such, tactics must be employed in pest management programs to decrease the detrimental economic and health implications that result from resistance.

Pesticide resistance management strives to avoid resistance in the field, delay the evolution of resistance development, and enhance the reversion of resistant populations to susceptiblity in order to keep the resistance level under a given threshold [134]. Understanding the physical properties of the insecticide along with the biochemical and genetic components of the resistance mechanism(s) are important considerations when choosing a strategy. Georghiou [135] has suggested three management strategies: 1) moderation, 2) saturation and 3) multiple attack.

Moderation works in populations that have not yet evolved resistance by using susceptible individuals as a dilution mechanism in the population. Under this strategy, infrequent applications of compounds that have short half-lives and low persistence are used at low dosages. Moderation works because applications are localized during an outbreak of the pest or directed at a particular life stage. Refugia are also a key component, as this provides a non-treated habitat for susceptible individuals to reproduce normally,

preserving susceptible alleles in the population [135].

Management by saturation can be used to control an invasive pest species that is newly introduced. This strategy renders the resistance allele functionally recessive by applying a high dose of insecticide (along with synergists to suppress detoxification mechanisms) in order to kill all individuals. The inheritance pattern is extremely important to know in this system, since heterozygotes will be nearly as susceptible as homozygous susceptible individuals if resistance is recessive. It is best to implement this strategy early in resistance development when homozygous resistant individuals are rare. If even a small number of homozygous resistant individuals are present, a highly resistant population will result after the intense selection. This approach also does not preserve natural enemies (like moderation) since they generally evolve resistance slower than the targeted pests [135].

Lastly, the multiple attack strategy makes use of insecticides with different modes of action either in mixture formulations or in rotation. In this case, two target sites will be saturated and the probability that an individual will be homozygous resistant for both is decreased. In cases where mixtures are used, the possibility of selection for both resistance mechanisms is possible, so synergists are employed to increase the potency of at least one compound [135]. Using compounds that exhibit negative cross-resistance may enhance this strategy such that one compound may increase the susceptibility to another compound used at the same time. An example of this is with pyrethroid resistance in house flies conferred by increased detoxification activity due to the cytochrome P450 monooxygenase, CYP6D1. This P450 may be able to metabolize chlorfenapyr and diazinon from the non-toxic form into

Table 1.3: Pyrethroid resistance levels in larvae of the *Culex pipiens* complex is widespread.

Location	Level of resistance	Year samples collected	Reference
Benin	<3-fold	2005	[142]
China	2.5- to >20-fold	2003	[143]
Cuba	3- and 20-fold	1995, 1986 & 1990	[144,145]
Martinique	10- to 2,800-fold	1991 & 1999	[146]
Saudi Arabia	2,500-fold	1984	[147]
Tanzania	5- to 10-fold	1988, 1993 & 1998	[139]
Tunisia	>7,100-fold	1990 to 1996	[148]
United States	Alabama: 100- to 940-fold	2002	[149]
	California: 3- to 18-fold	2001	[150]
	Florida: 13- to 50-fold	1998	[151]
	New York: 6- to 7-fold	2003	[60]
West Africa	9- to 82-fold	1994 to 1995	[152]

its toxic "-oxon" form [136]. This strategy is often employed to control mosquitoes by using multiple insecticides on bed nets [137–141].

Resistance can also occur as a byproduct of agrochemical applications. Some evidence towards this conclusion is that resistance appears prior to the application of insecticides used against mosquito populations, resistance levels are higher in agricultural areas, and mosquito resistance levels cycle with periods of agricultural spraying. In addition, resistance has been found in some species of mosquito larvae which are not directly targeted for control with insecticides (Table 1.3).

Efforts to decrease indirect exposure of mosquitoes to insecticides used in agricultural settings often involve integrative control techniques, such as water source reduction to eliminate mosquito breeding sites and the controlled use of larvicides and adulticides [153]. Some ways to accomplish more effective integrative measures are by increasing collaboration between health and agricultural agencies to identify compounds that could be used exclusively for public health, introducing comprehensive pest management practices between vector control and agriculture, and placing a greater emphasis on comprehensive mosquito control measures, such as habitat modification [154].

1.6 Mechanisms of resistance

1.6.1 Physiological - Modified pharmacodynamics

Reduced cuticular penetration (*pen*)

Reduced cuticular penetration (*pen*) has been shown to delay the entry of certain compounds (DDT, dieldrin, parathion, carbaryl, diazinon, malathion, chlorthion, and naphthalene [155]) through the cuticle [156]. It offers limited protection from poisoning with a 2-3-fold level of resistance (measured as a resistance ratio = LC_{50} resistant strain/LC_{50} susceptible strain). The gene(s) responsible for this mechanism have not been identified, but the factor(s) responsible have been mapped to autosome 3 in house flies [157]. Pyrethroid resistant house flies accumulated less [^{14}C]permethrin than their susceptible counterparts, with the greatest difference at 30 minutes post-treatment [158]. Malathion resistance in *Ae. aegypti* was shown to result from decreased absorption into the larvae [159].

1.6.2 Physiological - Altered target site

Knockdown resistance (*kdr*)

Knockdown resistance (*kdr*) is a single point mutation in the voltage sensitive sodium channel gene which produces a channel with decreased sensitivity to DDT and pyrethroids. This phenotype was described as the reduced potential to be knocked-down and killed by DDT [160] and was determined to be a recessive factor mapped to autosome 3 in house flies [161,162]. There was no difference in the affinity (K_D) or the total number of binding sites (B_{MAX}) between a resistant and susceptible strain of *Blattella germanica* [163]. Therefore, resistance is due to qualitative changes in the sodium channel function. Electrophysiology studies in house flies showed that *kdr* reduced sensitivity in the nervous system to DDT and pyrethroid treatments [164].

The electrophysiology data indicated that the gene involved in DDT and pyrethoid resistance was that of the sodium channel. The sequence of the *para* locus from *Drosophila melanogaster* was sequenced and had over 50% amino acid homology with rat sodium channels [165]. This sequence was used as a model in order to attain the sequences of *para*-like sodium channels in other species. *Kdr*-type resistance has been identified in numerous species, including *B. germanica* [166], *An. stephensi* [167], *Cx. tritaeniorhynchus* [168], *Cx. p. quinquefasciatus* [169], *Spodoptera littoralis* [170], *P. xylostella* [171], and *Heliothis virescens* [172].

Upon examination of the *para*-orthologous sodium gene of the house fly (*Vssc1*), mutations were found to be linked to pyrethroid resistance [173,174]. Sequence comparisons of house fly strains identified two nucleotide mutations associated with the resistance phenotype: leucine to phenylalanine at position

1014 (L1014F) in all *kdr* and *super-kdr* strains, and the second mutation of methionine to threonine at postion 918 (M918T) only in the *super-kdr* strains. The L1014F mutation is in the transmembrane region of segment 6 (S6) of domain II allowing a resistant individual to be 40 times less sensitive to cismethrin than sensitive channels [175], while the M918T mutation is located in a cytoplasmic linker between segments 4 and 5 of domain II [176]. The *super-kdr* allele (L1014F + M918T) confers more than 10-fold higher resistance levels than *kdr* alone [177,178]. Table 1.4 lists the *para*-sodium channel mutations currently identified in pyrethroid resistant insects.

The L1014F mutation shifts the voltage-dependence of activation in house fly sodium channels 5.9 mV in the positive direction while shifting the voltage-dependence of inactivation 3.4 mV in the positive direction. This suggests that an overall higher change in voltage is necessary to activate the channels which results in reduced neural excitability [178]. Studies with pyrethroid resistant *H. virescens* identified a second possible mutation at the 1014 position, the replacement of leucine with histidine (L1014H) [190]. This mutation was subsequently found in house flies [195,196].

In mosquitoes, the L1014F mutation is present in *Cx. pipiens* [187], *An. stephensi* [197], and the molecular S (Savannah) form of *An. gambiae* from west Africa [180] and Nigeria [198]. It is absent in the molecular M (Mopti) form of *An. gambiae* [180] and *Ae. aegypti* [199]. In the case of *An. gambiae*, the two molecular forms occur in partial sympatry. It was originally observed that the *kdr* mutation was only present in the S form and not the M form, providing evidence which suggests that the two forms are reproductively isolated, and that the M form has an additional resistance mechanism to pyrethroids [200]. It was later shown that M populations from Benin [201], Burkina Faso [202],

Table 1.4: *para*-sodium channel mutations found in resistant insect strains (modified from [169]).

Species	Mutations
Anopheles arabiensis	L1014F, L1014S [179]
Anopheles gambiae	L1014F, L1014S [180, 181]
Anopheles sacharovi	L1014F, L1014S, L1014L [182]
Bemisia tabaci	M918V, L925I , T929V [183]
Blattella germanica	L1014F, L993F, D58G, E434K, C764R, P1880L [184, 185]
Boophilus microplus	F1538I
Ctenocephalides felis	T929I, T929V, L1014F [186]
Culex pipiens	L1014F, L1014S [187]
Culex pipiens pallens	L1014F [143]
Cydia pomonella	L1014F [188]
Drosophila melanogaster	I253N, A1410V, A1494V, M1524I
Frankliniella occidentalis	L1014F [189]
Helicoverpa armigera	D1549V+E1533G
Heliothis virescens	L1014H [190]
Hematobia irritans	L1014F+M918T [191, 192]
Leptinotarsa decemlineata	L1014F
Leptinotarsa huidobrensis	L1014F [193]
Leptinotarsa sativae	L1014F [193]
Leptinotarsa trifolii	L1014H [193]
Musca domestica	L1014F [194], L1014F+M918T, L1014H [175, 176, 195]
Myzus persicae	L1014F [194]
Pediculus capitis	T929I+L932F
Plutella xylostella	L1014F+T929I [171, 194]

Equatorial Guinea [203] and Ghana [204] had low frequencies of *kdr*. In *Ae. aegypti*, the L1014F mutation was absent and four novel mutations were found from thirteen field strains collected in South America and southeast Asia [199].

Altered acetylcholinesterase

Insensitivity of acetylcholinesterase (AChE) is a major mechanism of resistance to OPs and carbamates. Since these compounds mimic the neurotransmitter acetylcholine (ACh), AChE is inhibited from its normal functions in the synapse [88]. The structure of AChE from the model organism *Torpedo californica* elucidated the residues composing the catalytic triad: Ser200, His440 and Glu327 [205, 206]. The decreased sensitivity of the resistant form of AChE was determined to be due to a reduced affinity of the enzyme for the inhibiting substrate (*i.e.*, insecticide compound) [207].

Since the first case of altered AChE activity conferring resistance in the spider mite [208], other species have been found to have this same mechanism (such as the house fly [209], *Anopheles* spp. [207, 210, 211] and *Cx. pipiens* [210]). In *D. melanogaster*, resistance to OPs was associated with the amount of AChE in the central nervous system [212] along with a single amino acid change resulting in F368Y. The substitution at position 368 is responsible for resistance in the single AChE gene [213], *Ace*, because it alters the catalytic properties of the enzyme [214]. Subsequently, four additional point mutations in *D. melanogaster Ace* were found: F115S, I199V, I199T and G303A. Like *Drosophila* where combinations of mutations gave significant levels of insensitivity [215], the same pattern was seen in the *Aedes* AChE [216].

In the mosquito species *Cx. pipiens*, *An. gambiae*, and *Ae. aegypti* [217] two forms of AChE exist, called AChE1 (*Ace-1* gene) and AChE2 (*Ace-2* gene) which likely resulted from a recent duplication event [218, 219]. Analysis of

other mosquito species, like, *An. stephensi*, *Cs. longeareolata* and *Cx. hortensis*, found that each of these species had only one form of AChE [220]. The *Ace-2* gene was found to be homologous to the single *Drosophila* gene, but no related sequence associates with the *Ace-1* gene. Insecticide resistance has been linked to mutations of the *Ace-1* gene [221]. The G119S mutation (in *Cx. pipiens*, *Cx. vishnui*, *An. gambiae* and *An. albimanus*) is located in the third coding exon which is part of the oxyanion hole [222], F290V in *Cx. pipiens* lines the active site [223], and F331W in *Cx. tritaeniorhynchus* is located in the acyl pocket next to the active site [224]. The *Ace-1*^{R} mutation has been found in *An. gambiae s.s.* M and S molecular forms in Burkina Faso. Mutation frequencies range from 25%-50% in the S form and 4%-13% in the M form and no resistant homozygotes were detected [225]. Unlike *Anopheles* spp. and *Cx. pipens*, only low levels of resistance have been observed in *Ae. aegypti* likely due to a "codon constraint." As such, in order for *Ae. aegypti* AChE1 to have the G119S mutation, two substitutions would be necessary since the glycine is encoded by GGR versus the glycine in the other mosquito species which is encoded by GGY and only necessitates one substitution [226].

Resistance to dieldrin

Cyclodiene resistance occurs in numerous species [128] and is due to a point mutation (G to T at nt 995) that replacing alanine 302 with a serine, called *Rdl* [227]. Resistance (100-fold insensitivity) to cyclodienes and picrotoxin were associated with this mutation by site-directed mutagenesis and functional expression in *Xenopus* oocytes. The amino acid change is in the second membrane-spanning domain of the GABA receptor and is thought to line the Cl^- ion channel pore [227].

Worldwide the A302S mutation is present in *D. melanogaster* [227], *D. simulans* [228], *Ae. aegypti* [229], *An. gambiae*, *An. arabiensis* [230], *Myzus persicae* [231], *B. germanica* [232] , *Tribolium castaneum* [233,234], *Hypothenemus hampei* [235], *Ctenocephalides felis* [236], *Bemisia tabaci* and *B. argentifolii* [237]. A second single nucleotide mutation conferred a second allele found only in *D. simulans* [228] and some *M. persicae* clones [231]. This second mutation replaced alanine 302 with glycine (C to G at nt 996) [228]. In *D. melanogaster*, *Rdl* is tightly linked to an *EcoR1* site, therefore it is believed that there was one mutation event that spread throughout the world. This is not the case for other species, like *T. castaneum*, in which multiple origins exist for *Rdl* since this tight linkage does not exist [238,239]. Overall, these two mutations confer a "dual resistance effect" by directly altering the insecticide binding site and indirectly destabilizing the preferred state of the receptor for insecticide binding [240].

1.6.3 Metabolic detoxification

Cytochrome P450 monooxygenases

Basic properties Cytochrome P450 monooxygenases (P450s) are a class of enzymes that are involved in the metabolism of insecticides (detoxification or bioactivation) and endogenous compounds. P450s are a hemoprotein and can be readily characterized by the conserved heme binding sequence FXXGXXXCXG [241,242] and an absorbance peak at 450 nm when the reduced form is combined with carbon monoxide [243]. Eukaryotic P450s (40-70 kDa) are membrane bound and are found in the endoplasmic reticulum and mitochondria. They catalyze the transfer of an atom of molecular oxygen to a substrate and reduce the other atom to water. To detoxify an insecticidal compound, P450s can perform a diverse array of reactions, such as oxidative ester cleavage and *O*-dealkylation. In cases of bioactivation, which mostly

occur with OPs, the P=S is desulfurated to the more toxic P=O [244]. The monooxygenase oxidation reaction requires substrate, P450, oxygen, NADPH, cytochrome P450 oxidoreductase (P450 reductase) and phospholipids [245] (Figure 1.12).

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

Figure 1.12: The overall reaction of cytochrome P450 monooxygenase metabolism of a substrate (RH).

P450 reductase is a microsomal flavoprotein that transfers reducing equivalents from NADPH to the microsomal P450 protein. Another potential electron donor in the reaction is cytochrome b_5 [246]. When cytochrome b_5 binds to the P450, it becomes a two electron acceptor which increases the rate of transfer for the second electron and substrate turnover [247]. These components are abundant in the proximal intestine, malpighian tubules, and fat bodies of insects [248,249].

Nomenclature Cytochrome P450s are present in a diverse array of organisms such as bacteria, plants, animals and insects. Universally, P450s are named CYP (for cytochrome P450), followed by a number, a letter and a number which represent the family, subfamily and isoform, respectively [250,251]. Alleles of a gene are designated *v1*, *v2*, etc [252]. P450s with >40% identity at the amino acid level are placed in the same family and those with >55% identity are placed in the same subfamily [251]. No detailed information about the function of a P450 can be assumed from its classification since it is possible for a single amino acid mutation to completely change the substrate specificity of the P450 [253]. To date insect P450s belong to six CYP families: the insect

Table 1.5: Cytochrome P450 monooxygenases in insects with sequenced genomes (modified from [255]). The number of genes (pseudogenes) within each P450 clan are listed.

Clan	*Apis mellifera*	*Drosophila melanogaster*	*Tribolium castaneum*	*Anopheles gambiae*	*Aedes aegypti*
CYP2	8(0)	7(0)	8(0)	10(-)	11(0)
CYP3	28(2)	36(4)	72(7)	41(-)	80(4)
CYP4	4(0)	32(0)	45(3)	45(-)	58(2)
Mitochondria	6(0)	12(0)	9(0)	9(-)	9(0)
Total	**46(2)**	**87(4)**	**134(10)**	**105(7)**	**158(6)**

specific CYP6, 9, 12, 18, and 28, and the family CYP4 which also contains sequences from vertebrates [241]. Clans are groups of P450 families that consistently cluster together on a phylogenetic tree. Insects currently have four clans, CYP2, CYP3, CYP4, and mitochondrial [254] (Table 1.5).

Often P450 genes are arranged in clusters throughout the genome of an insect. In *D. melanogaster* the largest cluster contains nine genes (eight *CYP6A* genes and *CYP317A1*) on chromosome 2 [256], while in *An. gambiae* the largest cluster contains 14 genes (all in the *CYP6* family) on chromosome 3 [244]. Clusters are thought to be the product of gene duplication, since many have high nucleotide identities and some are transcribed together. The high amount of independent gene duplications could be the source of apparent pseudogenes and allelic variants in the P450 enzyme family in different species [244]. Only ten orthologous P450 gene pairs have been found between *Drosophila* and *An. gambiae*, of which five were mitochondrial P450s [257] which makes it highly unlikely that identical P450 isoforms will be responsible for resistance to the same insecticide in two different species [258].

Purification methods Many methods have been used to successfully study single insect P450s (isoforms): protein purification, gene cloning, PCR with degenerate primers and genome sequencing. Protein purification was first successfully used on the LPR strain of house fly, which showed high resistance to permethrin, deltamethrin, cypermethrin and other pyrethroids. This method has had few successes because there is a need for a large amount of starting protein and there is difficulty in designing a purification methodology that targets the P450(s) which confers the observed resistance. In LPR there was a 4-fold increase in total P450 content relative to the susceptible strain [259]. The abundance of P450s in the microsomal fractions allowed for HPLC purification of the specific P450 associated with resistance, CYP6D1 [260]. Microsomes from LPR flies were able to metabolize 24% of the deltamethrin, while only 3% of the deltamethrin was metabolized when anti-CYP6D1 anti-serum was added [261]. In a semi-quantitative Western blot it was shown that the concentration of CYP6D1 was approximately 8-fold higher in LPR than the susceptible strain [262] and that CYP6D1 mRNA is present only in adult house flies [263]. Follow-up experiments on *CYP6D1* have shown that there is increased transcription due to factors on autosomes 1 and 2 [264] and that a 15 bp insertion in the promoter region where the transcriptional repressor *Gfi-1* binds is likely to cause increased expression [265].

The first insect P450 (*CYP6A1*) was cloned by screening a cDNA expression library prepared from phenobarbital induced house flies with an anti-serum raised against a partially purified P450 [266]. The success of this approach is highly dependant on the specificity of the anti-sera. Anti-CYP6D1 shows no cross-reactivity to P450s in other species [267]. Conversely, anti-CYP6A2 in *D. melanogaster* is cross-reactive to *CYP9A1* in *H. virescens* [268].

Constructing degenerate primers to obtain pure P450 isoforms by PCR has provided the opportunity to obtain gene sequences. In general, the primers are based on the conserved heme sequence and CYP family specific sequences. This technique obtained 17 new partial sequences from family CYP4 in the mosquito *An. albimanus* [269], 97 gene sequences from *Drosophila* [270] and *CYP6E1* from the mosquito *Cx. p. quinquefasciatus* [271].

Genome sequencing is an extraordinary tool in determining which P450 isoforms are responsible for resistance. Genome sequencing revealed 83 functional P450 genes and 7 apparent pseudogenes in *D. melanogaster* [256], 83 putative P450s in *Caenorhabditis elegans*, 58 in humans, 48 in *Apis mellifera*, approximately 80 (with 1 pseudogene) in *D. pseudoobscura* [272] and 111 in *An. gambiae* [257]. The genome information can be used to perform microarrays (*i.e.*, comparing the relative abundance of P450 mRNA in resistant and susceptible strains). Using this approach, Daborn, et al. [273] determined that *Cyp6g1* conferred DDT resistance found in *D. melanogaster* worldwide [273]. Studies have shown that CYP6G1 expressed in *Nicotiana tabacum* cell cultures are capable of metabolizing DDT into DDD as well as metabolizing imidacloprid into 4-hydroxyimidacloprid [274]. This conclusion has been challenged by subsequent studies, which have shown that Cyp6g1 mRNA levels are low in some resistant strains [275–277] and therefore, *Cyp6g1* alone could not explain the DDT resistance in all of the tested strains. To confirm that *Cyp6g1* does not need to be over-expressed for a strain to be resistant, Kuruganti et al. [278] synthesized recombination lines in which a susceptible *Cyp6g1* allele was placed in a resistant strain. The expression level of *Cyp6g1* in the recombined resistant strain was low, but the resistance remained high [278].

In an array of 19,680 ESTs, three P450s were overexpressed in resistant *An. gambiae*: CYP314A1 (1.4-fold), CYP325A3 (1.7-fold), and CYP6Z1 (1.3-fold) [279–281]. There were no accompanying reports of the resistance levels or mechanisms involved which makes it difficult to reconcile the involvement of these P450s in resistance. A microarray analysis of a deltamethrin resistant strain of *Cx. p. pallens* determined that five P450s were expressed 3-8-fold higher than the susceptible. Results from a Northern blot were similar to the microarray analysis, indicating that members of CYP4 may be associated with resistance [282]. The microarray approach can give a complete examination of the molecular biology associated with insecticide resistance, either by studying the expression of known detoxifying genes or to aid in the search for candidate genes. One consistent conclusion is that over-expression of P450 enzymes is not due to gene amplification.

Induction and inhibition

Induction Induction of P450s by chemicals can alter an organism's ability to metabolize insecticides. Induction is often seen in Lepidoptera that must respond to plant toxins. The plant defense signal molecules jasmonate and salicylate induced *CYP6B8*, *CYP6B9*, *CYP6B27*, and *CYP6B28* in the midguts and fat bodies of *H. zea* [283].

In mosquitoes, induction of detoxification enzymes has been primarily investigated in *Aedes* species. When *Ae. albopictus* larvae were pre-exposed to leachate compounds of automobile tires, survival after exposure to the insecticides carbaryl, rotenone and temephos increased [284]. When the leachates were mixed with piperonyl butoxide, (PBO, a P450 inhibitor), mortality increased [284]. *Ae. aegypti* larvae exposed to copper, fluoranthene, and permethrin exhibited P450 activity levels that were significantly higher

than the control. Overall, induction did not occur when larvae were exposed to atrazine or temephos [285]. Conversely, mortality did decrease in *Ae. aegypti* when treated with atrazine and then subsequently exposed to *Bti*, indicating in this case that atrazine is an inducer [286].

Inhibition Inhibitors of P450s are valuable tools as they can be mixed into formulations to increase the control success of the insecticide. Insecticide synergists are inhibitors that are widely used in commercial products. PBO, along with other related benzodioxole compounds, involve an initial metabolic activation by the P450 that irreversibly leads to the formation of a carbene-iron complex. Some P450s have low affinities for binding to benzodioxole or do not readily metabolize to the inhibited form. Therefore, synergists are not universal inhibitors [244], although PBO remains a highly useful tool for detecting P450-mediated metabolism.

Mosquitoes Increased insecticide detoxification can result from elevated expression of one or more P450s or possibly from mutations that change the P450 protein. Table 1.6 is a list of experimentally derived P450s over-expressed in different mosquito species.

Cytochrome P450 monooxygenases were found to be a major mechanism of pyrethroid resistance in a strain, JPal-per (also commonly referred to as JPAL), of *Cx. p. quinquefasciatus* [147] collected from Saudi Arabia [295]. This strain was selected in the laboratory for 20 generations until it had 2,500-fold resistance as compared to the susceptible strain. This resistance decreased to 43-fold when the animals were treated with PBO. JPAL had approximately 2.5-fold higher levels of total P450s and b_5 relative to the susceptible strain [147]. Metabolism of the P450 mechanism of JPAL was

Table 1.6: Over-expressed cytochrome P450 monooxygenases in pyrethroid resistant mosquito populations.

Mosquito species	P450 (CYP)	Reference
Culex pipiens pallens	6F1, 4H21, 4H22v1, 4H23v2, 4J4v2, 4J6v1, and 4J6v2	[282,287]
Culex quinquefasciatus	6F1, 9K1, 9F2	[288,289]
Anopheles funestus	6P9	[290]
Anopheles gambiae	6Z1, 314A1, 325A3, 6Z2, 6M2	[279–281,291]
Anopheles minimus	6AA2, 6AA3, 6P7, 325C1, 325A3	[292–294]

studied using gut microsomes from 4th instar larvae with and without NADPH. Microsomes isolated from JPAL were able to metabolize 62% of [^{14}C] *trans*-permethrin versus the susceptible strain, which metabolized 12%. The major metabolite was 4'-OH-permethrin, followed by PB alcohol, PB acid and two unknowns. When JPAL gut microsomal enzymes were inhibited with either PBO or PTPE, the amount of permethrin metabolized was less than 10% [147].

In order to determine the P450 isoform(s) responsible for pyrethroid resistance in JPAL, degenerate primers based on the conserved heme binding region and RT-PCR methods were employed. In total, 11 novel P450s from families 4 and 6 were isolated. Two new P450s, *CYP6E1* and *CYP6F1* were promising candidates. *CYP6E1* was not implicated in the role of conferring resistance since its expression levels were not statistically different from the susceptible strain [271,296]. *CYP6F1* was expressed more strongly in the JPAL strain and contains important P450 conserved domains such as a putative

membrane-anchoring signal, putative reductase binding sites, and a heme-binding site [288]. Phylogenetic analysis shows that CYP6F1 is closely related to CYP6D1, though there is no direct evidence to show that this P450 in mosquitoes is the one that imparts permethrin resistance [288].

Esterases/hydrolases

Esterases are within subgroup 1 of hydrolases which are primarily involved in the detoxification of OPs and minorly involved in pyrethroid resistance [297]. These enzymes hydrolyze carboxylester groups and phosphate ester bonds through the addition of water (Figure 1.13). Resistance via carboxylesterases may arise because a pest population expresses a mutant

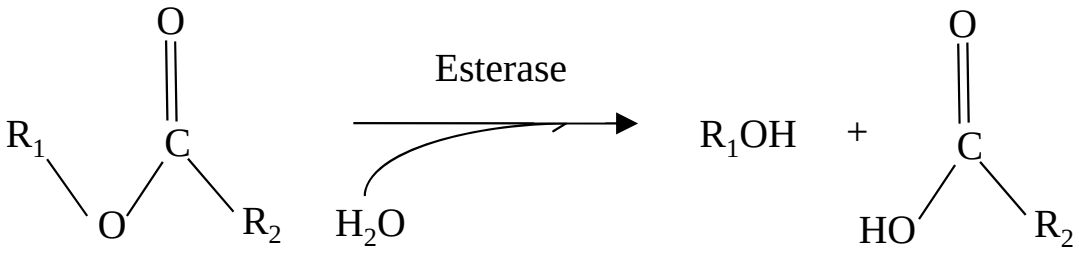


Figure 1.13: Hydrolysis mechanism of esterases (modified from [298]).

enzyme [298] or because it possesses and expresses multiple copies of a gene for carboxylesterase (gene amplification) [298]. Early work with a malathion resistant strain of house fly lead to the theory of "mutant aliesterase" in which a single mutation (designated *a*) in the structural gene for a non-specific esterase produced an altered enzyme that cleaved malathion rapidly [299]. This theory was confirmed with results obtained in the *Lucilia cuprina* system in which *Lcα-E7* gene which encodes the E3 esterase in resistant flies contained a single point mutation. This mutation resulted in a gain of activity towards

OP compounds and abolished aliesterase activity [300]. Esterase gene amplification, which results in the overall increase in carboylesterases, has been clearly demonstrated in the green peach aphid, *Myzus persicae* and *Culex* mosquitoes.

In *M. persicae*, esterase proteins act to sequester the insecticidal compounds (rather than detoxify them) and these esterases may account for over 3% of an insect's total body weight [301,302]. High levels of resistance have been correlated with amplified *E4*, while low resistance has been associated with the amplification of *FE4*. Resistance from *E4* is from 20-80 gene copies at one locus and *FE4* conferred resistance results from a smaller number of genes more widely distributed in the genome [303].

In *Cx. pipiens* mosquitoes, OP resistance is commonly conferred by the over-expression of *estα2* and *estβ2* genes which account for 0.4% of the total soluble protein in 4th instar larvae [304]. These two esterase genes are co-amplified on the same amplicon with 80-fold more copies in resistant strains than susceptible strains. Overall, in 100 ng of gDNA, the copy number of *estα*2 in the susceptible and resistant strain was 8.0×10^3 and 1.09×10^6, respectively. The copy number of *estβ*2 in the two strains was 1.9×10^4 and 1.03×10^6, respectively [304]. Despite the co-amplification of esterase A and B, they are differentially regulated, resulting in three times more of *estβ*2 produced than *estα*2 [305].

In an OP resistant strain of *Cx. p. quinquefasciatus*, esterase B1 (*estβ1*) is over-expressed 250-fold as compared to a susceptible strain. In general, the over-expression of esterase B1 is localized in the gut, malpighian tubules and ganglia [306]. In this strain, a novel esterase gene, *estα3* was found to be co-amplified with *estβ1*. This implies that either this strain contains two

different *estβ1* amplicons or another strain that does not have *estα3*, secondarily lost it [307]. In this strain the "-oxon" forms of OPs are highly efficient inhibitors, whereas chlorpyrifos, parathion, parathion-methyl, malathion, aldicarb and aldicarb sulfoxide were poor inhibitors [308]. The *Cx. tarsalis* homologue to *estβ2* and *estβ1* is *estβ3*. In contrast to *estβ2* and *estβ1*, *estβ3* is a single copy gene in all of the resistant strains and is not over-expressed. This esterase is therefore probably not involved in OP resistance [309].

At the esterase A locus A1, A2 and A4 are alleles, and at the esterase B locus B1, B2 and B4 are alleles. A1, A4-B4 and A2-B2 functionally behave as alleles of the tightly linked esterase A and B supergene [313]. Esterase A1 was first described in southern France and subsequently spread to the entire Mediterranean area [314,315]. Esterase B1 was first described in California and is now found in North America, South America and China [316]. *Estβ2* and *estα2* were co-amplified once and spread worldwide through migration [310,317]. In China, additional esterase alleles, B6 from Foshan [318], B7 from Chingdu [318] and A8-B8 from Guangzhou [319], have been found. In Vietnam, no additional esterases have been found and only A2-B2 has been collected in resistant populations [320]. A comprehensive geographic distribution listing of *Ester* alleles is in Table 1.7. Recently it has been determined that an inverse correlation exists between esterase activity and filiarial burden in *Cx. p. quinquefasciatus* [321]. This indicates that high amounts of esterase negatively affect the development and survival of *W. bancrofti*, which could affect transmission of this disease [322] in endemic areas.

In *Culex* species, resistance conferred by overproduced esterases is due to sequestering [308,323], which is the rapid binding and slow turnover of insecticide. In *Anopheles*, esterase-mediated resistance is due to changes in

Table 1.7: Geographic distribution of *Ester* alleles in *Culex pipiens* (modified from [310–312]).

Allele	Amplified gene(s)	Enzyme(s)	Current distribution			
			Americas	Western Eurasia	Eastern Eurasia	Africa
Ester^{1}	*Est-2* and *Est-3*	A1		France, Spain, Italy	Israel	
Ester^{2}	*Est-2* and *Est-3*	A2-B2	Most countries	France, Spain, Italy	Most countries	Most countries
Ester^{4}	*Est-2* and *Est-3*	A4-B4		France, Spain, Italy		North Africa
Ester^{5}	*Est-2* and *Est-3*	A5-B5		Italy	Mediterranean	Tunisia
Ester^{8}	*Est-2* and *Est-3*	A8-B8			China	
Ester^{9}	*Est-2* and *Est-3*	A9-B9			China	
Ester^{11}	*Est-2* and *Est-3*	A11-B11			China	
Ester^{B1}	*Est-2*	B1	Most countries		China, Japan	
Ester^{B6}	*Est-2*	B6			China	
Ester^{B7}	*Est-2*	B7			China	
Ester^{B10}	*Est-2*	A10			China	

substrate specificity and rapid hydrolysis [305,324].

Conjugation enzymes

Glutathione S-transferases GSTs are a family of detoxifying enzymes which confer resistance to all the major classes of insecticides [326] by catalyzing the conjugation of electrophilic compounds with the thiol group of reduced glutathione or by their reductive dehydrochlorination [325]. There are two types of GSTs: microsomal and cytosolic. The microsomal GSTs have not been linked to resistance [325]. Two classes of insect cytosolic GSTs were isolated and purified from *M. domestica* and designated as GST1 (composed of isoforms with isoelectric points from 4 to 9) and GST2 (all isoforms have acidic isoelectric points) [327]. Class I (or delta [328]) insect GSTs consist of a large complex gene family in which heterogeneity is increased due to alternative splicing in *An. gambiae* [329] and fusion genes present in *M. domestica* [330]. These genes are tightly clustered in *D. melanogaster* [331] and *An. gambiae* [329], but scattered throughout the *M. domestica* genome [332]. GST2 (or sigma [328]) consists of only one gene in the three species mentioned above [333,334]. An additional class (class III or Epsilon) has been added to the insect GSTs based on work using *An. gambiae* [335]. Table 1.8 gives the classifications of the currently known insect GST classes.

GST activity is associated with an increase in GST1 mRNA and protein levels in an OP and carbamate resistant strain of *M. domestica* [336] possibly due to over-transcription [327]. The first association of GST over-expression to resistance was in the diamondback moth, *P. xylostella*, in which the increased expression of *PxGST3* was responsible for organophosphate insecticide resistance [337]. DDTase has been identified as a resistance mechanism in many insects including *D. melanogaster* [338], *An. gambiae* [335,339], and *Ae.*

Table 1.8: Glutathione S-transferase classes currently known in insects (modified from [325]).

GST class	Organism	# putative transcripts in *An. gambiae*	# putative transcripts in *D. melanogaster*
Delta (Class I)	Insects	15	11
Epsilon (Class III)	Insects	8	14
Omega	Insects, mammals, nematodes	1	5
Theta	Insects, mammals, plants	2	4
Sigma (Class II)	Helminths, insects, mammals, mollusks, nematodes	1	1
Zeta	Insects, mammals, nematodes, plants	1	2

aegypti [340] where it has been shown to be a GST [341].

1.6.4 Molecular genetics

Increased transcription

Molecular mechanisms of metabolic detoxification include increased transcription of the detoxifying enzyme. In house flies, high levels of pyrethroid resistance in the LPR strain are due to the P450, CYP6D1 [260]. This P450 is controlled by factors on autosome 1 and 2 [342] which result in 8-fold higher expression levels of mRNA and protein in the resistant strain versus the susceptible strain [343]. Southern blot analysis indicated this difference was not due to gene amplification [344] and inhibition of transcription using actinomycin-D indicated over-expression was not due to mRNA stability [264]. Nuclear run-on assays indicated that over-expression of *CYP6D1* in LPR was due to an increase in the rate of transcription [264]. This mechanism of resistance is present in field caught house flies from New York and Georgia as well [345]. Repression of CYP6D1 by a *Gfi-1*-like protein is reduced in resistant animals because a 15 bp insertion does not allow for proper binding to the 5' regulatory sequence [346].

DDT resistance (DDT-R) in *D. melanogaster* is approaching global fixation for the P450 that confers resistance [347]. DDT resistance in all currently researched strains is due to the over-expression (10-100 times as much mRNA in the resistant strains) of *Cyp6g1* [348]. Resistance was produced in transgenic flies that had *Cyp6g1* inserted via *P*-element insertion. The results lead to the conclusion that over-transcription of *Cyp6g1* alone was "sufficient and necessary" for DDT resistance [273], which was later disputed [277,349]. Interestingly, this gene contains an *Accord* transposable element in the 5' region [273] that contains a long terminal repeat [350] which has not been

directly shown to produce the over-transcription of *Cyp6g1*. It is therefore unknown whether the *Accord* element was swept through the population by insecticide selection or if it is a beneficial pleiotropic mutation. There is no strong evidence to support that the *Accord* element was swept through *D. melanogaster* via insecticide selection. Conversely, the evidence that a beneficial mutation was the driver is the observation that heterozygosity is lacking at closely linked sites [349]. The frequency of the *Accord* insertion in non-African populations is 85-100%, while the frequency in African populations is 32-55% [347]. Since DDT was used worldwide, especially in malaria endemic parts of Africa, the insertion should be present at high frequencies in both populations. This leads to the conclusion that the insertion might have originally been driven through the populations by some other factor besides insecticide resistance and selection.

Gene amplification

Gene amplification is a major mechanism of resistance to OPs in aphids and *Culex* mosquitoes. Gene amplification is the expression of multiple copies of a gene, such as carboxylesterase [298]. In *M. persicae*, the esterases sequester rather than direct detoxify the insecticide since the proteins account for approximately 3% of the insect's total body weight [301]. In *Culex* mosquitoes, esterase A and B are co-amplified as a supergene with more than 80 more copies in resistant animals as compared to susceptible animals [304].

Premature stop codon

Cadherin genes are the primary source for "mode 1" resistance to *Bt* found in Lepidoptera. Mode 1 resistance is high resistance (over 500-fold) to at least one Cry1A toxin, recessive inheritance, reduced binding of at least one

Cry1A toxin, and little cross-resistance to Cry1C [351]. Therefore, modifications of the major target sites (such as cadherin) decrease binding of Cry1A toxins in homozygous resistant individuals, but do not effect the sustainability of heterozygotes. The *BtR* gene in a resistant strain of pink bollworm, *Pectinophora gossypiella*, has three alleles (*r1*, *r2* and *r3*), each with a unique and large deletion. Allele *r1* has a 24 bp deletion resulting in two amino acid substitutions (G1136R and T1137R), *r2* has a 202 bp deletion resulting in a frameshift mutation and premature stop codon, and *r3* has a 126 bp deletion. Individuals with two copies of any of the *r* alleles were resistant, while those with one copy or none were susceptible [51]. Resistance via premature stop codon in cadherin has also been found in laboratory selected *H. virescens* [352], and *H. armigera* [353], but not in field sampled *P. xylostella* [354].

Aminopeptidase N gene silencing

Aminopeptidase N (APN) has been implicated as a putative Cry1 toxin receptor protein in Lepidopteran insects. In *Bombyx mori* the binding site of Cry1Aa is located in the region between 135-Ile and 198-Pro, which is an amino acid structure that does not contain a sugar chain. This high binding affinity was not seen with Cry1Ac. In *Manduca sexta*, it has been reported that the Cry1Ac toxin has different affinities to the two APN-binding sites and shares only one of those sites with Cry1Aa [355]. In laboratory experiments on *Bt* resistant *Spodoptera litura*, a mutation in the regulatory region of the APN gene (*slapn*) may silence the gene so that protein production is inhibited. By using RNAi, the reduction in *slapn* transcript levels correlated very well with a reduction in the expression of APN and decreased the sensitivity of larvae to Cry1C proteins [356].

1.6.5 Behavioral

Along with biochemical alterations and mutations increasing in frequency to allow populations to survive in an environment treated with insecticide, alterations in behavior have also resulted. While the classification of these alterations as a mechanism of resistance is still controversial, there have been observations of insects changing their behaviors to decrease exposure to lethal compounds. "Behavioristic avoidance/resistance" occurs in a stimulus-dependent and stimulus-independent fashion.

Stimulus-dependent behavioristic resistance selects for increased irritability and increased repellency. Evidence for increased irritability resulted from a laboratory experiment in which *An. atroparvus* were selected for 10 generations for hypo- and hyperirritability to DDT-treated surfaces [357]. There is a negative correlation between behavioral resistance and biochemical-physiological resistance, such that a sensitive mosquito will escape and survive exposure to DDT or pyrethroid compound and increase the frequency of biochemical susceptibility. If the biochemically resistant individuals are also behaviorally resistant, then the frequency of resistant animals will increase and eventually predominate [155]. Increased repellency is also selected such that survival is conferred to individuals which can detect repellent components of the insecticide [155,358]. Mosquitoes can exhibit "excito-repellent" effects when they make physical contact with DDT or pyrethroids, but pyrethroids are classified as an irritant while DDT is considered a repellent to mosquitoes [359]. Stimulus-independent behavioristic resistance can select for increase exophily in mosquitoes [155]. In southern Rhodesia, BHC was used for control of *An. gambiae*. Since no behavioristic avoidance was observed to be associated with BHC in that region, conclusions

were that irritability was not a factor in the increased exophily [360].

1.6.6 Interactions between resistance mechanisms

In an insecticide treated environment, insect pests can evolve one or more resistance mechanisms. Multigenic resistance is frequently observed. Epistasis is the non-additive interaction between independent loci which contribute to a phenotype [361–363]. Independent loci conferring insecticide resistance can exhibit joint action in a less than additive manner (*i.e.*, antagonistic epistasis), an additive manner, or in a greater than additive manner (*i.e.*, synergistic epistasis) [364]. Two null hypotheses can be used to determine deviations from additivity by plotting the total phenotypic value associated with the genotype combinations of the two independent loci. Along the x-axis are the three possible genotypes of locus A and lines are constructed for each of the genotypes of locus B. The first null hypothesis examines genotypes versus phenotype values on a linear scale, where non-parallel lines indicate departures from additivity. The second examines genotypes versus logarithmically-transformed phenotype values, where parallel lines indicate a multiplicative type interaction [365, 366].

According to Raymond et al. [367], heterozygotes in conditions where the environmental concentration of the insecticide is constant and the detoxification mechanism is saturated will give an additive interaction relationship described by, $RR_{3\&4} = RR_3 + RR_4 - 1$, where RR are the resistance ratios. Multiplicative interactions, described by $RR_{3\&4} = RR_3 \times RR_4$, occur when there is a constant concentration of the insecticide in the environment, but the detoxification mechanism is not fully saturated [367].

Synergistic epistasis (linear analysis) or multiplicative (logrithmic analysis) interaction was observed in the dieldrin-resistant (>500-fold) house

fly strain SKA [368]. The degree of resistance conferred by each linkage group was minor compared to the overall resistance. The resistance for linkage groups 2, 3, and 5 were 2-fold, 14-fold, and 14-fold, respectively. When the linkage factors are analyzed in an additive manner, the total resistance calculates to 30-fold versus 392-fold when analyzed in a multiplicative manner. Either multiplicative interactions occur between all three mechanisms or between two major mechanisms with the minor factor acting as a modifier [368].

In the highly pyrethroid-resistant LPR house flies, Liu and Scott [369] determined that a multiplicative interaction existed between the mechanisms located on autosome 1 and autosome 2 only when both factors were in the homozygous state. Very little (1 to 2-fold) resistance was conferred in flies that were either heterozygous for both autosomal factors, and when each factor was homozygous but existed alone [369]. In fact, in LPR it was determined that in the heterozygous state, antagonistic epistasis (linear analysis) was observed in individuals with resistance factors in three chromosomal combinations, 2+3, 1+3+5, and 2+5 [364].

Antagonistic epistasis (linear analysis) was also observed in a strain of house flies from Japan [370]. The resistant flies showed moderate resistance to *d*-tetramethrin (RR = 62), but a low level of resistance to permethrin (RR = 13). Despite this low level, the linkage analysis was done with respect to permethrin resistance. They found that some interactions occurred between the 2^{nd}, 3^{rd} and 5^{th} chromosomes. While each linkage group independently had a significant resistance factor (18, 27, and 13, respectively), all observed chromosomal combinations resulted in less-than-additive levels of resistance [370].

1.6.7 Genetics and evolution of resistance

Commonly, when a new insecticide compound is introduced that has a novel mode of action, it is effective for the first few years of use. Development of resistance can be rapid and may occur even more rapidly when the gene(s) has been selected from previous insecticide exposure. The speed in which a pest population can become resistant has led to the "preadaptation hypothesis." Under this hypothesis, insecticides do not induce mutations or increase the mutation rate, rather the genetic variant (mutation) conferring resistance is present in the pest population at very low frequencies [126] and is selected for in insecticide-treated environments. However, this mutation (or polymorphism) need not always be rare.

Organophosphate resistance in blowflies, *L. cuprina*, is due to mutations of *αE7* (encoding the E3 enzyme). One mutation called diazinon resistance is due to a G137D mutation and is at a frequency of 90% in Australia and New Zealand, while the minor mutation (5-10%) called malathion resistance is because of a W251L mutation. Interestingly, the mutation at position 251 was found in extant flies collected post-OP use and in pinned specimens collected before the use of OPs. The major mutation, diazinon resistance, was found only in extant insects collected post-OP use and was confined to the Australasia region. These two mutations also occur in the sibling species *L. sericata*, suggesting convergent evolution occurred on *αE7* in both species. Therefore, there is evidence that a preadaptive condition was present with respect to malathion resistance, but that a mutation which postdates the introduction of insecticide pressure exists for diazinon resistance in the blowfly. Hence, the W251L mutation may have originally provided some resistance to diazinon but now the more effective G137D mutation is

predominating [371]. Many factors such as pest genetics, pest distribution, and insecticide application frequency can influence the rate of resistance evolution.

A dominant pattern of inheritance will allow for one individual to be resistant if it possesses an effective mutation. Within as little as two generations resistant homozygotes can be selected because they will be able to survive while heterozygotes and susceptible homozygotes will decrease in the population. Recessive inheritance slows the evolution of resistance because it takes many generations for resistant homozygotes to be selected since the F_1s will have lower levels of resistance [155].

Fitness can also affect the evolutionary rate of resistance and also the persistence of the resistance phenotype in untreated environments. In cases where there is a high fitness cost in the absence of insecticide selective pressure, the resistance gene(s) will quickly decrease in frequency in the population. In some cases the resistance gene(s) are not costly and consequently do not decrease in frequency. Since resistance can remain in untreated areas, it could compromise some resistance management practices, such as refugia in agricultural crop systems [155].

1.7 Fitness effects of resistance

Environmental persistence and selection pressure intensity of the insecticide, dominance, inheritance and loci number (monogenic versus polygenic) of resistance gene(s), effective population size, dispersal potential and geographic range of the pest, initial frequencies of the resistance allele along with fitness costs or benefits associated with resistance are a few of the key factors that can determine the evolutionary trajectory of resistance in the field [372]. Resistance allele frequencies have been estimated to range from 10^{-2} [371,373] to 10^{-13} [310] based on the mutation-selection equilibrium

theory [372].

Early predictions on the speed at which resistance would evolve in a population were based on observations that mutations conferring resistance were rare and often occurred in a single individual [126,374]. Resistant individuals might suffer a fitness cost in the absence of insecticide selective pressures leading to a declining frequency in a population and possibly resulting in a regression to susceptibility. The rate of regression is dependant on whether a fitness trait was selected and swept through the population along with insecticide resistance. If this did not occur, reversion would be fast since there would be a dramatic reduction in fitness to the resistant individuals [374]. Knowing the fitness of a pest species in a treated and untreated environment is an important component for successful and sustainable integrative resistance management and for maintaining insecticide effectiveness [375].

Identification of the fitness parameter, such as overwintering ability, reproductive output, or predator avoidance, that is affected by the resistance allele is very difficult and requires careful experimentation and observations. Failure to detect a cost can be due to the fact that there is no cost, the correlated antagonistic pleiotropy (*i.e.*, multiple phenotypes associated with a single gene) is undetectable, the physiological cost might only manifest in specific environments that are not present in the experimental set-up [376], or the cost may affect characters that are not examined [377]. Limits of fitness studies include the need to make comparisons between related strains and to ensure that experiments are not conducted in optimal conditions. Some strains, especially susceptible strains which are usually maintained in laboratory conditions for decades [378], are well adapted to the laboratory as compared to field strains analyzed in the laboratory. If one strain, especially for unrelated

strains, is more adapted to laboratory conditions, the fitness study will be biased. Only experiments that do not suffer from these flaws will be discussed here.

Two approaches can be used to attain strains with related genetic backgrounds for fitness studies. The first way is to isolate homozygotes (both resistant or susceptible) from a heterogeneous field population. This family selection method is simpler for obtaining a resistant strain, but can be difficult for isolating susceptible strains. The second way moves major resistance genes into a susceptible genetic background through repeated backcrossing and selection, resulting in isogenic strains. This method is preferred as it also tests if co-adaptation of resistance and fitness modification factors exist. Theoretically, the frequency of modifiers would reduce with each backcross and the fitness of the resistance gene would correspondingly decrease [378].

Fitness studies can measure the cost of either a resistance allele or a different gene that is tightly linked to the resistance gene (pleiotropy). As described above, during the construction of related strains, the genome of the resistant strain is diluted into the genetic background of the susceptible strain. The resistance phenotype is maintained in the new strain by selecting with insecticide for the resistance allele(s). Despite the high relatedness of the strains upon completion of the backcrosses, there is still potential for non-resistant alleles to be carried through the dilutions. If the cost/benefit is associated with the resistance allele alone, then it would be a universal cost/benefit. This means that the resistance gene would impart the same cost/benefit to all organisms that possess it. If a tightly linked gene is what is imparting the cost/benefit (*i.e.*, modifiers in the case of a fitness benefit), then there will be a variable cost amongst organisms depending on which genes are linked [379].

Fitness studies attempt to determine how a resistance allele will evolve in a natural ecological setting. An important component in the experimental design, especially in laboratory studies, is to assure that the conditions attempt to reflect field conditions [380]. While this is often difficult, the laboratory setting allows for more precise determinations of fitness components by excluding extraneous variables. In this way, a laboratory study can be conducted on related laboratory strains to evaluate where the biological cost exists, and then this knowledge can be applied to analysis of a field strain in the laboratory or in a field study.

Generally fitness experiments have focused on differences between individuals of the R/R and S/S genotypes [372]. While these distinctions are very important, heterozygotes must also be observed since they will be common in the early generations of a field population recently under selection [195,372]. Fitness studies in natural settings or laboratory simulated field settings are often done by observing changes in allele frequencies in non-treated versus insecticide treated populations, called the "population cage" [372] method. Another method is to compare physiological parameters such as female fecundity, developmental time, longevity, and mating competitiveness [378]. Sometimes costs may only be observed if alleles are in competition [381], when the pest is subjected to stressful conditions, such as high densities or poor food sources [382], or when frequencies are measured over time [383,384] and/or distance [383,385] relative to insecticide treatment.

1.7.1 Fitness costs

Less successful overwintering

Cyclodiene resistant blowflies possessing the *Rdl* mutation, which alters GABA-gated chloride channels, were less successful at overwintering than

their susceptible counterparts. This was determined by first observing the frequency of *Rdl* in the fall versus the spring. Caged experiments were then conducted in which resistant and susceptible wandering larvae were placed in the ground during the last part of summer during which time this species usually overwinters. Resistant individuals had only a 2.8-6.0% chance of reaching the adult stage, while susceptible individuals had 78.0-96.0% chance. There was an inverse relationship between the frequency of *Rdl* and amount of time spent in the ground in the overwintering stage [386].

Overwintering costs have also been measured in *Cx. pipiens* resistant to OPs. These mosquitoes possess two loci responsible for resistance: *Ace.1* (acetylcholinesterase) and *Ester* (esterases A and B). *Ace.1* locus has three alleles: $Ace.1^{R}$ (insensitive AChE), $Ace.1^{S}$ (sensitive AChE), $Ace.1^{R/S}$ (a duplication containing both sensitive and insensitive AChE). *Ester* is a "super locus" containing both esterases A and B, because they are closely linked. *Ester* has three resistance alleles: $Ester^{1}$ (increased esterase A1 expression), $Ester^{2}$ (increased expression of A2-B2) and $Ester^{4}$ (increased expression of A4-B4). During the first 20 days of sampling, susceptible mosquitoes at both loci entered overwintering caves before any resistant individuals. There was a significant decrease in *Ester* resistance phenotypes between collections occurring from mid October until early December (0.72) and a second collection (0.59) which occurred from early December until early March. It is possible that this difference was due to a survival cost during the first period or due to a migration cost [387]. The migration cost could have operated while resistant mosquitoes with *Ester* were trying to find other overwintering caves. Since these two possibilities were unable to be directly measured, a "generic designation of survival cost" was given and estimated to be 0.42. For *Ace.1*

there was a survival cost in overwintering sites that was calculated at approximately 7% and 2.5% per day for resistant homozygotes and heterozygotes, respectively [387].

Reduced reproductive output

One of the primary fitness parameters measured is reproductive potential, which encompasses female fecundity (number of eggs per female) and male competitiveness (access to a female and paternal success) [388]. Berticat et al. [389] used strains sharing the same susceptible genetic background to assess how each of the resistance alleles (*Ester*^{1}, *Ester*^{4}, *Ace.1*^{R}) individually influenced male paternity success in competitive environments (*i.e.*, two males for every female). In all possible mating combinations, susceptible males had a mating advantage when competing against any of the resistant males. In competitions among the resistant males, there was no difference in paternity success. Female genotype did not affect male paternity, except when the female was *Ester*^{1}. In this mating, *Ace.1*^{R} appeared more costly than the *Ester* genotypes [389].

Increased susceptibility to predation or parasitism

Resistance to DDT and pyrethroids of *M. persicae*, conferred by *kdr* is strongly associated with a reduced responsiveness to alarm pheromone. The mean proportional responses ±S.E. of each *kdr* genotype were: S/S=0.58±0.01, S/R=0.18±0.01 and R/R=0.11±0.01. Resistant homozygotes and heterozygotes were significantly less likely to respond to increasing amounts of alarm pheromone, increasing the risk of predation and parasitism [390].

Experiments were conducted to determine if a predation cost exists for larval or adult OP resistant *Cx. pipiens*. All resistance genes (*Ace.1* and *Ester*)

increased the chance of predation at either the larval or adult stage, or both. For the *Ace.1* locus, the predation cost was restricted to the second instar larval stage. *Ace.1* is most highly expressed in first instar larvae with the lowest expression level in adults [391]. The *Ester*^{1} allele induced a cost only in the adult stage, and *Ester*^{4} induced a cost both in the larval and adult stages [392]. Esterase activity in a strain of *Cx. p. quinquefasciatus* to malathion increased with mosquito age with the highest levels in the pupal and adult female stages [393].

Parasitism has the potential to influence the biology of individuals in the field and in the case of mosquitoes it can influence the epidemiology of diseases. Agnew et al. [394] determined that parasitism by *Vavraia culicis*, an obligate microsporidian found in natural mosquito populations, was costly to *Cx. p. quinquefasciatus* fitness. Mosquitoes with the *Ester*^{1} allele had increased cost when parasitized. Approximately 40% of *Ester*^{1} larvae died within 24 hrs of parasitism versus less than 10% mortality for the other resistance alleles. In all infected mosquito strains, emergence was earlier than for uninfected mosquitoes due to mortality of the more slowly developing infected individuals. Infection with *V. culicis* significantly reduced the longevity of the susceptible genotype, but increased the relative fitness of this parameter in mosquitoes with *Ester*^{4} and *Ace.1*^{R} [394].

Infection by *Wolbachia* causes manipulations in the reproduction of the insect host. One of the most frequent manipulations is cytoplasmic incompatibility, which leads to the death of early embryos in crosses of infected males with uninfected females. Therefore, in heterogeneous populations, uninfected females are at a reproductive disadvantage. The relationship of OP resistance in *Cx. pipiens* and *Wolbachia* was determined by

examining many life-history parameters. *Wolbachia* infection resulted in an increase in preimaginal mortality in strains with $Ester^1$ and $Ester^4$, but not in the susceptible or $Ace.1^R$ strains. Infected resistant individuals were more likely to be preyed upon than infected susceptible individuals. There was no detectable difference in mating competition between any of the strains and for infected versus uninfected individuals [395].

In natural populations

While studies in the laboratory have been conducted in order to determine the fitness of resistant populations in the absence of insecticides, few studies have tried to correlate fitness costs directly in natural populations. Bourguet et al. [380] conducted experiments on OP resistant *Cx. pipiens* from an insecticide treated area in France and a field site outside the treated area. They determined that resistance alleles were present in both breeding sites. In the insecticide treated area, overall frequencies of phenotype 1 ($Ester^0Ester^1$ and $Ester^1Ester^1$), phenotype 4 ($Ester^0Ester^4$ and $Ester^4Ester^4$) and phenotype 14 ($Ester^1Ester^4$) were 17.5%, 56.6%, and 5.4%, respectively. Frequencies of *Ace.1* at the treated field site were: $Ace.1^{R/S}$=52.5% and $Ace.1^{R/R}$=38.6%. In the untreated site, overall frequencies of phenotype 1, phenotype 4 and phenotype 14 were 8.9%, 30.2%, and 0%, respectively. Frequencies of $Ace.1^{R/S}$ and $Ace.1^{R/R}$ were 15.5% and 1%, respectively. Individuals that had either *Ester* or *Ace.1* took significantly longer to develop, and had significantly smaller wing lengths [380]. The smaller wing length can be directly correlated to a decrease in fecundity [388]. Comparisons between the two resistance alleles revealed that the overall cost of *Ace.1* is larger than that of *Ester* [380].

In the model organism *D. melanogaster*, fitness components related to OP resistance were measured for isofemale lines derived from one field

population. In this system, resistance to three OPs (malathion, prothiophos and fenitrothion) were negatively correlated to productivity (*i.e.*, number of offspring produced by one pair of an adult male and female in each day). The negative Pearson correlation coefficient, a statistical measure of the direction and strength of the linear relationship between two variables, related to productivity was traced to chromosome 3. With related chromosome substituted lines, *D. melanogaster* gave a significant positive effect on resistance to all of the tested OPs. As such, it can be inferred that some factor on chromosome 3 within natural populations of *D. melanogaster* positively correlated to OP resistance and negatively correlated to productivity [396].

Population genetics

Fitness parameters and the affect of resistance on an individual are important factors in determining the population genetics and dynamics of resistant insect populations. Data obtained from laboratory studies and field sampling can aid control management programs and epidemiologists, in the case of vector pests. Some informative work has been conducted on *Cx. pipiens* mosquitoes from around the world. Population studies conclude that each of the *Ester* alleles originated from one of approximately eight independent mutation events. Each of the alleles has a different degree of worldwide spread, with A1 and A2-B2 having the largest distribution. The wider geographical distribution of these two alleles results either from their age, the higher fitness they potentially provide, or long distance migration of resistant individuals into susceptible areas. There is evidence that migration is common and is the driving force in shaping the evolutionary spread of OP resistance [310,397].

While there is evidence of fitness influencing the evolution of resistance worldwide, there are even clearer examples occurring in local populations of

OP resistant mosquitoes. In southern France, the land along the Mediterranean coast has been controlled with OPs and more recently *Bacillus* spp. A geographical cline exists for each of the resistance alleles with high frequencies close to the water (where insecticides are sprayed) and decreasing frequencies further inland [103]. Spatio-temporal frequency differences for each of the OP resistance alleles also exist. In 1972, A1 was the only allele present and exhibited a steep cline along this transect. Five years later the presence of $Ace.1^R$ was detected, but when A4-B4 appeared in 1986, the A1 cline became more shallow and the maximum frequency dropped to 15%. Unlike A1, A4-B4 did not display a cline, but was homogenous throughout the area with a frequency of 5%. In 10 years time, A4-B4 completely replaced the A1 allele [310,397]. Therefore, the older more costly resistance allele was replaced by a younger less costly allele. For the *Ace.1* loci, this replacement pattern is even more dramatic. In 1993, $Ace.1^{R/S}$ was absent from the population, but by 1995 the frequency was up to 30%. Subsequent analysis of these alleles revealed that the fitness cost of $Ace.1^{R/S}$ is approximately 3-6% lower than that of $Ace.1^R$ [310,397]. These observations show that the population genetics of resistant insects are directly correlated to the insecticide regimen and fitness associated with the resistance mechanisms.

1.7.2 Fitness benefits

Although there is a large body of work which supports that resistance has deleterious fitness effects, there have been some experiments which revealed that resistance can actually enhance fitness resulting in the increase of resistance allele frequencies in the absence of insecticide. A well-studied system in which a fitness advantage is associated with resistance is in *T. castaneum* resistant to malathion. Beeman and Nanis [381] observed that the

resistance allele (R^{mal}) was stable in a laboratory population in the absence of malathion for 6 generations [381]. Subsequently, fitness tests have been conducted on this insect to determine why the resistance allele is maintained in an insecticide-free environment. In the studies that used related resistant and susceptible strains, the increased fitness was due to the increased success of mating competition in male beetles. In competition experiments for females, R^{mal} males had a higher relative fertilization success than the susceptible males [398]. To clarify what factor was producing the higher fertilization success, Arnaud et al. [399] examined the positive relationship in the framework of sperm competition. In this experiment the rate of successful insemination was determined by the presence of a spermatophore in the female's spermatheca. Resistant males (39% of mating attempts resulted successful insemination of females) were comparable with susceptible males (46%) at inseminating females, but were superior in fertilization. This could be the result of resistant males having a larger ejaculate size (*i.e.*, more sperm) and/or larger sperm size [399]. It would be assumed that the R allele would fix in the population since it is beneficial in both the presence and absence of insecticide. This observation is not observed and not commented on, though it could be due to factors associated with rearing or the laboratory environment.

DDT resistance (DDT-R) in *D. melanogaster* is approaching fixation globally after sampling over 600 field strains for the P450 that confers resistance [347]. Resistance in all currently researched strains is due to the over-expression of a single allele of *Cyp6g1* which contains an *Accord* transposable element in the 5′ region [273]. The conclusion that over-transcription of *Cyp6g1* alone was "sufficient and necessary" for DDT resistance [273] was subsequently disputed [277,349]. McCart et al. [400]

examined the fitness of resistant individuals along with heterozygotes. Interestingly, when the resistance allele was inherited via the female (*R/S*) there was an increase in development time in the larval and pupal stages, an increase in adult fecundity and viability of eggs and larvae, as compared to heterozygotes that inherited the resistance allele from the male (*S/R*) [400]. A possible explanation for this observation is that a modifier is female-linked and is associated with increased fitness, though this hypothesis has not been proven. The validity of this conclusion is hard to reconcile due to the controversy surrounding the resistance mechanism.

1.7.3 Neutral fitness

Neutral fitness associated with resistance genes maintains the resistance allele in the population by providing an overall increase in fitness of the resistant animal that counteracts the cost associated with an insecticide-free environment. In the western corn rootworm, *Diabrotica virgifera virgifera*, control consists mainly of cyclodiene insecticides used as soil treatments. Resistance has persisted in field populations where the cyclodiene compounds have not been used for years and in laboratory populations in the absence of selection (5-6 generations). The susceptibilities of the related field and laboratory populations were not different for aldrin or methyl-parathion [401].

There are three possible explanations for how resistance genes can remain steady in a population through time. One is that the resistance factors are homozygous in the population so reversion is impossible since no susceptible alleles exist. The second is that the resistance genes are truly neutral. Finally, the third explanation is that the resistance mutations have combined with compensatory mutations that act as fitness modifiers. These fitness modifiers switch a biologically deleterious feature into one that is

favored [374].

1.7.4 Fitness cost modifiers

While resistance may confer increased or decreased biological fitness to an individual, there is also the potential for a modifier to reduce the deleterious effects of a mechanism. There are four ways that compensation can occur: 1) restoration of structure and function of an altered protein or RNA by intragenic mutations, 2) restoration to a mutated multi-subunit complex (*i.e.*, RNA polymerase) by intergenic mutations, 3) demand for mutated function reduced via alternative pathways, or by 4) increasing the amount of mutated enzyme [402]. While these compensations are common in bacteria, evidence of compensatory mutations (modifiers) in insects is limited (see insect examples below).

The primary example of an insecticide resistance fitness modifier is in the Australian sheep blowfly, *L. cuprina*. In the mid-1970s a field survey was conducted which found that the resistance allele to diazinon was close to fixation in many populations independent of diazinon use. McKenzie et al. [403] determined that when the resistance alleles were introgressed into a wild-type field genetic population the R/R genotype increased over time. When the same backcrossing methods were used to insert the resistance genes into a laboratory susceptible strain, the R/R and R/S genotypes decreased in frequency [372, 403]. From these results it was concluded that modifications of the genetic background decreased the deleterious outcomes associated with the resistance allele. Follow-up experiments determined that the fitness modification factor segregated with a marker on the left arm of chromosome 3 [404]. When the marker was wild type (resistant) at this position, the S/S genotype remained stable through time, whereas the S/S genotype increased

significantly if the marker was present (susceptible) at this position. Single generation fitness estimates were conducted and it was determined that developmental time was significantly reduced in the presence of the modifier and egg hatch was marginally significant. S/S genotypes were not influenced at all by the presence of the fitness modifier region, therefore modifiers are at a selective advantage only in the presence of a resistance allele [404]. This is an example of compensation via altered pathway because the Australian sheep blowfly acquired a mutation in the gene (*Scl*) involved in the determination of cell fate throughout development to compensate for the decreased fitness that results from the G137D substitution in the carboxylesterase (*Rop-1* allele) [402].

An example of compensation resulting from increasing enzyme amounts is in the mosquito *Cx. pipiens* resistant to OPs [402]. In this mosquito species, the resistant mosquitoes (with $Ace.1^{R}$) express a defective acetylcholinesterase (AChE), which causes the malfunction of the cholinergic synapses in the central nervous system. The fitness disadvantage of the mutated AChE was decreased by a duplication of the AChE gene ($Ace.1^{R/S}$). This allows one copy to confer resistance and the other copy to maintain wild-type enzyme activity in the synapse. Thus the susceptible copy is a type of modifier for the cost generated by the resistant copy [310]. It was also found in *Cx. p. quinquefasciatus* which had multigenic resistance, where one resistance allele could compensate for the costs assocated with the other resistance allele present within the same animal [405].

CHAPTER 2

RESEARCH GOALS

2.1 General research objective

The goals of my Ph.D. research were to examine the cytochrome P450-mediated detoxification resistance mechanism in the southern house mosquito, *Culex pipiens quinquefasciatus*. Cytochrome P450-monoxygenase (P450) mediated resistance is a vital biochemical enzymatic system that metabolizes xenobiotics and regulates titers of endogenous compounds (such as hormones). Resistance is a major hurdle in controlling medically important pests and has the potential to directly impact the re-emergence of vector-borne diseases [406]. Much is known about *kdr* and esterase insecticide resistance mechanisms in the mosquito, including molecular tools for determining frequencies of resistance alleles in field populations and fitness effects of individual alleles. Currently, very little is known about the P450-mediated detoxification mechanism of insecticide resistance in mosquitoes. The P450 gene(s) that confers resistance to permethrin is unknown and there is no information about the fitness of individuals which possess the P450 resistance allele.

The specific objectives of this study were to:

1. Determine the inheritance, dominance and cross-resistance patterns to pyrethroids (other than permethrin) and organophosphates in a permethrin-resistant strain containing only the cytochrome P450 detoxification mechanism;
2. Investigate interactions between P450-mediated detoxification and *kdr* mechanisms of permethrin resistance;
3. Determine the fitness effects of P450-mediated detoxification;

4. Characterize a potential candidate P450 gene associated with permethrin resistance

Greater knowledge about this resistance mechanism and its evolutionary trajectory will be imperative for controlling *Cx. p. quinquefasciatus* and possibly other medically important vectors of disease. Results from these experiments will lead to the development of critical tools necessary for monitoring P450-mediated resistance which are currently lacking. I hope that my dissertation research can contribute vital knowledge about P450s and provide practical information for vector control programs.

2.2 Specific objectives

2.2.1 Objective 1

My first objective was to characterize the genetic basis (*i.e.*, mode of inheritance and dominance) of the cytochrome P450 mechanism present in a permethrin-resistant strain of mosquito originally collected in the field (called JPAL, which also contained the *kdr* mechanism of permethrin resistance). In order to determine these genetic components, it was necessary to create a resistant strain that was highly related to a susceptible strain (SLAB) but contained only the P450 detoxification mechanism. This strain (which was named ISOP450) was successfully created through repeatedly crossing male progeny that survived selection with permethrin to SLAB females [389]. The initial cross was started from SLAB females mated to JPAL males.

I then analyzed the cross-resistance pattern of ISOP450 to other pyrethroids and organophosphates that have previously been used in mosquito control programs. Cytochrome P450s generally detoxify pyrethroid compounds but can either detoxify or bioactivate (*i.e.*, create more toxic metabolites) organophosphates. Previous research [407] looked at the

cross-resistance spectrum of JPAL, so I evaluated the specific contribution provided by the P450 detoxification mechanism. The data from ISOP450 provided greater insight into the substrate specificity of the P450(s) present in these two resistant mosquito strains and how *kdr* can alter the cross-resistance pattern.

2.2.2 Objective 2

Determining the level of resistance conferred by one mechanism is important, but more importantly is how multiple resistance mechanisms act together such that the final phenotype (*i.e.*, resistance level) is dramatically altered. I examined the individual resistance contribution of the P450 detoxification mechanism and *kdr* as resistant homozygotes and heterozygotes, respectively. Resistance determinations in mosquitoes which contained the two mechanisms in various genotypic combinations were also performed. It was then possible to compare the observed phenotype of the genotype combination crosses to null models that test for additive joint action of two independent loci. Epistasis is the non-additive interaction between differing loci which contribute to a phenotype [361,362]. Epistasis between mechanisms of insecticide resistance can shape the rate that resistance evolves and can dictate the ultimate level of resistance in the field. Therefore, using models [367,408] to test for departures from additivity, I was able to determine what interaction was occurring between the *kdr* and P450 detoxification mechanisms.

2.2.3 Objective 3

In order to determine how an insecticide resistance allele evolves, it is necessary to determine the fitness associated with the allele. Many studies have found, using measures such as allele frequencies through time or

space [310] or measurements of biological parameters [409], that resistance mechanisms carry a cost. Though, this is not the case for all mechanisms [410].

I examined parameters associated with fitness using a population cage experimental set-up where the P450 resistance allele was placed in competition with the susceptible allele and subjected to different laboratory created environments. These environments included a standard laboratory condition, temephos exposure and cold temperatures. I expected that the standard laboratory condition would be the least costly since it provided the "best" laboratory environment (an abundance of food and space). If a cost was associated with the resistance allele, the allele frequency would decrease in the standard laboratory environment since the insecticide selection pressure was absent without the addition of other environmental manipulations.

I also measured fitness indices associated with the P450 resistance allele by examining biological parameters and energy reserves of ISOP450 versus SLAB. Since P450s are also involved in the regulation of endogenous compounds, over-expression to confer resistance may cause the mosquito to divert resources used for development or survival. The cost may then be more prominently measured by examining biological parameters such as larval development time, larval survival, adult longevity and energy obtained from nutritional reserves at different life stages.

2.2.4 Objective 4

I used a quantitative real-time PCR approach in order to compare the relative transcript levels for two over-expressed P450 cDNA sequences obtained from JPAL (*CYP4H34* and *CYP9M10*) between ISOP450 and SLAB. In order to determine if the P450s found to be over-expressed in JPAL were actively involved in permethrin resistance, it was imperative that the

comparisons be conducted between highly related strains. Since the P450 mechanism was determined to be monogenic and SLAB and ISOP450 strains are highly related, I expected that the P450 which was highly over-expressed in JPAL would also be highly over-expressed in ISOP450, and not in SLAB. This cytochrome P450 gene would also show larger expression levels in larvae versus the adults of ISOP450 due to the larval-specific resistance determined in the experimentation of Objective 1.

CHAPTER 3

CYTOCHROME P450 MONOOXYGENASE-MEDIATED PERMETHRIN RESISTANCE CONFERS LIMITED AND LARVAL SPECIFIC CROSS-RESISTANCE IN *CULEX PIPIENS QUINQUEFASCIATUS* *

3.1 Introduction

Southern house mosquitoes, *Culex pipiens quinquefasciatus*, are able to transmit a multitude of pathogens that infect both humans and other animals [4,19,20,411]. For mosquito control, pyrethroid insecticides are mainly used as adulticides to treat bed nets and as aerial sprays [31,312], while OPs and IGRs are used as larvicides [22]. Some pyrethroid and pyrethroid-like compounds (*i.e.*, etofenprox) are used as larvicides in Japan (personal communication, T. Kozaki), but substantial larval exposure to pyrethroids may occur as a byproduct of agrochemical applications [153].

Insecticide resistance is an important problem in controlling medically important pest populations and is extremely detrimental in the on-going struggle to control or eradicate vectors of disease [129]. Pyrethroid resistance in the *Culex pipiens* complex [60,139,142–148,150–152] has been recorded worldwide. Understanding the genetic basis of resistance, as well as the patterns of cross-resistance, is important to develop effective resistance management strategies [412]. Resistance to pyrethroids in *Culex* mosquitoes occurs due to detoxification by cytochrome P450 monooxygenases [147], as well as target site insensitivity (*i.e.*, *kdr*) [187].

* Presented with minor modifications from the originally published article Hardstone, M. C., C. A. Leichter, L. C. Harrington, S. Kasai, T. Tomita and J. G. Scott. (2007). "Cytochrome P450 monooxygenase-mediated permethrin resistance confers limited and larval specific cross-resistance in the southern house mosquito, *Culex pipiens quinquefasciatus*." Pestic. Biochem. Physiol. 89:175-184.

Cytochrome P450 monooxygenases (P450s) are an important biochemical system involved in the metabolism of xenobiotics and endogenous compounds. Insect P450s are important because they are involved in the detoxification (*i.e.*, limiting the toxicity) and bioactivation (*i.e.*, produce more toxic metabolites) of insecticides. P450s are particularly interesting because they are able to metabolize diverse substrates and are capable of catalyzing a large array of reactions [413–415]. The extent of possible substrates metabolized by monooxygenases is due partially to the large number of isoforms and to the broad substrate specificity of some isoforms. For example, CYP1A1 can metabolize more than 20 substrates [415] and CYP6D1 can carry out a wide range of reactions on structurally diverse substrates [261,416,417]. Not all P450s are able to metabolize a broad range of substrates. For example, CYP7A1 has only one known substrate [415]. Certain P450s have overlapping substrate specificity (*i.e.*, CYP2C subfamily in humans) [415] such that a single compound may be metabolized by multiple P450s. Additionally, some P450s can attack multiple sites of a substrate and produce multiple metabolites. Amino acid similarity of two P450s need not indicate they metabolize similar compounds, as a single amino acid mutation can change the P450 substrate specificity [253]. It has also been shown that a small change in substrate structure can dramatically change the turnover rate of insecticides [418].

Cytochrome P450s are one of the major mechanisms of insecticide resistance in insects [419]. JPAL is a strain of *Cx. p. quinquefasciatus* collected from Saudi Arabia and selected with permethrin for 20 generations [295]. JPAL larvae exhibited 2,500-fold resistance to permethrin due to P450 detoxification and *kdr* [147]. Metabolism studies showed that NADPH-dependent metabolism of permethrin to 4′-OH-permethrin was greater in JPAL compared

to a susceptible strain [147]. The P450 responsible for this resistance has not been identified.

Weerasinghe, et al. [407] previously examined cross-resistance patterns to pyrethroid insecticides in JPAL. However, since JPAL has two major mechanisms of resistance (P450 detoxification and *kdr*) understanding the contribution of each mechanism to the resulting cross-resistance patterns is difficult. In order to more completely identify the cross-resistance conferred specifically by P450-mediated resistance, I studied a resistant strain of *Cx. p. quinquefasciatus* (ISOP450) (produced by repeated backcrossing of JPAL to a susceptible strain), that contains P450-mediated resistance, but lacks *kdr*. This was the first time in mosquitoes that P450 monooxygenase involvement in pyrethroid resistance had been isolated and studied without the confounding effects of *kdr*. ISOP450 allowed me to answer some previously intractable questions about P450-mediated resistance in mosquitoes, by investigating the genetic basis of the mechanism as well as cross-resistance to pyrethroids and OP insecticides commonly used in mosquito control programs.

3.2 Materials and methods

3.2.1 Mosquito strains

Three strains of *Cx. p. quinquefasciatus* Say were used. SLAB, a standard susceptible strain [420]. JPAL, which originated from Saudi Arabia [295] and is highly resistant to permethrin due to *kdr* and P450-mediated (P450) detoxification [147]. Standard backcrossing methods [389] were used to produce ISOP450, a resistant strain with the genetic background of SLAB but with the P450 resistance mechanism and lacking *kdr*. To construct ISOP450, SLAB females were crossed with JPAL males *en masse*, and F_1 males were backcrossed to SLAB females. Fourth instar progeny were treated (see below)

with permethrin (0.02 μg/ml, LC_{25} for progeny of SLAB female x JPAL male) to select for resistant heterozygotes. Surviving males were then backcrossed to SLAB females and this procedure was repeated for 11 generations (Table 3.1). For generations 12, 13 and 14, the reciprocal cross of surviving backcross females x SLAB males was included. Survivors were combined for the next backcross cycle.

Table 3.1: Construction of the ISOP450 strain.

Generation	Cross (female x male)	n [a]	Mortality (%)
BC1	SLAB (317) [b] x SLABxJPAL F_1 (201)	1,200	66
BC2	SLAB (255) x BC1 (287)	1,180	76
BC3	SLAB (175) x BC2 (233)	540	53
BC4	SLAB (117) x BC3 (80)	1,200	60
BC5	SLAB (81) x BC4 (36)	240	56
BC6	SLAB x BC5	1,500	50
BC7	SLAB (190) x BC6 (150)	590	51
BC8	SLAB (292) x BC7 (60)	880	49
BC9	SLAB x BC8	949	79
BC10	SLAB x BC9	625	57
BC11	SLAB x BC10	1,060	62
BC12	SLAB x BC11	2,250	59
	BC11 x SLAB	1,025	48
BC13	SLAB x BC12	1,500	69
	BC12 x SLAB	1,780	60
BC14	SLAB x BC13	1,400	60
	BC13 x SLAB	1,200	62

[a] Number of fourth instar larvae treated with permethrin at 0.02 μg/ml.

[b] Number of individuals included in the cross.

The resulting backcross strain was then selected with permethrin for eight generations to produce a homozygous resistant strain (Table 3.2).

Table 3.2: Selection of the ISOP450 strain.

Generation	Concentration [a]	n [b]	Mortality (%)
G1[c]	0.02	2,200	79
G2	0.1	3,000	89
G3	1.0	2,580	85
G4	1.0	10,180	95
G5	1.0	3,600	65
G6	1.0	960	66
	5.0	1,200	97
G7	1.0	489	84
	3.0	4,880	93.5
G8	5.0	15,400	96

[a] Units: μg/ml

[b] Number of fourth instar larvae treated.

[c] BC14 F_1 survivors (see Table 3.1).

All strains were reared by standard methods. Larvae were reared with ample development space in plastic trays containing 2000 ml distilled water for every 400 larvae. Larvae were provided abundant food consisting of a mixture of ground TetraFin® goldfish flakes, rabbit pellets and liver powder (1:2:1) in distilled water. Adults were provided a 20% sugar solution *ad libitum*, and provided a chicken for 30 min two times per week (Cornell University Animal Use Protocol # 01-56). All life stages were maintained at 27±1°C, 80% RH, and photoperiod of 14 hr:10 hr (L:D) including 2 hr of simulated dawn and 2 hr simulated dusk.

3.2.2 Chemicals

Deltamethrin (99%) was from Roussel UCLAF and 1-*R-trans* fenfluthrin was from Bayer CropScience (Kansas City, MO). Insecticides obtained from Chem Service (Westchester, PA) included: permethrin (98%), cypermethrin (98%), *S*-bioallethrin (99%), tetramethrin (99.5%), bifenthrin (99.7%), temephos (98%), malathion (99.2%), diazinon (99.5%), and methyl-parathion (98.9%). Fenitrothion was from Sumitomo Chemical (Tokyo, Japan). The synergist PBO (90%) was from Sigma-Aldrich, Inc. (St. Louis, MO).

3.2.3 Larval bioassays

For larval bioassays, batches of 20 fourth instars were placed in 4 oz. waxed paper cups (Sweetheart Cup Co., Owings Mills, MD) with 99 ml of distilled water and 1 ml of insecticide (in acetone) solution (or just acetone for the controls). To evaluate the role of P450s, bioassays were run as described above, except that 1 ml of PBO solution (0.1 mg/ml) was added. Preliminary experiments indicated that 1 μg/ml (final concentration) was the maximum sublethal concentration of PBO. Mortality was assessed after 24 hrs, and larvae were considered dead if they failed to move or resurface after being probed.

3.2.4 Adult bioassays

Adult mosquito bioassays were conducted in glass jars (230 ml, internal surface area of 180 cm^2) treated with 1 ml of insecticide solution (or 1 ml of acetone for controls), which was evenly coated on the inner walls. Jars were held (for acetone to completely evaporate) for 30 min under a fume hood and then 10 adult females (3 dyo virgins) were placed in each jar, and the opening was covered with mesh. Adults were provided cotton wicks saturated with 20% sugar water throughout the assay. Adults were considered dead if after 24

hrs they were ataxic. Adult bioassays with PBO (1 mg/ml) were also performed.

3.2.5 Statistical analysis

Each bioassay consisted of at least three replicates per concentration and at least three concentrations of insecticide were tested, giving greater than 0% and less than 100% kill. All bioassays (larval and adult) were run at 25°C and replicated a minimum of five times. Bioassay data were pooled and analyzed by standard probit analysis [421], as adapted to personal computer use [422] using Abbott's correction [423] for control mortality.

3.2.6 Isolation of gDNA

Genomic DNA was extracted from a single adult male mosquito based on the protocol developed by J. Rehm, Berkeley Drosophila Genome Project (http://www.fruitfly.org). An individual mosquito was completely homogenized in 200 μl Buffer A (100 mM Tris-HCl, pH 7.5; 100 mM EDTA; 100 mM NaCl; 0.5% SDS) with a disposable tissue grinder (Kontes, Vineland, NJ). The homogenate was incubated at 65°C for 30 min, then 400 μl of LiCl/KAc solution (5M KAc: 6M LiCl) was added and the sample was incubated on ice for 10 min. The mixture was centrifuged for 15 min at room temperature, and 0.5 ml of supernatant was transferred into a new tube. To precipitate the gDNA, 300 μl isopropanol was added, mixed and centrifuged at room temperature for 15 min. The supernatant was then aspirated and the DNA pellet was washed with 70% ethanol. All ethanol was aspirated and the pellet was dried for 5 min under a fume hood. DNA was dissolved in 30 μl ddH_2O and stored at −20°C.

3.2.7 PCR and sequencing

A partial genomic sequence of the voltage sensitive sodium channel (*VSSC*), including the *kdr* mutation site and adjacent intron, was amplified by PCR using the following primers: forward primer CulexkdrF (5′ GGAACTTCACCGACTTCATGC 3′) and reverse primer CulexkdrR (5′ CGCCGACAGACTTGAGGAACC 3′). *Taq* polymerase was obtained from New England Biolabs (Beverly, MA). Amplification reactions were performed using the following thermal cycler conditions: 94°C for 5 min, 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions and sequenced at the Cornell University Biotechnology Resource Center.

3.2.8 Degree of dominance and mode of inheritance bioassays

To determine the degree of dominance and mode of inheritance for permethrin resistance in JPAL and ISOP450, standard backcross methods were used [424]. Pupae from each strain were individually isolated in plastic tubes. Upon eclosion, the respective sex and strain were released into a cage to create the desired crosses. The adults were released *en masse* with an approximately 3:1 (female:male) ratio, with at least 200 females used. The following reciprocal crosses were created: SLAB females x ISOP450 males, and ISOP450 females x SLAB males. The backcrosses were constructed using 300 virgin F_1 females (progeny of SLAB females x ISOP450 males and progeny of ISOP450 females x SLAB males) mated to 100 SLAB males.

3.2.9 Degree of dominance and mode of inheritance analysis

Using probit regression lines of the parental strains (SLAB and ISOP450) and reciprocal cross progeny, the degree of dominance (*D*) for the P450 mechanism was calculated using Stone's [425] equation,

$$D = (2X_2 - X_1 - X_3)/(X_1 - X_3)$$

where X_1 is the $\log_{10}LC_{50}$ of homozygous resistant strain (ISOP450), X_2 is the $\log_{10}LC_{50}$ of the heterozygous F_1 progeny of each reciprocal cross, and X_3 is the $\log_{10}LC_{50}$ of the homozygous susceptible strain (SLAB).

The standard backcrossing method tests the null hypothesis that one locus controls resistance (monofactorial inheritance). To determine if the backcross progeny in this experiment differed significantly from the null hypothesis, the following equation was used [424] to calculate the expected mortality of the backcross offspring (Y_x) at a specific concentration (x),

$$Y_x = 0.50\ (W_{R/S} + W_{S/S})$$

where $W_{R/S}$ is the mortality of the heterozygote (F_1) genotype at concentration x, and $W_{S/S}$ is the mortality of the susceptible parental strain at concentration x.

Chi-squared statistical analysis was conducted to determine significance between the calculated backcross progeny mortality and the observed mortality by this equation [424],

$$\chi^2 = (F_1 - pn)^2\ /\ pqn$$

where F_1 is the observed backcross progeny mortality at concentration x, p is the expected backcross progeny mortality, q=1-p, and n is the number of backcross progeny assayed at concentration x. The monofactorial null hypothesis is rejected if $p<0.05$.

3.3 Results

3.3.1 ISOP450 strain construction

After 14 generations of backcrosses and permethrin selection followed by an additional 8 generations of permethrin selection, I isolated a strain (highly related to SLAB) called ISOP450. Larvae of ISOP450 were 1,300-fold resistant to permethrin (Table 3.3) relative to SLAB. This is lower than the levels found in JPAL (29,000-fold), suggesting a resistance factor present in JPAL may have been lost during isolation of the ISOP450 strain. Resistance to permethrin dropped to 5.4-fold when ISOP450 larvae were treated with PBO. This suggested that the major resistance mechanism in ISOP450 was inhibited by PBO. JPAL larvae treated with PBO had 70-fold resistance to permethrin. When treated with PBO, ISOP450 had significantly lower resistance to permethrin than JPAL. This indicates that a mechanism (one which could not be overcome by PBO (*i.e.*, *kdr*)) in the JPAL strain had been lost in ISOP450 (Figure 3.1).

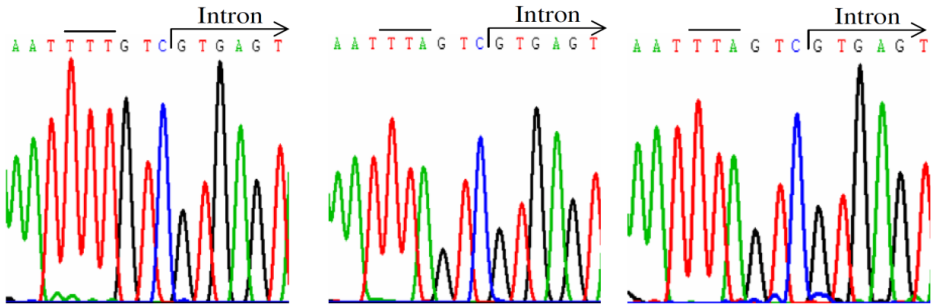


Figure 3.1: Genotyping of *VSSC* in SLAB, ISOP450 and JPAL. Accession numbers BI918677, BI918687, and BI91976, respectively. TTA (Leucine) = susceptible and TTT (Phenylalanine) = *kdr*.

Table 3.3: Comparative insecticide screening of mosquito strains to determine mechanism of resistance in ISOP450.

Life stage	Insecticide	SLAB			JPAL				ISOP450			
		n [a]	slope (SE)	LC_{50} [b] (95% CI)	n [a]	slope (SE)	LC_{50} [b] (95% CI)	RR [c]	n [a]	slope (SE)	LC_{50} [b] (95% CI)	RR [c]
Larvae	permethrin	320	4.5 (0.6)	1.7 (1.5-1.9)	480	4.6 (0.4)	49,000 (45,000-54,000)	29,000	860	1.8 (0.1)	2,100 (1,900-2,500)	1,300
Larvae	permethrin +PBO	480	8.0 (2.8)	1.1 (0.9-1.4)	240	3.5 (1.4)	77 (49-120)	70	960	3.0 (0.2)	5.9 (5.3-6.5)	5.4
Adult	permethrin	670	2.5 (0.2)	1.6 (1.4-1.9)	637	2.1 (0.2)	72 (61-83)	45	360	1.9 (0.2)	7.2 (5.6-8.3)	4.5
Adult	permethrin +PBO	680	1.6 (0.1)	0.12 (0.10-0.14)	470	4.0 (0.5)	9.3 (8.3-10)	77	460	1.1 (0.3)	0.19 (0.11-0.33)	1.6

[a] Total number of animals treated.

[b] Units for larvae: ng/ml, units for adult: ng/cm^2.

[c] Resistance ratio = LC_{50} resistant strain/LC_{50} susceptible strain.

To confirm the bioassay results (that ISOP450 lacked *kdr*), I genotyped individual mosquitoes for the *kdr* mutation and found that all (20/20) ISOP450 individuals contained the susceptible allele (Figure 3.1).

Adult JPAL and ISOP450 were less resistant to permethrin compared to larvae, being only 45- and 4.5-fold resistant to permethrin, respectively (Table 3.3). The addition of PBO resulted in 13-fold synergism of permethrin toxicity in SLAB, suggesting there is some P450-mediated detoxification of permethrin in this susceptible strain, but that it is stronger in adults than in larvae. This contrasts with *Ae. aegypti*, where the opposite pattern was observed [111]. For both the JPAL and ISOP450 strains, resistance in adults is comparable to the resistance ratios of the respective larvae treated with permethrin + PBO. These results suggest that the P450 mechanism present in JPAL and ISOP450 larvae is not expressed in the adult stage and is larval specific.

3.3.2 Inheritance of permethrin resistance in ISOP450

To evaluate the mode of inheritance of cytochrome P450-mediated permethrin resistance in ISOP450 larvae, I calculated the degree of dominance (*D*) for the heterozygote progeny of SLAB females x ISOP450 males (F_1a) and ISOP450 females x SLAB females (F_1b). The *D* value for the F_1 progeny of both crosses was +0.3 (incompletely dominant) [426]. The F_1 responses did not differ significantly between the reciprocal crosses, indicating that there were no cytoplasmic influences and that resistance was due to a chromosomal genetic factor [426]. I also found no differences in susceptibility between the sexes of the F_1s, indicating resistance was not sex-linked [426].

To determine if resistance was monofactorial or polyfactorial, backcrosses were conducted. The resulting lines of BCa (F_1a females x SLAB males) and BCb (F_1b females x SLAB males) displayed distinct plateaus at 50%

mortality (Figures 3.2 and 3.3). Chi-squared analysis of backcross progeny indicated that the null hypothesis of monofactorial inheritance could not be rejected (BCa: χ^2=13.76, df=11, p>0.1; BCb: χ^2=11.51, df=12, p>0.1).

3.3.3 Cross-resistance to pyrethroids in ISOP450

The ISOP450 strain provided a unique opportunity in which to determine the level of resistance conferred solely by P450-mediated permethrin resistance to pyrethroid insecticides with various structures. LC_{50} values of the seven pyrethroid insecticides to SLAB and ISOP450 strains are listed in Table 3.4. The resistance ratios were dramatically lowered (compared to permethrin) when either an α-cyano group was present (deltamethrin RR=11 and cypermethrin RR=6.6) or when the pyrethroid lacked a 3-phenoxybenzyl moiety (tetramethrin RR=12, fenfluthrin RR=7.7, bioallethrin RR=4.9 and bifenthrin RR=1.5) (Figure 3.4).

3.3.4 Cross-resistance to organophosphates in ISOP450

Comparisons between SLAB and ISOP450 also allowed me to determine if any cross-resistance was conferred by the P450 mechanism to OP insecticides commonly used in mosquito control (Table 3.5 and Figure 3.5). ISOP450 was 8.1-fold resistant to fenitrothion as compared to SLAB. ISOP450 was then bioassayed with a structurally similar organophosphate, methyl-parathion, which does not contain a methyl substituent at the *meta* position on the benzene ring. The absence of the methyl group at that metabolic site eliminated the resistance (RR=0.55) (Table 3.5). I found small, but significant, levels of negative cross-resistance (*i.e.*, bioactivation) to temephos (RR=0.73) and malathion (RR=0.84) as well as significantly low levels of cross-resistance to diazinon (RR=1.7). It has been previously shown that isogenic strains can have

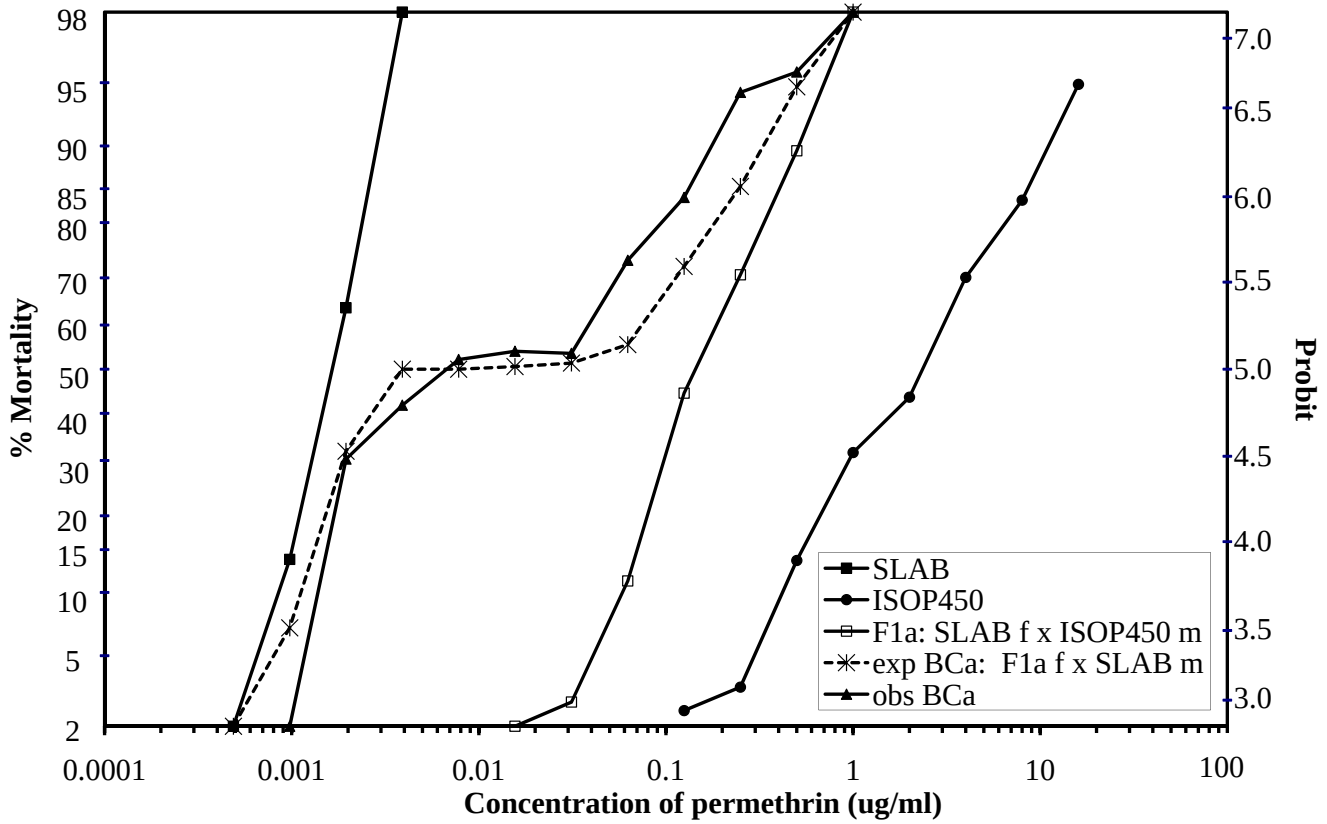


Figure 3.2: Probit graph for mode of inheritance determination of the cytochrome P450 resistance mechanism in ISOP450, reciprocal cross A: SLAB f (female) x ISOP450 m (male). Obs, observed; exp., expected.

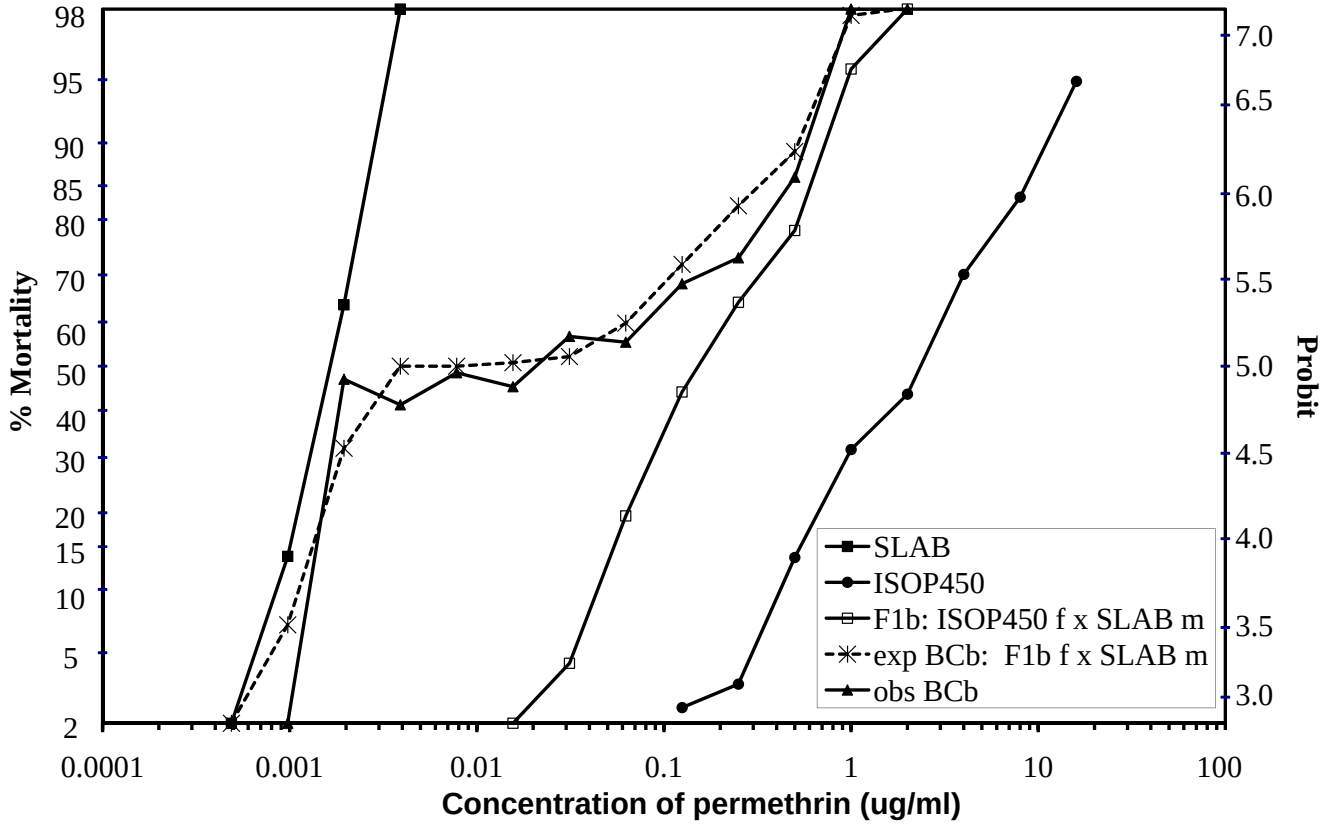


Figure 3.3: Probit graph for mode of inheritance determination of the cytochrome P450 resistance mechanism in ISOP450, reciprocal cross B: ISOP450 f (female) x SLAB m (male). Obs, observed; exp., expected.

LC_{50}s that differ [427]. Thus, while the levels of cross-resistance in ISOP450 are significantly different for these three OPs, the biological significance is unclear.

3.4 Discussion

Results from this experiment show that P450-mediated permethrin resistance in the ISOP450 strain of *Cx. p. quinquefasciatus* is monofactorial. Similarly, monofactorial inheritance was found in *H. armigera* resistant to fenvalerate due to metabolic factors [428]. My results differ from the polygenic nature of P450-mediated pyrethroid resistance in house flies [342, 364, 429, 430].

This study has demonstrated that the P450 responsible for resistance in the ISOP450 strain has a narrow substrate specificity, with a limited ability to detoxify pyrethroids containing an α-cyano substituent or lacking a 3-phenoxybenzyl group. For example, permethrin resistance in ISOP450 larvae was 1,300-fold, while cypermethrin resistance was 6.6-fold (Table 3.4). This pattern was also observed in JPAL larvae, where resistance to permethrin was 2,500-fold and resistance to cypermethrin was 47-fold [407]. In JPAL, resistance to pyrethroids with an α-cyano substituent was moderate (RR=56-39) and resistance to those without a 3-phenoxybenzyl group was low (RR=6-9) [407]. The resistance in ISOP450 does not have exactly the same pattern as JPAL. Resistance in ISOP450 is low to pyrethroids with an α-cyano substituent (RR=6.6 and 11) as well as to pyrethroids without a 3-phenoxybenzyl group (RR=1.5-12). It has been shown that *kdr* confers different levels of resistance to various pyrethroids [431]. The higher resistance to α-cyano pyrethroids found in JPAL, relative to ISOP450, may be due to the presence of *kdr* in JPAL.

LPR is a strain of house fly which is highly resistant to pyrethroids (>6,000-fold) [432] due to *kdr* and detoxification mediated by CYP6D1 [430]. LPR showed high levels of resistance to pyrethroids with or without an

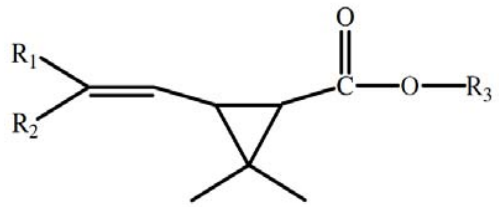
Table 3.4: LC_{50} values of seven structurally different pyrethroid insecticides to susceptible (SLAB) and resistant (ISOP450) strains of *Cx. p. quinquefasciatus* fourth instar larvae.

Insecticide	SLAB			ISOP450			
	n [a]	slope (SE)	LC_{50} [b] (95% CI)	n [a]	slope (SE)	LC_{50} [b] (95% CI)	RR [c]
permethrin	320	4.5 (0.6)	1.7 (1.5-1.9)	860	1.8 (0.1)	2,100 (1,900-2,500)	1,300
tetramethrin	960	9.4 (0.6)	47 (46-48)	640	4.3 (1.3)	560 (450-700)	12
fenfluthrin	960	5.7 (0.7)	0.68 (0.61-0.75)	1,180	2.2 (0.2)	5.2 (4.8-5.8)	7.7
bioallethrin	900	2.5 (0.2)	67 (61-79)	1,000	7.5 (1.8)	330 (280-370)	4.9
bifenthrin	480	3.7 (0.4)	3.5 (3.1-4.0)	600	4.1 (0.9)	5.2 (3.9-6.8)	1.5
deltamethrin	720	3.6 (0.2)	0.26 (0.32-0.28)	600	4.0 (0.3)	2.9 (2.7-3.2)	11
cypermethrin	500	7.3 (0.8)	0.79 (0.74-0.85)	480	5.1 (0.4)	5.2 (4.8-5.7)	6.6

[a] Total number of animals treated.

[b] Final concentration in ng/ml.

[c] Resistance ratio = LC_{50} resistant strain/LC_{50} susceptible strain.



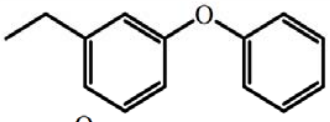
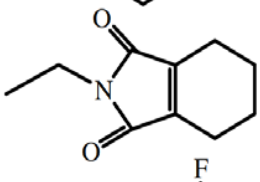
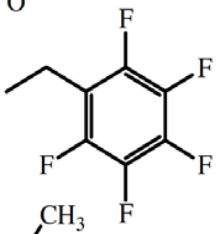
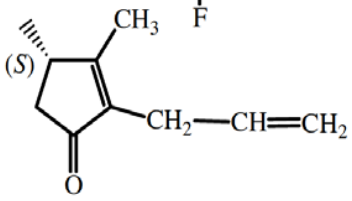
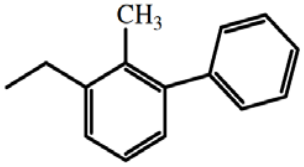
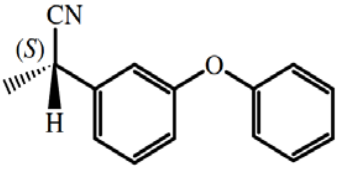
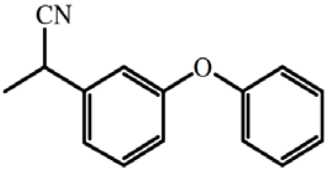
Insecticide	R_1	R_2	R_3	RR
permethrin	Cl	Cl		1,300
tetramethrin	CH_3	CH_3		12
fenfluthrin	Cl	Cl		7.7
S-bioallethrin (1*R-trans*)	CH_3	CH_3		4.9
bifenthrin	CF_3	Cl		1.5
deltamethrin (1*R-cis*)	Br	Br		11
cypermethrin	Cl	Cl		6.6

Figure 3.4: Levels of resistance to seven pyrethroid insecticides in larvae of the ISOP450 strain.

Table 3.5: Toxicities of five organophosphate insecticides to susceptible (SLAB) and resistant (ISOP450) strains of *Cx. p. quinquefasciatus* fourth instar larvae.

Insecticide	SLAB			ISOP450			
	n [a]	slope (SE)	LC_{50} [b] (99% CI)	n [a]	slope (SE)	LC_{50} [b] (99% CI)	RR [c]
temephos	1,420	11 (0.56)	3.0 (3.0-3.1)	1,080	15 (0.82)	2.2 (2.2-2.3)	0.73
fenitrothion	1,100	7.7 (0.42)	4.7 (4.6-4.9)	1,060	8.9 (0.52)	38 (36-39)	8.1
malathion	980	8.5 (1.3)	77 (71-83)	1,140	8.9 (1.0)	65 (60-70)	0.84
diazinon	1,040	13 (1.6)	83 (79-88)	850	10 (0.65)	140 (140-150)	1.7
methyl-parathion	800	7.5 (1.9)	4.0 (3.2-5.0)	800	8.3 (3.0)	2.2 (1.7-2.9)	0.55

[a] Total number of animals treated.

[b] Final concentration in ng/ml.

[c] Resistance ratio = LC_{50} resistant strain/LC_{50} susceptible strain.

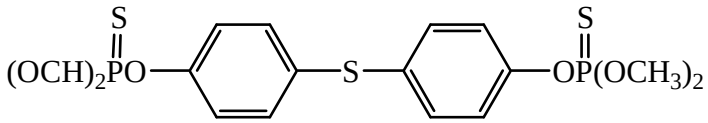
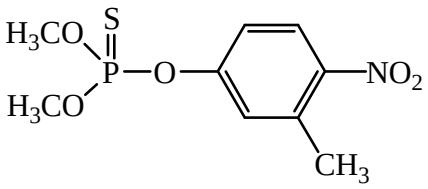
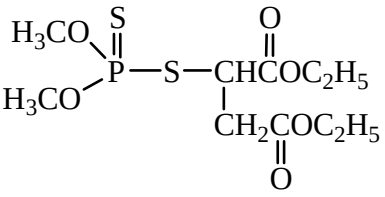
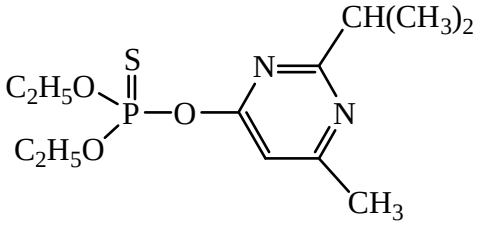
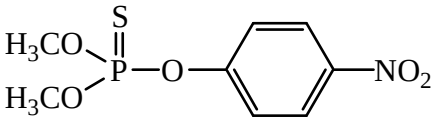
Insecticide	Structure	RR
temephos		0.73
fenitrothion		8.1
malathion		0.84
diazinon		1.7
methyl-parathion		0.55

Figure 3.5: Levels of resistance to five organophosphate insecticides in larvae of the ISOP450 strain.

α-cyano group [430]. This pattern contrasts that observed in ISOP450 and JPAL [407], where the presence of an α-cyano group decreases the level of resistance. Thus, even though CYP6D1 and the P450 responsible for permethrin metabolism in JPAL (and ISOP450) metabolize the same site of permethrin (and produce the same metabolite; 4'-OH-permethrin) [147,416], they differ in their substrate specificity to α-cyano pyrethroids, with the former

being able to metabolize these pyrethroid structures and the latter being much less able to metabolize them. Similar to ISOP450 and JPAL, resistance to pyrethroids lacking an unsubstituted phenoxybenzyl group was decreased in LPR (relative to pyrethroids which possessed an unsubstituted phenoxybenzyl group) [430].

Interestingly, ISOP450 was 8.1-fold cross-resistant to fenitrothion, but was not cross-resistant to methyl-parathion. Given that these two insecticides differ only in the presence of a *meta* methyl group (in fenitrothion, but lacking in methyl-parathion) suggests that the cross-resistance is due to metabolism of the methyl group. This suggests that the P450 responsible for permethrin resistance (due to aromatic hydroxylation) is also capable of carrying out the alkyl hydroxylation of fenitrothion. Identification of the P450 responsible for permethrin resistance in ISOP450 will allow this hypothesis to be tested.

In both ISOP450 and JPAL, the larvae exhibit high levels of permethrin resistance (1,300-fold and 29,000-fold, respectively). The adults of these strains have significantly lower levels of permethrin resistance than the larvae (Table 3.3). This is similar to the recent report of high levels of pyrethroid (deltamethrin) resistance in larvae, but not adults, of *Ae. aegypti* [433]. In vector control programs pyrethroids are used as aerial sprays and on bed nets [31,312] where adults are targeted. Interestingly, the P450 mechanism present in ISOP450 and JPAL does not seem to be expressed in the adults. Thus, it appears that either *kdr* is sufficient to survive insecticide exposure as an adult, or that high levels of resistance has not yet evolved in adults of these strains of *Cx. p. quinquefasciatus*. Further studies on pyrethroid resistance levels in adult *Cx. p. quinquefasciatus* would help clarify this.

Pyrethroid resistance in mosquito larvae is documented worldwide. Substantial insecticidal pressures in the environment of the larvae exist possibly due to agricultural runoff or misuse. This inadvertent exposure may be selecting for resistance mechanisms essential for larvae to survive constant insecticidal exposure in a body of water, but unnecessary for adults to survive in their terrestrial environment. This may be why P450-mediated detoxification in JPAL and ISOP450 is exclusively in the larval stage. Since high levels of resistance in ISOP450 seems to be restricted to permethrin, most other pyrethroids, as well as most OPs, could still be effective control tools against mosquitoes with this resistance mechanism.

CHAPTER 4

MULTIPLICATIVE INTERACTION BETWEEN THE TWO MAJOR MECHANISMS OF PERMETHRIN RESISTANCE, *KDR* AND CYTOCHROME P450-MONOOXYGENASE DETOXIFICATION, IN MOSQUITOES *

4.1 Introduction

Epistasis is the non-additive interaction between different loci which contribute to a phenotype [361–363]. Independent loci conferring insecticide resistance can exhibit joint action in a less than additive manner (*i.e.*, antagonistic epistasis), an additive manner, or in a greater than additive manner (*i.e.*, synergistic epistasis) [364]. Evolutionarily, epistasis favors natural selection to purge populations of deleterious mutations [434–436], affects changes in allele frequency [437], helps maintain equilibrium but with increased genetic variation decreasing the problem of genetic load [438], and alters fitness effects associated with a genotype [437].

Studies examining the presence or absence of epistasis between multiple loci have been conducted on an assortment of organisms including bacteria, fungi [439], plants [440], and animals [441]. In general, these studies have found that the type of epistasis (or lack thereof) between independent loci is dependent on the environment [442–444] in which the organism evolves, the phenotype measured [442, 445], and the genetic backgrounds [445–447] of the organism. For example, examination of fitness costs associated with multiple resistance mechanisms present in *E. coli* to bacteriophages showed that

* Presented with minor modifications from the originally published article Hardstone, M. C., C. A. Leichter and J. G. Scott. (2009). "Multiplicative interaction between the two major mechanisms of permethrin resistance, cytochrome P450- monooxygenase detoxification and *kdr*, in mosquitoes." J. Evol. Biol. 22:416-423.

epistasis played a role in decreasing the total cost associated with the mechanisms in a trehalose rich environment versus a glucose environment [443]. In *Arabidopsis*, synergistic epistasis occurs only between the phenotypes trichome density and indole glucosinolate concentration (unlinked plant resistance mechanisms to herbivory) in a field experiment when general herbivory is measured [442]. In human dizygotic twin pairs, those with the *ibs*C variable, compared to those with the *ibs*T variable, showed significance of an interaction between the *Xmn*I-Gγ genotype and linkage to a region on chromosome 8, which influences the expression of fetal haemoglobin [446].

Like a variety of phenotypes in many organisms, the evolution of insecticide resistance in a population results from the balance between the fitness advantage resistance alleles confer in the presence of insecticides and their fitness cost in the absence of insecticides. In a population with multiple resistance mechanisms, the evolutionary outcome of each is additionally shaped by the relative interaction of the resistance alleles in both environments. Therefore, to fully assess the evolution of resistance, the type of interaction between the resistance loci must be considered. It would be expected that an interaction between resistance loci that provided protection greater than either loci alone would be favored in the presence of insecticides.

Given that there are a limited number of mechanisms responsible for insecticide resistance, it might be possible to establish general rules for interactions between loci that would be useful in the development of resistance management strategies. Studies of the interactions between resistance loci have been most commonly conducted in house flies [364,370,448–454]. Generally, a greater than additive interaction is observed between two loci that are both homozygous resistant, while additivity (lack of epistasis) occurs between two

loci that are both heterozygous. A complication to understanding interactions between resistance mechanisms is that homozygous and heterozygous resistant individuals exist in field populations [195] and often confer differing levels of resistance. Thus, it is desirable to know the type of interaction occurring between resistance loci when they are present in both homozygous and heterozygous conditions. Herein, I examined the interaction of two pyrethroid resistance loci in both the homozygous and heterozygous states. Determining if an interaction is present between loci of resistance is important to investigate because it may play a role in the evolution and maintenance of multigenic resistance in the field, consequently dictating the effectiveness of a chemical control campaign.

In the current study I determined if and how *kdr* interacted with a P450-mediated resistance (detoxification) locus in *Cx. p. quinquefasciatus*. My hypothesis was that in an insecticide treated environment, a greater than additive interaction between resistance loci would be observed, whether present as homozygotes or heterozygotes. This hypothesis was tested by conducting bioassays on one insecticide susceptible strain, two pyrethroid resistant strains (with known resistance mechanisms) and crosses from these strains. The observed resistance levels were tested against null models to detect interactions.

4.2 Materials and methods

4.2.1 Mosquito strains

Three strains of *Cx. p. quinquefasciatus* Say were used as previously described (Chapter 3). SLAB is a laboratory susceptible strain [420]. JPAL [147,295,407] is a permethrin resistant strain containing *kdr* and P450 detoxification. ISOP450 [455] is a permethrin resistant strain (1,300-fold)

related to SLAB containing the P450 resistance mechanism present in JPAL, but lacking *kdr*.

4.2.2 Bioassays

Resistance levels to permethrin (50:50, cis:trans, Chem Service, Westchester, PA) for the parental strains and progeny from the crosses were determined by larval bioassay. In order to determine the resistance conferred by the P450 detoxification mechanism as well as *kdr* individually, insecticide solutions were prepared with either acetone or in acetone containing PBO (Sigma-Aldrich, Inc., St. Louis, MO). Treatment with PBO plus the insecticide allows for detection and measurements of non-P450 mediated resistance mechanisms (*i.e., kdr*). It was previously determined that the maximum sublethal concentration of PBO was 1 mg/ml [455]. For each bioassay, batches of 20 fourth instar larvae were placed in a 4 oz. wax coated paper cup (Sweetheart Cup Co., Owings Mills, MD) with 99 ml of distilled water and 1 ml of insecticide solution. Control cups contained 99 ml of distilled water with 1 ml of acetone or 1 ml of PBO in acetone solution. All tests were run at 25°C. Each bioassay consisted of at least 12 replicates per concentration and at least 3 (usually 4-6) concentrations of insecticide were tested that gave greater than 0% and less than 100% kill. Mortality was assessed after 24 hrs. Larvae were considered dead if they failed to move or resurface after being probed. Bioassay results were pooled and analyzed by standard probit analysis [421], as adapted to personal computer use [422], using Abbott's correction [423] for control mortality.

In order to obtain mosquitoes with various combinations of genotypes, crosses were set up between existing strains. Individual pupae from each strain were kept in separate tubes. Upon eclosion, the respective sex and strain

were released into a cage to create the desired cross. The adults were released *en masse* with a ~ 3:1 female:male ratio, with at least 200 females used. The following crosses were created: SLAB female x ISOP450 male, ISOP450 female x SLAB male, SLAB female x JPAL male and JPAL female x SLAB male. Bioassays (see above) of each parental strain along with the progeny of the crosses were conducted.

4.2.3 Analysis of interaction between *kdr* and P450 detoxification resistance mechanisms

In order to determine if and what type of interaction was occurring between the two resistance loci (*kdr* and P450 detoxification) I first determined the toxicity of permethrin and permethrin + PBO in all mosquito strains and cross progeny. The toxicity information was used to determine the resistance contribution of each locus and the observed resistance levels were compared to the values expected from the models.

I tested for interactions between *kdr* and P450 detoxification loci using a null additive mathematical model. For this model, RR_1 and RR_2 are the resistance ratios conferred by the *kdr* and P450 detoxification mechanism, respectively, and RR_{12} is the resistance ratio of the two loci in combination. The additive model gives $RR_{12} = RR_1 + RR_2 - 1$ and I calculated the expected resistance conferred for each of the genotype combinations using the resistances measured for the individual mechanisms [367,443]. Additionally, interactions between the independent resistance loci were examined in models where the total two-locus genotypic contribution was plotted in relation to the phenotypic values (resistance ratios) on a linear scale and by logarithmically transforming the phenotype values (see Appendix B).

4.3 Results

When the observed resistance ratios of each resistance loci were subjected to the three models to test for interactions (additive mathematical model, linear scaled plot, and logarithmic-transformation) (for plot details see Appendix B), multiplicative interactions were found for all tested genotype combinations.

The levels of permethrin and permethrin + PBO toxicity in larvae of the parental strain and the progeny of the crosses are listed in Tables 4.1 and 4.2, respectively. When treated with permethrin, all reciprocal cross (SLAB female x ISOP450 male versus ISOP450 female x SLAB male and JPAL female x ISOP450 male versus ISOP450 female x JPAL male) LC_{50} values were not significantly

Table 4.1: Toxicity of permethrin to parental mosquito strains and F_1 progeny of crosses.

Strain (cross:female x male)	LC_{50} [a] (95% CI)	Slope (SD)	n [b]
SLAB	0.0017 (0.0015-0.0019)	4.5 (0.6)	320
JPAL	49 (45-54)	4.6 (0.4)	480
ISOP450	2.1 (1.9-2.5)	1.8 (0.1)	860
SLAB x JPAL	1.3 (1.1-1.6)	2.1 (0.2)	480
SLAB x ISOP450	0.16 (0.15-0.17)	2.6 (0.1)	1,440
ISOP450 x SLAB	0.17 (0.15-0.19)	2.1 (0.1)	1,800
ISOP450 x JPAL	11 (8.4-13)	2.3 (0.3)	1,440
JPAL x ISOP450	16 (10-25)	3.4 (1.2)	1,120

[a] Units: μg/ml.

[b] Total number of treated fourth instar larvae.

Table 4.2: Toxicity of permethrin + PBO to parental mosquito strains and progeny of crosses.

Strain (cross:female x male)	LC_{50} [a] (95% CI)	Slope (SD)	n [b]
SLAB	0.0011 (0.00090-0.0014)	7.9 (2.8)	480
JPAL	0.077 (0.049-0.12)	3.5 (1.4)	240
ISOP450	0.0059 (0.0053-0.0065)	3.0 (0.20)	960
SLAB x JPAL	0.011 (0.0094-0.012)	5.0 (0.54)	300
ISOP450 x JPAL	0.053 (0.048-0.058)	2.7 (0.2)	1,310
JPAL x ISOP450	0.026 (0.020-0.034)	2.6 (0.4)	1,540

[a] Units: μg/ml.

[b] Total number of treated fourth instar larvae.

different based on overlap of 95% confidence intervals. When larvae were treated with permethrin + PBO, the reciprocal crosses of JPAL female x ISOP450 male versus ISOP450 female x JPAL male were minor, but statistically different. The biological significance of the differing LC_{50} values is unknown though variation of this magnitude has been found amongst near isogenic strains [427] and *kdr* has previously been shown not to be sex-linked [162].

When treated with permethrin + PBO, resistance in JPAL was reduced to 70-fold. Resistance in ISOP450 treated with permethrin + PBO was 5.4-fold versus 1,300-fold when treated with permethrin only. This indicates that the major mechanism for permethrin resistance in the JPAL and ISOP450 strains was highly PBO suppressible (*i.e.*, P450-mediated detoxification). These results are expected since previous studies showed JPAL contains both P450 detoxification and *kdr*, while ISOP450 contains only the P450 mechanism [455].

PBO treatment was able to suppress >99.5% of the resistance in the ISOP450 strain. Therefore, the deduced relative contribution of *kdr* (Table 4.3) obtained from the bioassay of JPAL treated with permethrin + PBO is quite accurate, but is slightly inflated since there is a minor contribution of unsuppressed P450 detoxification to the resistance level. This is consistent with the 70-fold resistance to permethrin + PBO in JPAL being higher than the 34-fold resistance to permethrin previously reported for a *kdr* strain of *Cx. p. quinquefasciatus* [259]. The small amount of P450 detoxification that is not inhibited by PBO is also seen when resistance ratios of *kdr* heterozygotes are compared (Table 4.3).

Table 4.3: Individual contribution of *kdr* and P450 detoxification mechanisms in permethrin resistance.

Genotype [a]		Treatment	Resulting phenotype		RR [b]	Strain
kdr	P450		*kdr*	P450		
S′/S′	R/R	permethrin	S′/S′	R/R	1,300	ISOP450
S′/S′	R/S	permethrin	S′/S′	R/S	94	SLAB x ISOP450
S′/S′	R/S	permethrin	S′/S′	R/S	100	ISOP450 x SLAB
R′/R′	R/R	permethrin + PBO	R′/R′	S/S[c]	70	JPAL
R′/S′	R/S	permethrin + PBO	R′/S′	S/S[c]	10	SLAB x JPAL
R′/S′	R/R	permethrin + PBO	R′/S′	S/S[c]	48	ISOP450 x JPAL
R′/S′	R/R	permethrin + PBO	R′/S′	S/S[c]	24	JPAL x ISOP450
S′/S′	R/R	permethrin + PBO	S′/S′	S/S[c]	5.4	ISOP450

[a] R/R = resistant homozygote, R/S = heterozygote, S/S = susceptible homozygote.

[b] RR (Resistance ratio) = LC_{50} resistant strain/LC_{50} susceptible strain.

[c] Resistance mechanism largely suppressed by PBO.

The permethrin resistance conferred by the homozygous P450 mechanism (1,300-fold) was greater than for homozygous *kdr* (70-fold). For both mechanisms, the heterozygotes conferred less resistance than the homozygotes. The P450 heterozygotes gave 94- to 100-fold resistance, and the *kdr* heterozygotes gave 10- to 48-fold permethrin resistance. Inability to completely inhibit the P450 mechanism with PBO likely contributes to the variation seen for the *kdr* heterozygotes. It is also possible that the variation seen for the *kdr* genotypes could be due to other differences present in the genetic backgrounds among the crosses.

When resistance levels were examined using the null additive mathematical model, all observed resistance ratios were greater than expected for additivity (Table 4.4). Since some genotypes of the two mechanisms had multiple resistance ratios, all are included in the analysis for a comprehensive picture of the expected range of interaction values present in this system. When homozygous for both characters, the observed resistance was 29,000-fold, while the mathematical model predicted a resistance of 1,369-fold. When homozygous for P450 detoxification and heterozygous for *kdr*, the observed relative resistances were 6,200 and 9,300-fold, while the mathematical model predicted resistance levels ranging from 1,309 to 1,347-fold. Finally, when both loci were heterozygous the observed resistance was 770-fold, but under the mathematical model the resistance levels were predicted to be from 103 to 147-fold. I was unable to obtain or isolate a strain that was heterozygous for the P450 character and homozygous for *kdr*, so I could not determine if the P450 heterozygote and *kdr* homozygote combination departed from additivity.

Table 4.4: Observed permethrin resistance compared to the expected resistance under the null additive mathematical model.

Genotype		Expected RR [a] from additive mathematical model [b]	Observed RR [a]	Strain(s) used for observed RR [a]	Observed interaction
kdr	P450				
R′/R′	R/R	1,300 + 70 - 1 = 1,369	29,000	JPAL	Non-additive
R′/S′	R/R	1,300 + (10 to 48) - 1 = 1,309 to 1,347	6,200 to 9,300	ISOP450 x JPAL & JPAL x ISOP450	Non-additive
R′/S′	R/S	(94 to 100) + (10 to 48) - 1 = 103 to 147	770	SLAB x JPAL	Non-additive

[a] RR (Resistance ratio) = LC_{50} resistant strain/LC_{50} susceptible strain.

[b] Additive mathematical interaction model: $RR_{12} = RR_1 + RR_2 - 1$. For mechanism genotypes with multiple RR (see Table 4.3), RR range using the multiple expected values are shown for a comprehensive analysis of expected values.

4.4 Discussion

In mosquitoes, permethrin resistance is wide spread and conferred by two major mechanisms, *kdr* and P450 detoxification. Interactions between resistance loci (whether additive or non-additive) are important to identify since these phenomena may contribute to high levels of resistance in the field; and understanding how mechanisms interact is important for more effective and targeted control strategies. I demonstrated that for *Cx. p. quinquefasciatus* there are multiplicative interactions between *kdr* and P450-mediated permethrin resistance mechanisms, whether the resistance alleles were homozygous or heterozygous.

The general trends reported in the literature for the interaction of insecticide resistance mechanisms are almost exclusively based on studies conducted on adult house flies (with a variety of resistance mechanisms) by topical application. Greater than additive interactions result when both resistance loci are homozygous [259,440,449,456–461] and when both loci are heterozygous, additivity is generally observed [364,370,448–454]. However, there are exceptions to these general patterns [457,459].

Studies from the insecticide resistance literature, along with literature from other organisms, which examine interactions between independent loci, have shown that all three types of joint action exist. My data show that there was significance for multiplicative interactions between *kdr* and P450 detoxification in all possible genotype combinations tested. Results from the current study follow the general trend found when both insecticide resistance mechanisms are in the homozygous state, but does not follow the trend found in house flies for interactions of heterozygous resistance alleles.

The single previous study conducted on the interaction between mechanisms of resistance (insensitive acetylcholinesterase, B1 esterase and an unidentified mechanism) in mosquitoes [367] found that when all loci were heterozygous there was epistasis. However, the epistasis was not strictly multiplicative (*i.e.*, $RR_{123} < RR_1 \times RR_2 \times RR_3$). Based on a pharmacokinetic model, they proposed the best fit to their results was a mixed interaction where resistance was equal to the unknown mechanism resistance ratio times the sum of the altered acetylcholinesterase and B1 esterase resistance ratios ($RR_{123} \approx RR_1 \times (RR_2 + RR_3)$).

The only other study that extensively examined combinations of resistance mechanism genotypes was Sawicki (1970) who investigated the interaction of *pen* and an unknown resistance gene (called gene *a*) in house flies. *Pen* alone provided 1.4-fold resistance to chlorthion-ethyl and 2.5-fold resistance to diazinon. Gene *a* alone provided 114-fold resistance to chlorthion-ethyl and 12.5-fold resistance to diazinon [449]. When both mechanisms were homozygous, there was a greater than additive interaction. Also, when either *pen* or gene *a* was homozygous with the other mechanism in the heterozygous condition, there was a greater than additive response. However, when both mechanisms were heterozygous, the results did not depart from the null additive model [449].

Conversely, I found all tested combinations of *kdr* and P450 detoxification resulted in multiplicative interactions. In an evolutionary context, it is possible that both resistance loci will be maintained in the mosquito population if the fitness costs of both resistance alleles (in the absence of insecticides) are low. Future studies will be needed to determine the fitness cost/benefit of the *kdr* and P450 detoxification resistance loci in the

absence of insecticide. By determining the presence (and direction) or absence of interactions occurring between resistance loci, it might be possible to better predict the evolutionary trajectory of resistance.

Overall, interactions between independent loci, including those of insecticide resistance mechanisms, are complex. Many variables, such as the type of resistance mechanisms present, the fitness costs and benefits associated with the mechanisms, the insecticide compound investigated, the insect species and the definition of epistasis used, complicate the ability to formulate general interaction trends. It is therefore important and necessary to analyze each system individually and to consider the interactions between all possible genotypes as well as how the fitness costs/benefits of the mechanisms can manipulate the observed interactions.

CHAPTER 5

THE EFFECT OF THREE ENVIRONMENTAL CONDITIONS ON THE FITNESS OF CYTOCHROME P450 MONOOXYGENASE-MEDIATED PERMETHRIN RESISTANCE IN *CULEX PIPIENS QUINQUEFASCIATUS* *

5.1 Introduction

Fitness of insecticide resistance mechanisms (*i.e.*, alleles) can impact the evolution of resistance in the field, as well as the persistence of the resistance phenotype in untreated environments [372]. Models of resistance evolution within a pest population are based on the premise that mutations which confer resistance are rare, but are strongly favored in insecticide treated environments. Conversely, if in the absence of insecticide selection pressures resistant individuals suffer a fitness cost, the frequency of the resistance allele will decrease, leading to a regression towards susceptibility [126,374]. In the absence of insecticide, resistant insects have been found to be less successful at overwintering [386,387,462], mating [389], predator avoidance and parasite resistance [390,394,395], and general development [380]. If no cost is associated with the resistance allele in the absence of insecticide, then it is possible for the resistance allele to become fixed in a population.

Determining the fitness of an insecticide resistance allele [374] is difficult, but can be undertaken using allele competition experiments between genetically related strains [378]. A number of methods and variables are used to assess fitness of a resistance allele. Fitness costs or benefits are most meaningful when genetically related strains are used, and may be observed

* Presented with minor modifications from the originally published article Hardstone, M. C., B. P. Lazzaro and J. G. Scott. (2009). "The effect of three environmental conditions on the fitness of cytochrome P450 monooxygenase-mediated permethrin resistance in *Culex pipiens quinquefasciatus*" BMC Evol. Biol. 9:42

more easily if alleles are in competition with each other [381]. As such, a common approach tracks allele frequencies of a population either in an untreated environment through time [195,383,384] or at one time point over a transect that includes areas that are insecticide treated and untreated [383,385]. The former of these two types of studies can be conducted in the laboratory using population cages [372]. In population cage experiments, when allele frequencies decrease, increase or remain constant through time, they are considered costly, beneficial or neutral, respectively. Failure to detect a fitness cost could be due to fitness associated with the focal allele truly being beneficial or neutral, evolution of a compensatory mutation that mitigates the cost of the resistance allele [402], or the correlated antagonistic pleiotropy maybe undetectable (either because the physiological cost might only be manifest in specific environments other than those tested [376], or the cost may affect characters that are not examined [377]). Fitness costs may be manifest under most conditions, or only under certain environments such as when populations are at high densities or when resources are poor [382].

Examining the fitness of a resistant pest species in different environments including an insecticide-free environment is important for creating successful and sustainable resistance management, and for maintaining insecticide effectiveness by elucidating the dynamics of the resistance allele and providing information for optimal timing of insecticide rotations [375]. I conducted a laboratory population cage study using genetically related strains of *Cx. p. quinquefasciatus* [380] subjected to environmental conditions that are relevant to the type of stresses this mosquito species could be exposed to in the field. Altering the environmental stresses in a laboratory setting allowed me to avoid confounding variables (such as food

availability, larval development space, and differences in microclimates) that could stress field mosquitoes.

I was interested in determining if, and how quickly, the P450 resistance allele frequency would change when it was placed in competition with the susceptible allele under environmental stress. My hypothesis was that under environmental stress the P450 detoxification resistance allele would be costly. Since this P450 detoxification mechanism was previously shown to be larval-specific (Chapter 3), measurements were conducted on larvae to see if associated costs were present. The environmental conditions imposed in the laboratory included insecticide-free standard rearing procedures, larval exposure to temephos (a bioactived OP [455]), as well as larval exposure to a colder temperature (16±1°C) representing the northern boundary for *Cx. p. quinquefasciatus* in its native geographic range. This experimental regime allowed me to examine the fitness associated with the P450-detoxification in ISOP450 in relation to controlled environmental stresses, as well as providing a greater understanding of resistance evolution when insecticide selection is relaxed.

5.2 Materials and methods

5.2.1 Mosquito strains and insecticides

Two strains of *Cx. p. quinquefasciatus* were used and reared as previously described (Chapter 3): SLAB (susceptible) and ISOP450 (permethrin resistant due to P450-mediated detoxification).

5.2.2 Selection line cages for fitness analysis of P450-mediated resistance

Selection line cages were initiated by randomly collecting at minimum 35 egg rafts from the main colony cages and rearing larvae under standard

conditions. Pupae from each strain were individually isolated into plastic tubes. Upon eclosion, the respective sex and strain were released into a cage simultaneously to create the desired crosses. The adults were released into a cage *en masse* with an approximately 3:1 (female:male) ratio, with at least 200 females used. The following reciprocal crosses were created: SLAB females x ISOP450 males (referred to as cross A), and ISOP450 females x SLAB males (referred to as cross B). Crosses were reared under standard conditions. The F_2 generation of both reciprocal crosses was split into two cage replicates (referred to as #1 and #2) for each of the three environmental conditions.

5.2.3 Standard laboratory conditions with no exposure to insecticides or other known selective pressures

In the standard laboratory environment, mosquitoes were reared under standard procedures with no exposure to insecticides or other known selective pressures which resulted in less than 1% mortality. At every generation, pupae were individually isolated so that virgin adults would be released into a new cage (minimum of 200 females and ratio of 2:1 females to males) within the same week and genotype frequencies were measured (see below). This cycle continued for a total of 12 generations.

5.2.4 Temephos exposed environmental condition

A temephos treatment generation occurred simultaneously for each replicate cage and consisted of a minimum of 300 larvae and an average of 1,600 larvae undergoing temephos selection. To apply the temephos stress, batches of 20 fourth instar larvae were placed in 4 oz. wax coated paper cups (Sweetheart Cup Co., Owings Mills, MD) with 99 ml of distilled water and 1 ml of temephos solution. The temephos concentration used throughout this

experiment (0.0003 mg/ml) resulted in 60% mortality of the F_2 generation. Control cups with 1 ml of acetone in 99 ml of distilled water were treated at the same time as treatment cups to insure that death in the treatment cups was due to temephos exposure. All cups were held at 25±1°C and larval mortality was assessed after 24 hours. Surviving treated larvae were then washed twice in clean distilled water and transferred to clean cups with 98 ml of distilled water and 2 ml of larval food. Surviving treated pupae were washed twice in clean distilled water and directly placed into individual tubes. Adults that emerged were released into a new cage that was placed in the standard rearing chamber. The succeeding generation to a treatment generation was started with a minimum of 100 surviving adult females and ratio of 2:1 females to males. Temephos treatment was performed for three consecutive generations. Genotype frequencies were measured (see below) every fourth generation and this generation would not undergo a temephos treatment. This cycle continued for a total of 12 generations.

5.2.5 Cold temperature environmental condition

A cold treatment generation occurred simultaneously for each replicate cage and consisted of a minimum of 600 larvae and an average of 1,300 larvae undergoing cold stress. To apply the cold temperature condition, batches of 20 fourth instar larvae were placed in 4 oz. wax coated paper cups with 98 ml of distilled water and 2 ml of larval food. The cups were then placed in a 16±1°C incubator for 4 consecutive days with a photoperiod of 14 hr:10 hr (L:D). These conditions resulted in an average of 60% mortality for each treatment generation. Simultaneously, cups serving as treatment controls were placed in a room temperature incubator (25±1°C) with a photoperiod of 14 hr:10 hr (L:D) to insure handling and food availability were not the cause of mortality in the

treatment. On day 4, cold-treated pupae were place into individual tubes to complete development to adult at 25±1°C. Adult emergence rates were recorded in the treatment and control cups to determine mortality (*i.e.*, selection pressure). The succeeding generation to a treatment generation was started with a minimum of 100 surviving adult females and ratio of 2:1 females to males. Cold treatment was performed for three consecutive generations. Genotype frequencies were measured (see below) every fourth generation and this generation would not undergo a cold treatment. This cycle continued for a total of 12 generations.

5.2.6 Measuring genotype frequencies

Genotype frequencies were determined by performing bioassays on fourth instar larvae. The diagnostic concentrations to ascertain genotype frequencies were determined from the concentration-mortality curve of the F_2 generation (Figure 5.1). The plateau at approximately 25% mortality (0.0078 μg/ml permethrin) clearly distinguished susceptible homozygotes (S/S) while the plateau at approximately 75% mortality (1 μg/ml permethrin) separated S/S and heterozygotes (R/S) from the resistant homozygotes (R/R).

For each larval bioassay, batches of 20 fourth instar larvae were placed in 4 oz. wax coated paper cups with 99 ml of distilled water and 1 ml of permethrin solution. Half of the available larvae were treated at each of the diagnostic permethrin concentrations such that mortality at 0.0078 μg/ml indicated the frequency of S/S genotypes, survivorship at 1 μg/ml specified the frequency of R/R genotypes and R/S genotype frequencies were determined by taking 1 - S/S genotype frequency - R/R genotype frequency. The total number of larvae used ranged from 80-1,800 (Tables 5.1, 5.2, 5.3) depending on the number of eggs laid in a cage. Test insecticide dilutions were

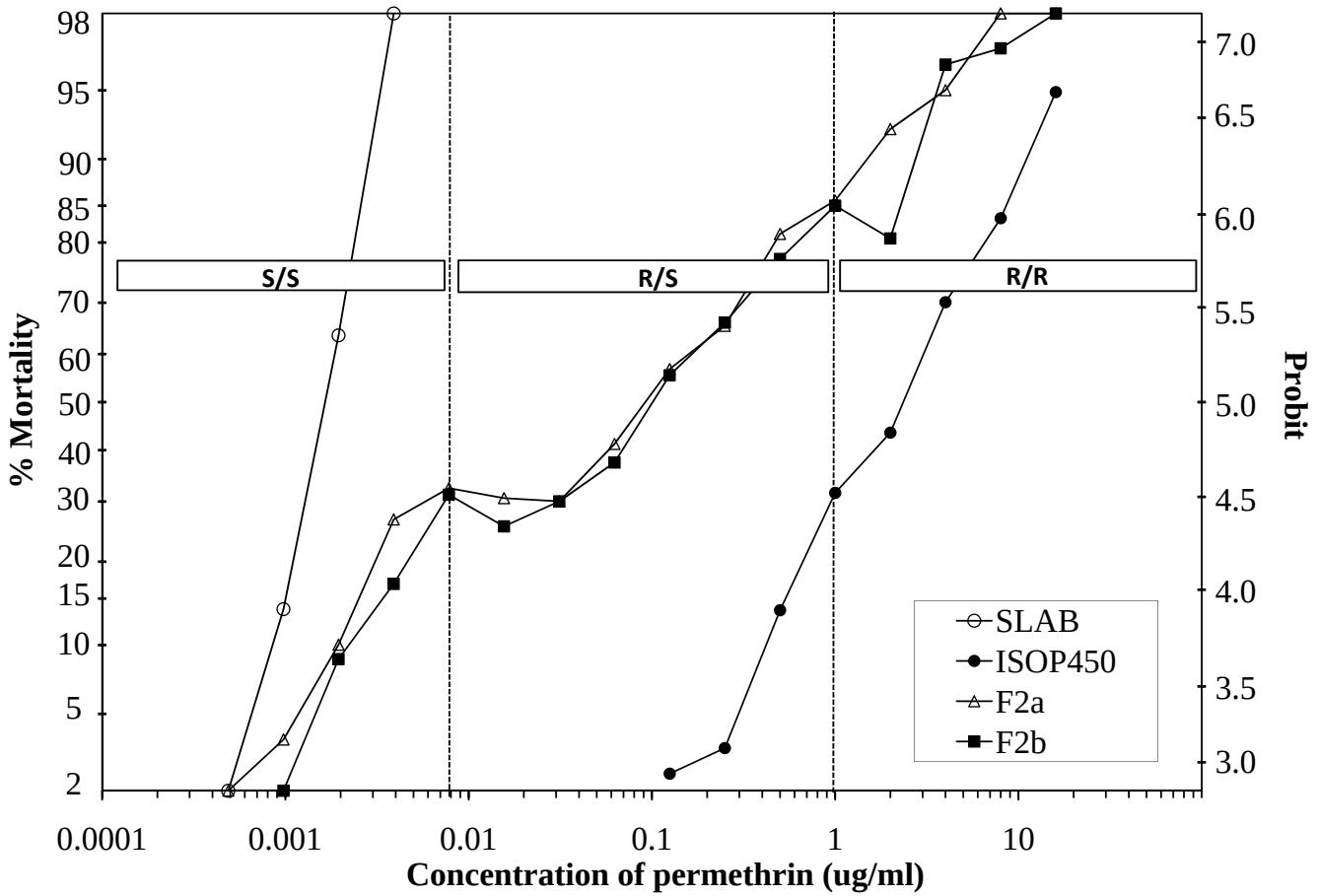


Figure 5.1: Concentration-mortality lines of parental SLAB and ISOP450 and reciprocal F_2 generations. Vertical dashed lines indicate diagnostic concentrations used to distinguish S/S, R/S and R/R genotypes (shown in boxes) of the P450 mechanism.

dissolved in acetone and control cups were treated with 1 ml of acetone in 99 ml of distilled water. Mortality was assessed after 24 hours, and larvae were considered dead if they failed to move or resurface after being probed.

5.2.7 Statistical and data analysis

Genotype frequencies were inferred from arc-sin transformed concentration dependent mortalities. Expected genotype frequencies (Tables 5.1, 5.2, 5.3) were calculated by using the observed allele frequencies from the previous generation. In all environments at all generations where genotype frequencies were measured, deviations from HWE were analyzed with a χ^2 test (p<0.05). To account for multiple testing, data were subjected to Bonferroni correction. Using Fisher's combined probabilities test, I determined a global p-value for a set of HWE tests associated with a particular environment.

The probability of R allele frequency changes due to genetic drift acting alone between generations was calculated from the binomial distribution. For the temephos and cold environments, the probability of R allele frequency changes due to drift over the 2 and 4 generation intervals were simulated using R (Appendix C). The model assumed a panmictic population with fixed size of 200 diploid individuals with allele frequencies defined by the empirical observation at the start of the interval and was run for 10,000 simulations. The average of five p-values was reported and subjected to Bonferroni correction. Fisher's combined probabilities test was used to determine a global p-value for a set of genetic drift tests from a particular environment.

For the determination of R allele frequency changes, all crosses and replicates of an environmental condition were combined (n = 4). Statistical differences between means were determined using Tukey's test (p<0.05).

5.3 Results

These results show that fitness costs can be associated with the cytochrome P450-mediated permethrin detoxification resistance allele in *Cx. p. quinquefasciatus* in the tested environmental conditions (Figure 5.2) and that the strength of the cost varies depending on the environmental conditions. When fitness is assessed using resistant (R) allele frequencies, P450 detoxification is most costly in the temephos exposed environment, minorly costly in the standard laboratory environment, and not costly in the cold temperature environment.

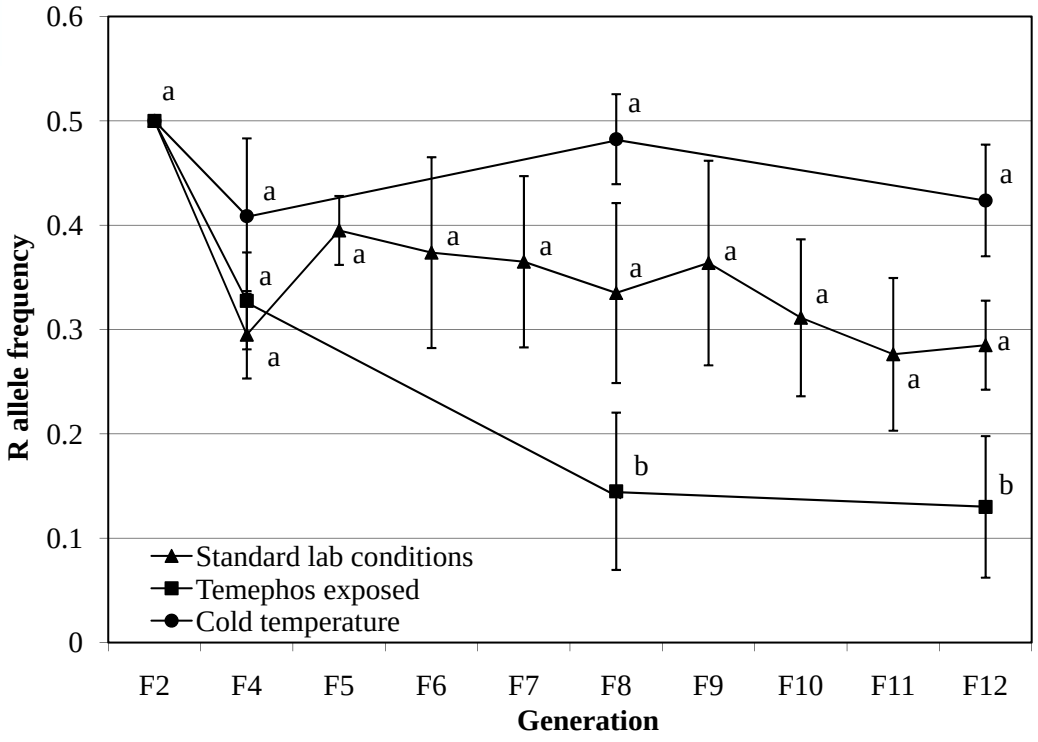


Figure 5.2: Frequencies of the P450-mediated detoxification R allele monitored through time in three environmental conditions: standard laboratory rearing, temephos exposure and cold temperature. Results are the averages of the four replicates for each environment. Bars are the S.E. Different letters indicate statistical differences (p<0.05) between means.

5.3.1 Standard laboratory rearing conditions

In the absence of insecticide, the R allele frequency changed from a starting frequency of 0.5 to 0.295 in F_{12} (Figure 5.2). While the frequency differences were not found to be statistically different due to the level of variation between replicates (Tukey's HSD, $p>0.05$), throughout the experiment the trend of a declining R allele frequency was steady with an exception at F_4 where a larger dip occurred. Examination of the genotypes shows that the homozygous resistant genotype (R/R) decreases, and for most generations stays below 0.1 (Figure 5.3). This trend was observed for both crosses (A and B) and for both replicates for each of these crosses, although there was some variation between generations (Table 5.1). This data indicates that the fitness cost of being R/R is greater than the cost of having a single R allele in this environment. Interestingly, the R/S genotype stays at a frequency of approximately 0.5, while the S/S genotype tends to increase in frequency over the 12 generations (Table 5.1 and Figure 5.3). Frequency changes in the three genotypes ultimately leads to the trend of a decreasing frequency of the R allele and an increase in the S allele frequency.

Across all replicates, there are multiple generations where the genotype frequencies are not in HWE indicating that selection or genetic drift are acting on these generations (Table 5.1). This is true for F_4 through F_{11} in cross A#1; F_4 through F_{12} in cross B#1; and F_4 through F_{12}, excluding F_9, in cross B#2. Using the allele frequency data, the null hypothesis of frequency changes being due to genetic drift can not be rejected for F_6, F_7, F_9 and F_{10} of cross A#1; F_4, F_5, F_7, F_9 and F_{12} of cross A#2; F_5, F_6, F_8, F_9, F_{10} and F_{12} of cross B#1; and F_6 and F_{10} in cross B#2. However, many of the same generations that reject genetic drift are also out of HWE (Table 5.1). This means that despite being in a laboratory

setting where many conditions are optimized (food, space, etc.), these populations were probably undergoing selection (and not genetic drift (Fisher's combined probability, χ^2_{72}=417, p<0.0001)) since the generations are out of HWE (Fisher's combined probability, χ^2_{72}=492, p<0.0001) and there was no assortative mating.

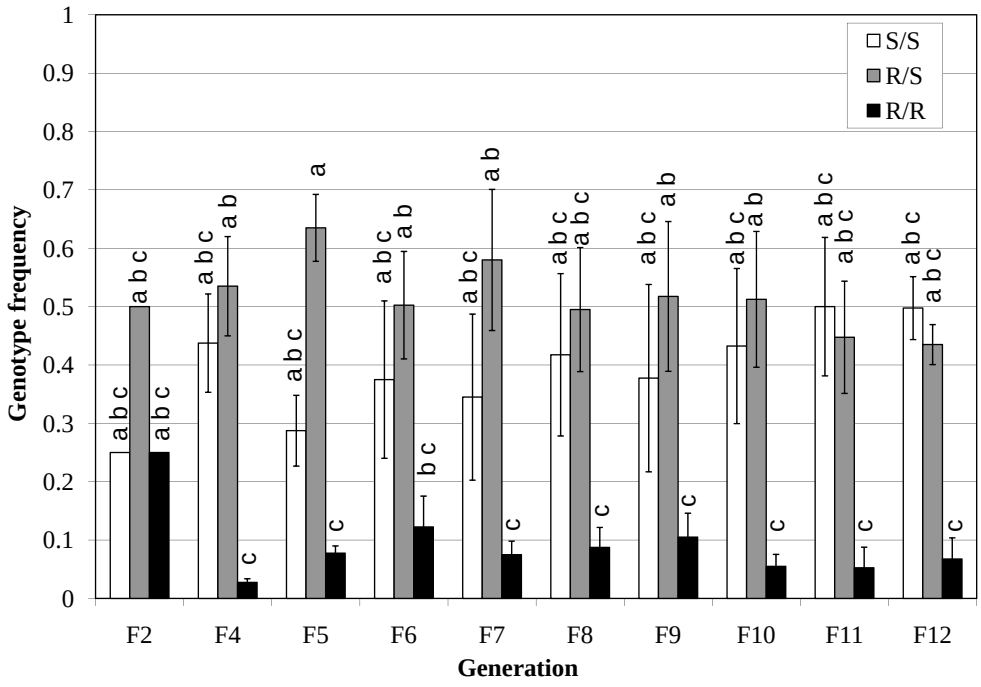


Figure 5.3: Frequencies of the P450-mediated detoxification genotypes monitored every generation for 12 generations in the standard laboratory environmental condition. Results are the mean ± S.E. (n=4). Different letters indicate statistical differences (p<0.05) between means.

Table 5.1: P450 genotype and allele frequencies in a standard laboratory environment.

Cross	Generation	Genotype frequency Observed (Expected)			Allele frequency		n [a]	HWE [b]	Drift [b]
		S/S	R/S	R/R	S	R			
A#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.69 (0.69)	0.28 (0.28)	0.03 (0.03)	0.83	0.17	120	0.9203	< 0.0001*
	F_5	0.31 (0.38)	0.62 (0.47)	0.07 (0.15)	0.62	0.38	960	< 0.0001*	< 0.0001*
	F_6 #	0.31 (0.34)	0.55 (0.49)	0.14 (0.17)	0.59	0.41	1,040	< 0.0001*	0.0105
	F_7 #	0.24 (0.33)	0.67 (0.49)	0.09 (0.18)	0.58	0.42	1,560	< 0.0001*	0.2039
	F_8	0.19 (0.27)	0.66 (0.50)	0.15 (0.23)	0.52	0.48	900	< 0.0001*	0.0004*
	F_9 #	0.17 (0.24)	0.63 (0.50)	0.20 (0.26)	0.49	0.51	1,640	< 0.0001*	0.0023
	F_{10} #	0.21 (0.30)	0.68 (0.50)	0.11 (0.20)	0.55	0.45	1,760	< 0.0001*	1
	F_{11}	0.24 (0.35)	0.70 (0.48)	0.06 (0.17)	0.59	0.41	1,520	< 0.0001*	0.0009*
	F_{12}	0.47 (0.46)	0.41 (0.43)	0.12 (0.11)	0.68	0.32	400	0.1900	0.0002*
A#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.35 (0.43)	0.61 (0.45)	0.04 (0.12)	0.66	0.34	80	0.0018	0.0024
	F_5	0.44 (0.46)	0.48 (0.44)	0.08 (0.10)	0.68	0.32	420	0.0349	0.1427
	F_6	0.76 (0.77)	0.23 (0.22)	0.01 (0.01)	0.88	0.12	1,200	0.0748	< 0.0001*

Table 5.1 (Continued):

	F_7	0.77 (0.77)	0.22 (0.22)	0.01 (0.01)	0.88	0.12	1,440	0.1138	0.2772
	F_8	0.79 (0.79)	0.20 (0.20)	0.01 (0.01)	0.89	0.11	900	0.5199	< 0.0001*
	F_9	0.83 (0.83)	0.17 (0.16)	0 (0.01)	0.92	0.08	560	0.0279	0.0252
	F_{10}	0.78 (0.78)	0.21 (0.21)	0.01 (0.01)	0.89	0.11	480	0.4884	< 0.0001*
	F_{11}	0.36 (0.36)	0.49 (0.48)	0.15 (0.16)	0.61	0.39	520	0.5651	< 0.0001*
	F_{12}	0.38 (0.38)	0.48 (0.48)	0.14 (0.14)	0.62	0.38	240	0.7719	0.3329
B#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.35 (0.44)	0.62 (0.45)	0.03 (0.11)	0.66	0.34	160	< 0.0001*	< 0.0001*
	F_5 #	0.25 (0.36)	0.70 (0.48)	0.05 (0.16)	0.60	0.40	320	< 0.0001*	0.0144
	F_6 #	0.13 (0.19)	0.61 (0.49)	0.26 (0.32)	0.44	0.56	760	< 0.0001*	1
	F_7	0.19 (0.29)	0.69 (0.50)	0.12 (0.21)	0.54	0.46	1,440	< 0.0001*	< 0.0001*
	F_8 #	0.22 (0.29)	0.64 (0.50)	0.14 (0.21)	0.54	0.46	800	< 0.0001*	0.4023
	F_9 #	0.13 (0.27)	0.77 (0.50)	0.10 (0.23)	0.52	0.48	1,640	< 0.0001*	0.0209
	F_{10} #	0.24 (0.35)	0.71 (0.48)	0.05 (0.16)	0.60	0.40	680	< 0.0001*	0.1192
	F_{11}	0.73 (0.75)	0.27 (0.23)	0 (0.02)	0.87	0.13	680	< 0.0001*	< 0.0001*
	F_{12} #	0.64 (0.66)	0.35 (0.30)	0.01 (0.04)	0.82	0.18	880	< 0.0001*	0.9999

Table 5.1 (Continued):

B#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.36 (0.46)	0.63 (0.44)	0.01 (0.10)	0.68	0.32	300	$< 0.0001^*$	$< 0.0001^*$
	F_5	0.15 (0.27)	0.74 (0.50)	0.11 (0.23)	0.52	0.48	880	$< 0.0001^*$	$< 0.0001^*$
	F_6 #	0.30 (0.37)	0.62 (0.48)	0.08 (0.15)	0.61	0.39	1,440	$< 0.0001^*$	1
	F_7	0.18 (0.30)	0.74 (0.50)	0.08 (0.20)	0.55	0.45	1,440	$< 0.0001^*$	$< 0.0001^*$
	F_8	0.47 (0.50)	0.48 (0.42)	0.05 (0.08)	0.71	0.29	520	0.0002^*	$< 0.0001^*$
	F_9	0.38 (0.40)	0.50 (0.47)	0.12 (0.13)	0.63	0.37	920	0.0279	$< 0.0001^*$
	F_{10} #	0.50 (0.53)	0.45 (0.40)	0.05 (0.07)	0.73	0.27	1,800	$< 0.0001^*$	1
	F_{11}	0.67 (0.70)	0.33 (0.28)	0 (0.02)	0.84	0.16	1,000	$< 0.0001^*$	$< 0.0001^*$
	F_{12}	0.50 (0.56)	0.50 (0.38)	0 (0.06)	0.75	0.25	1,700	$< 0.0001^*$	$< 0.0001^*$

[a] number of fourth instar larvae used in genotype monitoring assay.

[b] Nominal *p*-values are reported for the probability that the data fit the null hypothesis of HWE or genetic drift.

* indicates tests that remain statistically significant after Bonferonni correction over the entire experiment.

# indicates generation is out of HWE likely due to drift.

5.3.2 Temephos exposed environmental condition

The temephos exposed environment proved to be extremely detrimental to the maintenance of the R allele and R/R genotype and more costly than the standard laboratory condition. The P450 detoxification R allele dramatically decreases from the F_2 to the F_8 generation where the frequency drops significantly to 0.13 from 0.5 (Figure 5.2). Between generations 8 and 12 the frequency plateaus, but if given enough time the R allele should be eliminated since at these two time points the R alleles are primarily in the heterozygotes (Table 5.2). The genotype pattern follows that expected from my original hypothesis. In the temephos exposed environment the R/R genotype almost completely disappears, the R/S genotype decreases from 0.5 (at F_2) to 0.25 (at F_8 and F_{12}), while the S/S genotype undergoes an increase in frequency (Figure 5.4) where two of the four cages become fixed for the S allele. In cage A#1, the R/R and R/S genotype are completely eliminated by generation F_4 and F_{12}, respectively and in cage A#2 by F_8 and F_{12}, respectively.

The elimination of the R allele is faster in the A cross versus the B cross, though the B cross has a matching pattern where the R allele decreases through time and the R/R genotype is eliminated from the population (Table 5.2). As expected, the temephos treated replicates are undergoing selection since they are out of HWE (Fisher's combined probability, χ^2_{24}=115, p<0.0001) and this is not due to drift (Fisher's combined probability, χ^2_{24}=137, p<0.0001). Previously, I determined that the P450 in ISOP450 bioactivates temephos, and that this P450 is tightly linked to (or identical to) the permethrin resistance allele. Thus, the R allele in this environment is even more costly to the animal and is eliminated at a faster rate than the R allele in the standard environment.

Table 5.2: P450 genotype and allele frequencies in a temephos treated environment.

Cross	Generation	Genotype frequency Observed (Expected)			Allele frequency		n [a]	HWE [b]	Drift [b]
		S/S	R/S	R/R	S	R			
A#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.44 (0.52)	0.56 (0.40)	0 (0.08)	0.72	0.28	180	< 0.0001*	< 0.0001*
	F_8	0.98 (0.98)	0.02 (0.02)	0 (0)	0.99	0.01	560	0.8113	< 0.0001*
	F_{12}	1 (1)	0 (0)	0 (0)	1	0	220	1	0.2175
A#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.35 (0.45)	0.64 (0.44)	0.01 (0.11)	0.67	0.33	280	< 0.0001*	< 0.0001*
	F_8	0.98 (0.98)	0.02 (0.02)	0 (0)	0.99	0.01	1,640	0.6828	< 0.0001*
	F_{12}	1 (1)	0 (0)	0 (0)	1	0	440	1	0.2207
B#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.19 (0.28)	0.67 (0.50)	0.14 (0.23)	0.53	0.34	200	< 0.0001*	0.2486
	F_8	0.66 (0.66)	0.31 (0.30)	0.03 (0.03)	0.82	0.18	1,060	0.3617	< 0.0001*
	F_{12}	0.60 (0.63)	0.39 (0.33)	0.01 (0.04)	0.80	0.20	1,040	< 0.0001*	0.3089
B#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.59 (0.60)	0.37 (0.35)	0.04 (0.05)	0.78	0.22	280	0.3078	< 0.0001*

Table 5.2 (Continued):

F_8	0.30 (0.39)	0.65 (0.47)	0.05 (0.14)	0.63	0.37	1,640	$< 0.0001^*$	0.0052
F_{12}	0.38 (0.47)	0.61 (0.43)	0.01 (0.10)	0.69	0.31	80	0.0002^*	0.1150

[a] total number of fourth instar larvae used in genotype monitoring assay.

[b] Nominal *p*-values are reported for the probability that the data fit the null hypothesis of HWE or genetic drift.

* indicates tests that remain statistically significant after Bonferonni correction over the entire experiment.

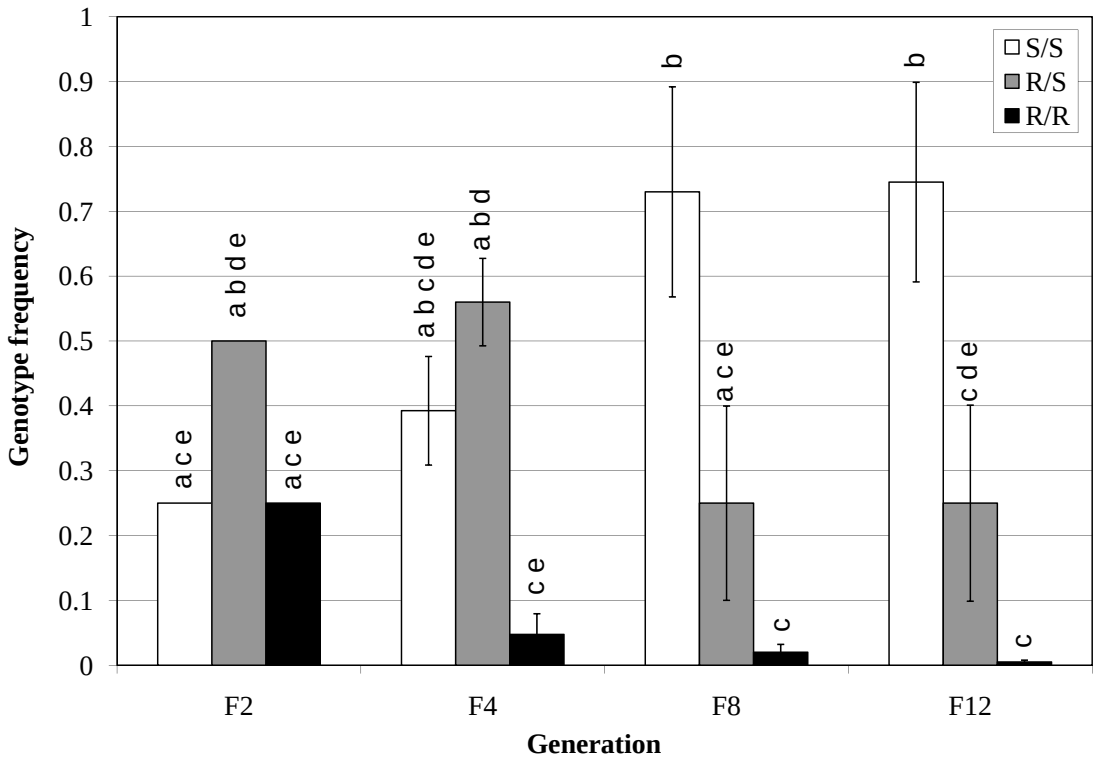


Figure 5.4: Frequencies of the P450-mediated detoxification genotypes monitored every 3 generations for a total of 12 generations in the temephos exposed environmental condition. Results are the mean ± S.E. (n=4). Different letters indicate statistical differences (p<0.05) between means.

5.3.3 Cold temperature environmental condition

In the cold temperature environment, no fitness cost of the P450 detoxification R allele frequency was observed. Despite frequency shifts between generations, the R allele stays in equilibrium around a frequency of 0.45 (Figure 5.2). This equilibrium frequency is not statistically different from the starting frequency of 0.5 (χ^2_7=0.816, p=0.997). In three of the four cages (A#1, B#1 and B#2), the R/R genotype decreases to frequencies of 0, 0.02 and 0.03, respectively. Surprisingly, there was a clear heterozygote advantage which

allowed the R allele to persist in the cold temperature treated population (Figure 5.5).

The S/S genotype remains at frequencies close to the F_2 frequency, ending in the F_{12} generation at 0.32, 0.27 and 0.23 for cages A#1, B#1 and B#2, respectively. Despite the A#2 cage R/R frequency in generation 12 being 0.16 and the S/S genotype frequency being 0, there was still a heterozygote advantage evident in this cage (Table 5.3). Almost all (11 of 12) of the monitored generations are out of HWE (Fisher's combined probability, χ^2_{24}=203, p<0.0001) and the null hypothesis that drift is acting on the generations was rejected (Fisher's combined probability, χ^2_{24}=99, p<0.0001) (Table 5.3), indicating that cold selection is driving the changes in genotype and allele frequencies.

5.4 Discussion and Conclusion

My results show that the fitness associated with cytochrome P450 monooxygenase-mediated permethrin resistance in *Cx. p. quinquefasciatus* varies according to the environment. There is a potential minor cost associated with this P450 detoxification mechanism when in competition with a susceptible allele under standard rearing conditions and a greater cost when exposed to temephos. I also determined that a heterozygote advantage is present when in the cold temperature environment.

Intriguingly, all replicates in the cold environment exhibited an excess of the heterozygote genotype. The R/R genotype did poorly (n = 4), indicating that having two R alleles is costly. A similar pattern was shown in *T. castaneum* where the S allele was placed in direct competition with an allele that conferred malathion resistance (R^{mal}). Over 10 generations, malathion resistant strains had a heterozygote advantage and the best fitness [410]. Observations of

Table 5.3: P450 genotype and allele frequencies in a cold temperature environment.

Cross	Generation	Genotype frequency Observed (Expected)			Allele frequency		n [a]	HWE [b]	Drift [b]
		S/S	R/S	R/R	S	R			
A#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.46 (0.53)	0.54 (0.39)	0 (0.08)	0.73	0.27	280	< 0.0001*	< 0.0001*
	F_8	0.26 (0.38)	0.71 (0.47)	0.03 (0.15)	0.62	0.38	920	< 0.0001*	0.0066
	F_{12}	0.32 (0.44)	0.68 (0.45)	0 (0.11)	0.66	0.34	160	< 0.0001*	0.1841
A#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.21 (0.21)	0.49 (0.49)	0.30 (0.30)	0.46	0.54	310	0.8320	0.1068
	F_8	0.04 (0.17)	0.73 (0.48)	0.23 (0.35)	0.41	0.0591	1,280	< 0.0001*	0.1716
	F_{12}	0 (0.17)	0.84 (0.49)	0.16 (0.34)	0.42	0.58	1,040	< 0.0001*	0.3875
B#1	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.12 (0.22)	0.70 (0.50)	0.18 (0.28)	0.47	0.53	140	< 0.0001*	0.2041
	F_8	0.07 (0.28)	0.86 (0.50)	0.04 (0.22)	0.53	0.47	1,160	< 0.0001*	0.1204
	F_{12}	0.27 (0.39)	0.71 (0.47)	0.02 (0.14)	0.63	0.37	560	< 0.0001*	0.0284
B#2	F_2	0.25	0.50	0.25	0.50	0.50			
	F_4	0.45 (0.50)	0.52 (0.41)	0.03 (0.09)	0.71	0.29	270	< 0.0001*	< 0.0001*

Table 5.3 (Continued):

F_8	0.19 (0.27)	0.66 (0.50)	0.15 (0.23)	0.52	0.48	840	$< 0.0001^*$	$< 0.0001^*$
F_{12}	0.23 (0.36)	0.74 (0.48)	0.03 (0.16)	0.60	0.40	800	$< 0.0001^*$	0.0529

[a] total number of fourth instar larvae used in genotype monitoring assay.

[b] Nominal *p*-values are reported for the probability that the data fit the null hypothesis of HWE or genetic drift.

* indicates tests that remain statistically significant after Bonferonni correction over the entire experiment.

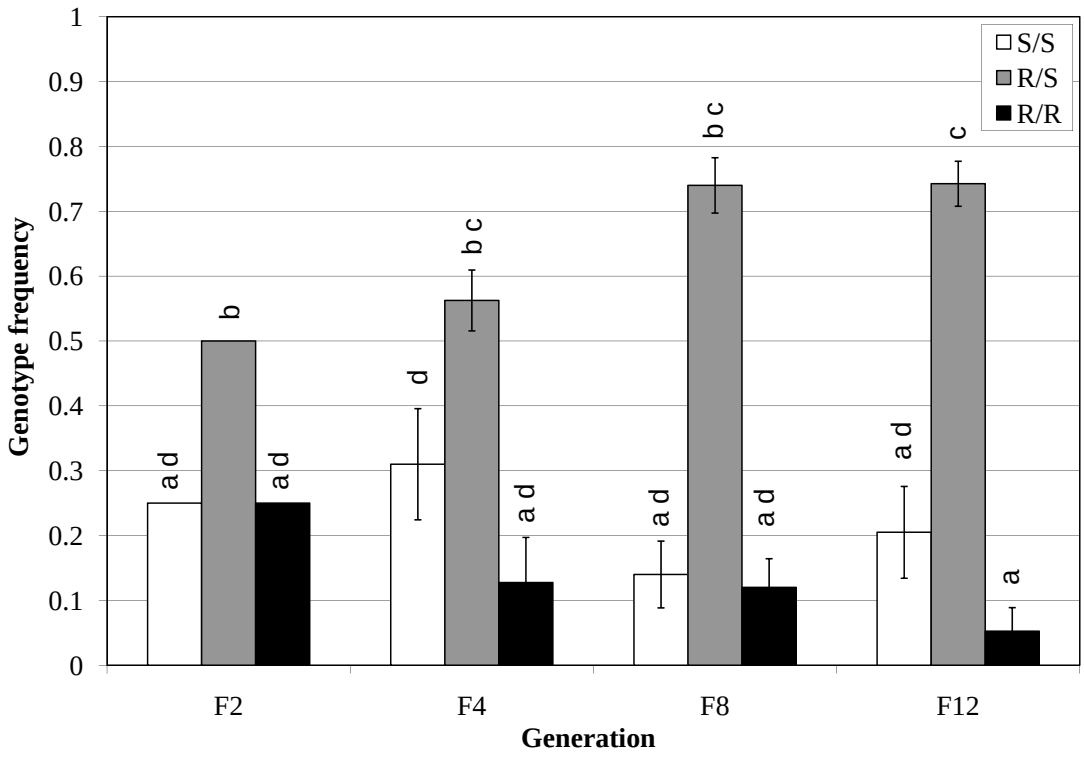


Figure 5.5: Frequencies of the P450-mediated detoxification genotypes monitored every 3 generations for a total of 12 generations in the cold temperature environmental condition. Results are the mean ± S.E. (n=4). Different letters indicate statistical differences (p<0.05) between means.

heterozygote advantage are rare and this phenomenon may contribute to the maintenance of genetic variation in populations [463].

Conversely, the P450 detoxification mechanism under temephos exposure was shown to be highly costly. In the temephos exposed environment, the R/R genotype frequency was entirely eliminated in two replicates and decreased to an average frequency of 0.0475 by the F_4 generation. Results from Chapter 3 demonstrated low (0.73-fold), but significant, levels of bioactivation to temephos in ISOP450 [455]. This is likely

one of the reasons underlying the fitness cost of the R allele in the temephos treated environment. This suggests that vector control efforts could use temephos in populations with this P450 allele since it may serve as a method to slow the evolution of permethrin resistance. In a temephos treated environment, a population with this R allele would not be favored and a shift towards susceptibility at this locus could occur over time.

Fitness studies on insecticide resistant insects have shown that resistance mechanisms are costly [383,385,390,392,464]. In this experiment I examined the fitness of the P450 resistance allele under multiple environmental conditions, whereas several previous population cage studies limited the environmental condition to a relaxation of the insecticide selection pressure [465–467]. For example, the R allele in a strain of *Cx. p. quinquefasciatus* which was resistant to OPs due to elevated levels of Esterase-2 declined steadily when left unselected for 3 generations [465].

Population cage studies do not always show that a fitness cost is associated with the insecticide R allele. Examination of malathion R alleles in *T. castaneum* under no known selection (including insecticide) pressure in a laboratory population cage experiment revealed the R allele is not costly and the frequency of this allele remained steady through time, independent of the starting R allele frequency [381]. Fluctuations in the R allele frequency between generations were concluded to be the result of random genetic drift [381].

Few studies have examined what happens to the R allele in a population cage study under sub-optimal environmental conditions. Raymond et al. (2005) placed two strains of diamondback moth (*P. xylostella*) with the same resistance mechanism conferring resistance to *Bti* in two different stressful environments in the lab. The two strains exhibited different fitness costs

relative to one another under the same stressor and the fitness cost varied in each environment for an individual strain [382]. In contrast, no fitness costs were observed in a diazinon-resistant strain of Australian sheep blowfly (*L. cuprina*) when reared under three larval densities or in a population cage competition experiment [403].

Resistance mechanisms due to metabolic processes are generally regarded as costly [468,469]. Some studies have proposed that the extent to which a metabolic resistance mechanism is costly depends on the relative expression of the protein in the R strain versus the S strain [384]. Insecticide resistance due to gene amplification or gene duplication of general esterases is highly costly due to the resulting amount of enzyme which is produced. For example, in highly resistant green peach aphids, esterase amounts to approximately 3% of the total body protein [470]. Contrastingly, costs associated with increased monooxygenase activity have been found to be modest or absent. This phenomena has been shown using biotic potential measures in the house fly [471], laboratory, glasshouse and field studies measuring life-table parameters, predator-prey interactions, and mating competitiveness of the predatory mite, *Metaseiulus occidentalis* [472,473], in *H. zea* after feeding on a diet with a P450 inducer and measuring food utilization parameters [474], and with P450-mediated resistance to DDT in *D. melanogaster* [400]. However, even when the same insecticide is used, there can be plasticity in which P450(s) are selected for in different populations [258]. This could lead to variation in fitness costs between populations, due to the different P450s conferring the resistance.

Determining the cost of resistance is imperative for implementing successful vector control strategies. As evident from the literature and these

results, resistance alleles are able to perform differentially (manifest varied levels of cost) in different environments. Additionally, in accordance with previous studies on insecticide resistance mechanisms, a minor fitness cost is associated with this P450 locus in an insecticide-free environment. It is also possible that differences present in the genetic backgrounds among the crosses could affect the fitness of the resistance allele. Understanding how resistant pest populations evolve under a spectrum of environmental conditions is essential if resistance management tactics are to be successful. Results from this study have implications for mosquito control strategies. For example, rotating permethrin and temephos insecticides should slow the evolution of P450-mediated permethrin resistance. Additionally, the permethrin R allele can be maintained within the colder upper boundary of the *Cx. p. quinquefasciatus* habitat, where a hybrid region with *Cx. p. pipiens* is present. This could lead to an introduction of the *Cx. p. quinquefasciatus* P450 R allele into populations of *Cx. p. pipiens* and impact the control of this species as well.

CHAPTER 6

DIFFERENCES IN DEVELOPMENT, GLYCOGEN AND LIPID CONTENT ASSOCIATED WITH CYTOCHROME P450-MEDIATED PERMETHRIN RESISTANCE IN *CULEX PIPIENS QUINQUEFASCIATUS*

6.1 Introduction

The southern house mosquito, *Cx. p. quinquefasciatus*, is found throughout the southern hemisphere and is the most important urban vector of the nematode, *Wuchereria bancrofti* (the causative agent of lymphatic filariasis), and other pathogens of humans and animals [4]. Controlling adults of this species and other insect vectors of disease relies almost exclusively on the use of insecticides and especially the pyrethroid permethrin [22,27]. Pyrethroid insecticides are used to treat bed nets and for indoor residual spraying campaigns. Resistance to pyrethroids in *Culex* mosquitoes is conferred primarily by enhanced detoxification due to cytochrome P450 monooxygenases and target site insensitivity (L1014F point mutation, called *kdr*) [147,455].

Fitness studies in the context of metabolic-based insecticide resistance mechanisms in mosquitoes have focused on characteristics associated with esterase-based resistance [310,380,383,475]. In these studies, fitness costs have been implied due to lowered reproductive output [389], decreased predator avoidance and parasite resistance [392,394,395] and lower overwintering survival [387]. Thus far, a single study in mosquitoes has been conducted to examine the fitness specifically associated with cytochrome P450-mediated resistance. In that study, a population cage experiment in which the R allele (from the ISOP450 strain) was placed in direct competition with the S allele (from the SLAB strain) were monitored through time. Under the standard

laboratory rearing procedures (with no exposure to insecticides), the R allele frequency decreased over time implying that a cost was associated with P450-mediated permethrin resistance [476].

The evolution of insecticide resistance in populations has been based on observations that mutations which confer resistance to insecticides are rare prior to insecticide application, increase in frequency only during insecticide application, and decrease in frequency in the absence of insecticide use [126,374]. Therefore these mutations are generally believed to be costly in the absence of insecticides. Fitness costs of resistance alleles in the absence of insecticides could be due to the reallocation of resources, changes in metabolic functions or alteration of developmental processes [372,374]. Since P450-mediated permethrin resistance in the resistant ISOP450 strain is known to be expressed in larvae [455], examination of larval parameters was pursued. While a myriad of parameters are available to examine fitness in the adult stage, particularly those associated with reproductive capabilities, many of these factors are not possible to measure in the larval stage. Consequently, in order to determine the physical condition (fitness) of the larvae, energetic resources were measured along with mortality and development time.

Food is metabolized to provide necessary energy reserves in the form of nutrients such as carbohydrates (glucose, trehalose and glycogen) and lipids for insects to complete development, perform basic metabolic processes, engage in flight and reproductive activities [477] and provide substrates for the normal functioning of the nervous system [478]. Glucose is not readily used by insects for direct energy utilization; rather it is primarily used as a precursor in the synthesis of glycogen and trehalose [479] and therefore is usually found in low concentrations [480]. Glycogen can also be metabolized into trehalose, a

storage sugar found in the hemolymph of insects, which provides immediate energy resources. Glycogen is used as an immediate source of energy in the early stages of flight [477] since it is stored within the cell and there is no time needed to transport the molecule [481]. During insect development and particularly during feeding phases, ingested carbohydrates are converted into lipids [481–483]. Lipids are utilized during molting, prolonged flight, oogenesis and normal body functions [484]. Therefore, energy reserves of larvae and adults can alter survival and energy reserves used for flight possibly altering mating, host seeking, and oviposition behaviors.

The aim of this study was to measure biological parameters associated with development and energy resources between a highly related (>99.9%) resistant (ISOP450) and susceptible (SLAB) strain. If ISOP450 larvae and adults were slower to develop, had increased mortality and accumulated lower energy reserves as compared to the susceptible SLAB strain, these patterns could explain the previously observed fitness cost present in the ISOP450 strain [476]. Differences in the biological parameters whereby SLAB out-performs ISOP450 are suggestive of a fitness cost being associated with the P450 resistance allele.

6.2 Materials and methods

6.2.1 Mosquito strains and experimental setup

SLAB is a susceptible laboratory strain of *Cx. p. quinquefasciatus* which has been in colony for ~40 years without exposure to insecticides [420]. JPAL is a permethrin resistant strain originally collected in 1984 from Saudi Arabia and subsequently was selected with permethrin for 20 generations. It contains P450-mediated detoxification and *kdr* [147]. ISOP450 is a strain of *Cx. p. quinquefasciatus* which has been in colony since 2005 and is highly related

(>99.9%) [380] to SLAB. ISOP450 was isolated through repeated backcrosses of JPAL to SLAB with permethrin selection of backcross progeny such that the final strain was 1,300-fold resistant to permethrin conferred by cytochrome P450 monooxygenase detoxification [455]. Permethrin resistance in ISOP450 is incompletely dominant, autosomally linked, monofactorially inherited, and expressed in the larvae, but not in adults [455]. Strains were maintained as previously described in Chapter 3 except colony cages were provided an anesthetized mouse for 30 min twice per week (Cornell University Approved Animal Use Protocol #2001-0056).

Mosquito body size determinations were made using an Olympus DF PLAPO dissecting microscope with an Olympus DP25 camera and DP2-B5W (Olympus) computer software. Fourth instar larvae were immobilized by placing them on a piece of filter paper and the width of the thorax was measured as an indication of body size [485]. Adult body size was determined by measuring the length of the right wing, removed and mounted on a glass microscope slide with clear tape, from the axillary incision to the apical margin, not including fringe [486].

6.2.2 Development and mortality parameters

Measurements of developmental parameters were made by placing 24 newly hatched first instar larvae individually into a 60 ml plastic cup (Fabri-Kal, Kalamazoo, MI) with 50 ml of distilled water and 18 mg of powered food consisting of a 1:2:1 mixture of ground TetraFin® goldfish flakes (Spectrum, Atlanta, GA), ground rabbit pellets (Hartz Mountain Corporation, Secaucus, NJ) and liver powder (MP Biomedicals, Solon, OH) for a total of 12 replicates. First instars were obtained from a minimum 14 egg rafts. Mortality or pupation of the larvae was recorded at 24 hr intervals. Pupae were

transferred to individual plastic test tubes containing 3 ml of distilled water. Tubes were monitored every 24 hrs for mortality or adult emergence. Newly eclosed adults were kept individually in 177 ml paper hot cups (International Paper, Memphis, TN) covered with mesh. Each sex was divided into two cohorts. One was continuously provided cotton saturated with 20% sugar water, while the other received distilled water. Adults were monitored at 24 hr intervals for mortality. Upon death, wings were removed and measured to estimate body size as described above. All life stages were maintained at 27±1°C.

6.2.3 Nutritional energetic measurements

Egg rafts were collected *en masse* from colony cages. To obtain large sized animals for experimentation within the range of large sizes collected in the field [487], 75 first instar larvae were placed into a plastic rearing tray (26.7 cm x 20.3 cm x 7.6 cm, Lock & Lock, New South Wales, Australia) containing 2 L of distilled water. Larvae were provided 200 mg of powered food (described above) daily. Pupae were placed individually into plastic tubes with 3 ml of distilled water to allow for adult eclosion. Emerged adults were sorted and maintained in same-sex cages. All life stages were maintained at 27±1°C. Glycogen, total sugars (primarily glucose and trehalose) and lipid measurements were obtained for fourth instar larvae (L4), teneral females (T, within 24 hrs of emergence), 2 day old (dyo) (A2S) and 4 dyo (A4S) virgin females with access to 20% sugar water, and 2 dyo (A2W) and 4 dyo (A4W) virgin females with access to distilled water. The adult life stages were chosen because 100% of adults provided distilled water died after 4 days and 2 dyo adults provided a data point between emergence and death. Seventy-five individuals were included per age group.

Mosquito samples were fixed in an oven for 30 min at 100±1°C and prepared following the methods of Van Handel and Day (1988) [488] as modified by Harrington et al. (2001) [489]. Briefly, samples were homogenized for 30 sec in 200 μl of 2% sodium sulfate solution followed by the addition of 1.5 ml chloroform:methanol (1:2) solution. Tubes were centrifuged for 1 min at 450 x g. Supernatant (containing total sugars and lipids) was transferred into two new glass test tubes. Precipitate (containing glycogen) was used for glycogen measurements. Tubes containing supernatant were evaporated under a fume hood for 24 hrs (until 200 μl remained) and used for total sugar analysis and for 72 hrs (dried completely) and used for lipid analysis. Serial dilutions of *D*-glucose (Fisher Scientific, Fair Lawn, NJ) were prepared in duplicate to obtain standard curves for glycogen and total sugars each day samples were prepared. Measurements of samples for glycogen and total sugars as well as the *D*-glucose standards were determined using the hot anthrone-based assay [490]. New standard curves to measure lipids were analyzed in duplicate using serial dilutions of peanut oil (nSpired Foods, San Leandro, CA) each day samples were measured. Lipid levels of samples and standards were determined using the vanillin reagent assay [491]. Optical densities were measured using a Beckman DU 640 Spectrophotometer (Beckman-Coulter, Fullerton, CA) at 625 nm for glycogen and total sugars and 525 nm for lipids.

Nutritional measurements of μg/mosquito were corrected for body size (by dividing μg/mosquito by mm thorax length of larvae or mm wing length of adults) and converted to calories (by multiplying μg total sugars by 0.004 and μg lipids by 0.009 [6,492]). These conversion rates account for the total amount of energy provided by the nutrient based on the Atwater system [493] and allowed for the direct comparison of energetic content provided by each

nutrient between the strains.

6.2.4 Statistical analyses

Development parameters were compared between SLAB and ISOP450 strains with ANOVA (JMP 7.0, SAS Institute Inc, Cary, NC). Larval mortality and pupal mortality were arc-sin transformed and statistically compared using ANOVA. Additionally, Kaplan-Meier analysis was conducted on the survival curves created for adults of both sexes maintained on the diet treatments. Tukey-Kramer HSD test was used to compare adult body size (mm wing length) from diet treatments.

Statistical comparisons of the energy content between SLAB and ISOP450 strains were made using an ANOVA random block design test with date of analysis as the random variable. Comparisons between age groups were made using Tukey-Kramer HSD test. Body size measurements between strains were compared using ANOVA.

6.3 Results

6.3.1 Development and mortality parameters

The resistant strain (ISOP450) had a significantly longer egg-to-adult female development time than the susceptible strain (SLAB) (F=10.43, df=1, p=0.002) when the strains were held at the same temperature. All other development and mortality parameters measured (larval development time, larval mortality, pupal duration, pupal mortality and egg-to-adult male development time) were not statistically different between the two strains (Table 6.1).

Survivorship curves of adult males and females of ISOP450 and SLAB under the two diet treatments are shown in Figure 6.1. As expected, the

Table 6.1: Comparisons of development and mortality parameters between susceptible (SLAB) and resistant (ISOP450) strains (24 individuals/replicate, 12 replicates) held at 27±1°C.

Life stage	Parameter	SLAB (mean ± S.E.)	ISOP450 (mean ± S.E.)
Larval	Development (days)	8.24 ± 0.05	8.35 ± 0.05
	Mortality (%)	7.39 ± 0.67	7.90 ± 0.77
Pupal	Development (days)	1.85 ± 0.03	1.83 ± 0.03
	Mortality (%)	9.84 ± 0.67	8.29 ± 0.41
Adult	Egg-to-adult male (days)	9.85 ± 0.05	9.82 ± 0.07
	Egg-to-adult female (days)	10.22 ± 0.05	10.51 ± 0.08*

* Statistically greater than SLAB ($p<0.05$) using ANOVA.

distilled water treatment resulted in significantly lower survivorship than the sugar water treatment (χ^2=462.38, df=1, $p<0.0001$). Within the distilled water treatment, no differences in male survival (SLAB median longevity=4 days, ISOP450 median=4 days, χ^2=0.519, df=1, p=0.471) or female survival (SLAB median=4 days, ISOP450 median=3 days, χ^2=0.356, df=1, p=0.55) were detected between strains. Within the sugar water treatment, between SLAB and ISOP450 there were no significant differences in survival between males (SLAB median=26 days, ISOP450 median=28 days, χ^2=1.41, df=1, p=0.235). Strains of females provided 20% sugar water were significantly different from one another (χ^2=8.17, df=1, p=0.004), with median longevity of SLAB being 26 days while that of ISOP450 was 30 days. Body size was correlated with sex as expected and did not differ between strains or water treatments (Table 6.2).

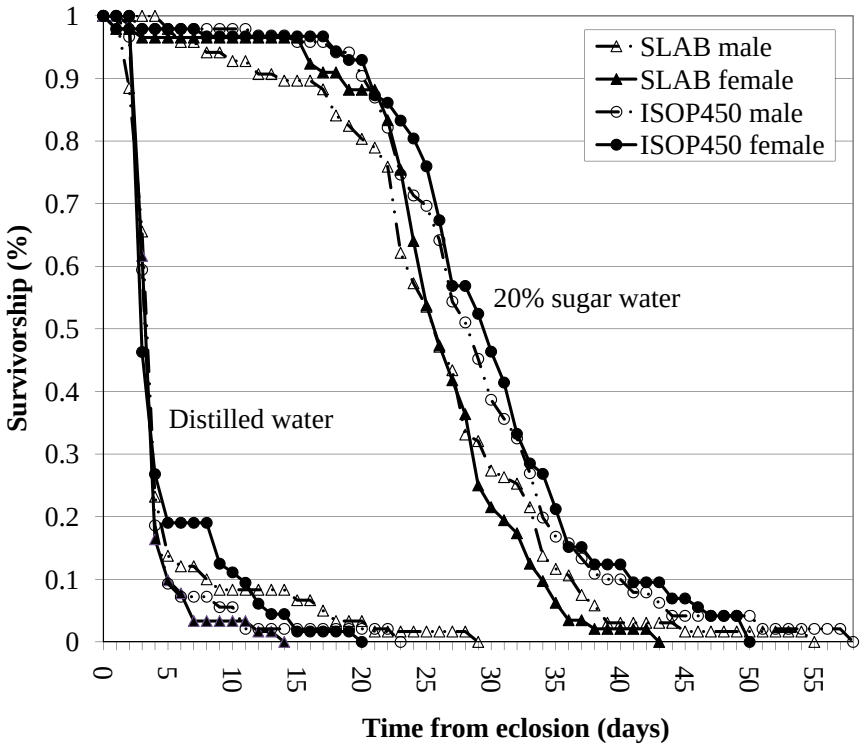


Figure 6.1: Survival curves of adult male and female SLAB and ISOP450 maintained on 20% sugar water or distilled water. Day 0 indicates day of eclosion.

Table 6.2: Wing sizes of surviving SLAB and ISOP450 adults maintained individually and provided 20% sugar water or distilled water for the duration of their lives. Values are mean ± S.E. (n in parethesis). Different letters indicate statistical differences (p<0.01) using Tukey-Kramer HSD test.

Treatment	sex	Wing size in mm (n)	
		SLAB	ISOP450
20% sugar water	male	2.62 ± 0.01 (58) a	2.64 ± 0.01 (58) a
20% sugar water	female	3.11 ± 0.02 (53) b	3.08 ± 0.02 (55) b
distilled water	male	2.62 ± 0.01 (59) a	2.63 ± 0.01 (58) a
distilled water	female	3.15 ± 0.02 (54) b	3.11 ± 0.02 (57) b

6.3.2 Nutritional energetic measurements

Table 6.3 lists the μg/mosquito values of glycogen, total sugars and lipid of SLAB and ISOP450 fourth instar larvae, teneral female adults, 2 dyo and 4 dyo females provided 20% sugar water and 2 dyo and 4 dyo females provided distilled water. In an environment where larvae were reared in groups, ISOP450 tended to be significantly smaller than SLAB (Table 6.4). Therefore, μg/mosquito measurements were corrected for body size (mm) for all age groups and then converted to calories (to obtain energy measurements). This allowed for direct comparison of energy obtained from each nutrient between the two strains.

Glycogen caloric content per mm body size was statistically different between SLAB and ISOP450 teneral female adults (F=6.85 df=1, 138, p=0.01), 2 dyo adults provided distilled water (F=10.66, df=1, 134, p=0.001) and 4 dyo adults provided sugar water (F=6.51, df=1, 143, p=0.012) where SLAB levels were greater than ISOP450. Calories/mm of glycogen in SLAB teneral adults, 2 dyo adults provided distilled water and 4 dyo adults provided sugar water were 0.028 ± 0.001, 0.014 ± 0.001 and 0.084 ± 0.004, respectively (Figure 6.2). Calories/mm of glycogen in ISOP450 were 0.025 ± 0.001, 0.010 ± 0.001 and 0.071 ± 0.004 in the teneral adults, 2 dyo adults provided distilled water and 4 dyo adults provided sugar water, respectively (Figure 6.2). Fourth instar larvae contained more glycogen than did any adult age group for both strains. Amongst the adult age groups, glycogen calorie levels tended to increase with age when adult mosquitoes had access to sugar water. From tenerals to 4 dyo adults provided sugar water, calories from glycogen increased by 300% in SLAB and 284% in ISOP450. The converse occurred to adults deprived of sugar; calories from glycogen tended to decrease with age (Figure 6.2) where

Table 6.3: Glycogen, total sugars and lipid content of different age groups of SLAB and ISOP450 strains. Values are mean ± S.E. (n=75 samples per age group).

Age group	Glycogen (μg/mosquito)		Total sugars (μg/mosquito)		Lipid (μg/mosquito)	
	SLAB	ISOP450	SLAB	ISOP450	SLAB	ISOP450
4th instar larvae	42.13 ± 1.92	45.18 ± 2.74	9.07 ± 1.59	9.86 ± 1.66	65.25 ± 4.01	58.14 ± 3.81
Teneral female	24.15 ± 0.94	20.67 ± 0.92	4.46 ± 0.63	3.98 ± 0.49	83.77 ± 3.21	79.76 ± 2.59
2 dyo female adults provided 20% sugar water	53.51 ± 3.29	46.18 ± 5.03	30.64 ± 2.32	33.34 ± 4.08	120.26 ± 4.77	95.33 ± 5.35
2 dyo female adults provided distilled water	11.55 ± 0.61	8.85 ± 0.64	1.46 ± 0.23	2.03 ± 0.35	39.75 ± 1.45	35.75 ± 1.22
4 dyo female adults provided 20% sugar water	70.36 ± 3.05	58.85 ± 2.90	23.02 ± 1.93	22.27 ± 1.80	163.46 ± 9.13	110.18 ± 5.51
4 dyo female adults provided distilled water	8.93 ± 0.44	8.23 ± 0.39	3.92 ± 0.54	2.70 ± 0.34	13.95 ± 0.97	12.39 ± 0.99

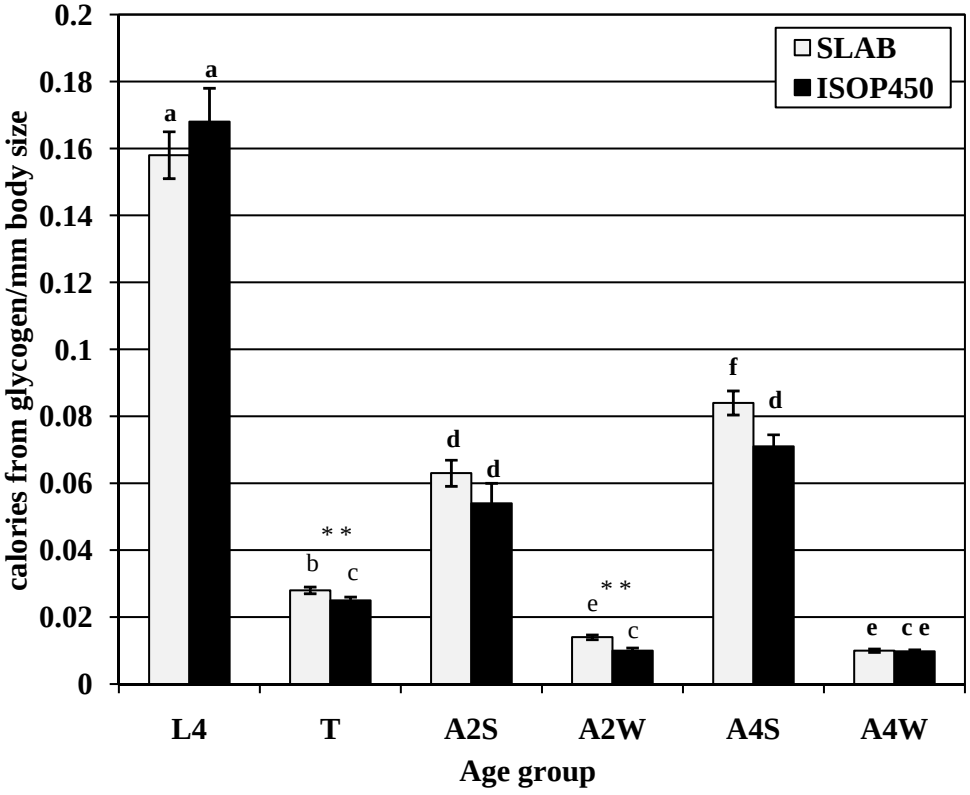


Figure 6.2: Glycogen energetic resources of SLAB and ISOP450 strains at different age groups. Values are mean ± S.E. (n=75 samples per age group). Letters which differ indicate statistical differences (p<0.05) and ** indicates significant differences at p<0.01 level. L4: 4th instar larvae, T: teneral female adults, A2S: 2 dyo female adults provided 20% sugar water, A2W: 2 dyo female adults provided distilled water, A4S: 4 dyo female adults provided 20% sugar water, A4W: 4 dyo female adults provided distilled water.

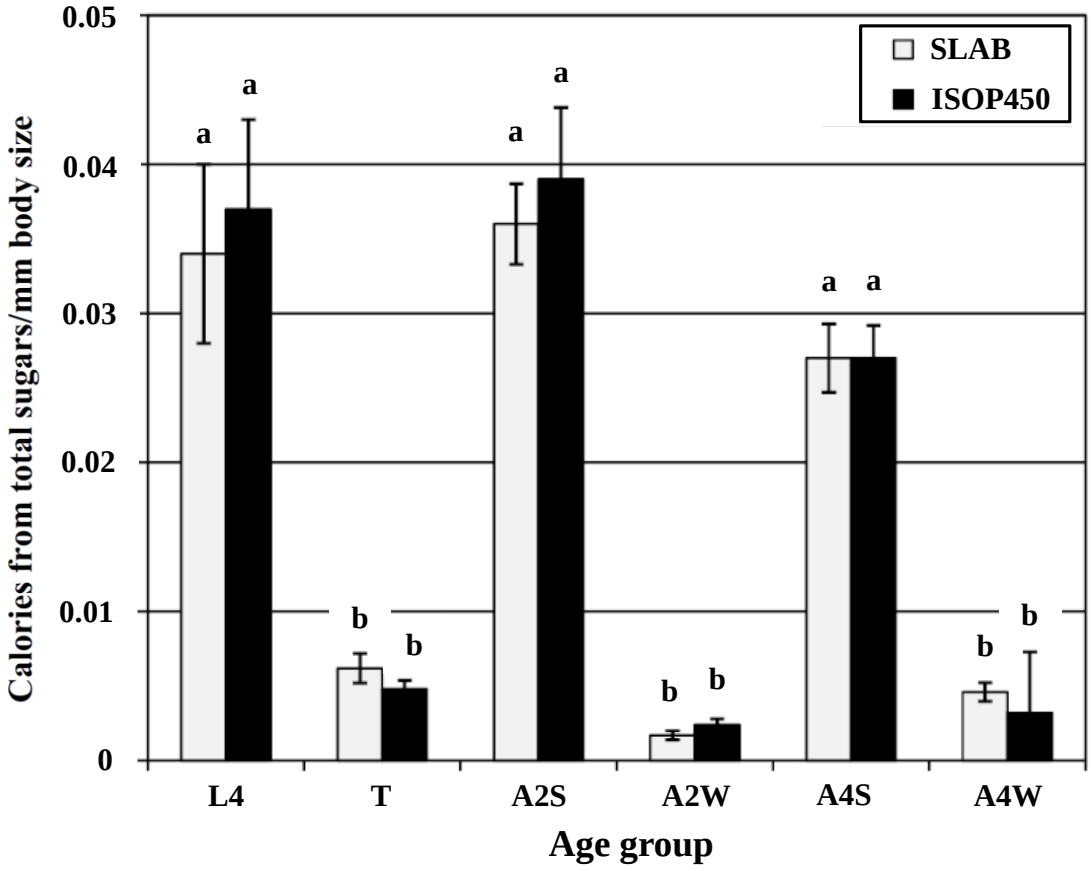


Figure 6.3: Energetic resources from total sugars of SLAB and ISOP450 strains at different age groups. Values are mean ± S.E. (n=75 samples per age group). Letters which differ indicate statistical differences (p<0.05) and ** indicates significant differences at p<0.01 level. L4: 4th instar larvae, T: teneral female adults, A2S: 2 dyo female adults provided 20% sugar water, A2W: 2 dyo female adults provided distilled water, A4S: 4 dyo female adults provided 20% sugar water, A4W: 4 dyo female adults provided distilled water.

Table 6.4: Body sizes of SLAB and ISOP450 reared in groups at different age groups. Values are mean ± S.E.

Age group	Body size (mm)	
	SLAB	ISOP450
4th instar larvae	1.06 ± 0.01	1.07 ± 0.01
Teneral female	3.41 ± 0.01	3.37 ± 0.01*
2 dyo female adults provided 20% sugar water	3.40 ± 0.01	3.43 ± 0.01
2 dyo female adults provided distilled water	3.42 ± 0.01	3.38 ± 0.01*
4 dyo female adults provided 20% sugar water	3.36 ± 0.01	3.29 ± 0.01*
4 dyo female adults provided distilled water	3.43 ± 0.01	3.35 ± 0.01*

* Statistically smaller than SLAB (p<0.05) using ANOVA.

64.3% and 60% of the glycogen measured in teneral adults had disappeared in SLAB and ISOP450 4 dyo adults provided distilled water, respectively.

Total sugar caloric content per mm body size was not statistically different between SLAB and ISOP450 in all age groups (Figure 6.3). Neither SLAB nor ISOP450 consistently had higher levels of calories from total sugars throughout the age groups. Calorie levels from total sugars were lower than glycogen calorie levels.

The greatest differences between SLAB and ISOP450 strains were measured in calories obtained from lipids. Energetic resources from lipids (calories/mm) were different between SLAB and ISOP450 in fourth instar larvae (F=5.88, df=1, 134, p=0.017), 2 dyo adults provided sugar water (F=12.04, df=0, 125, p=0.001), 4 dyo adults provided sugar water (F=56.26, df=1, 142, p<0.0001) and 4 dyo adults provided distilled water (F=5.82, df=1, 144, p=0.017) wherein SLAB levels were larger than ISOP450. Similar to the pattern observed with glycogen, calories/mm from lipids within the adult age groups

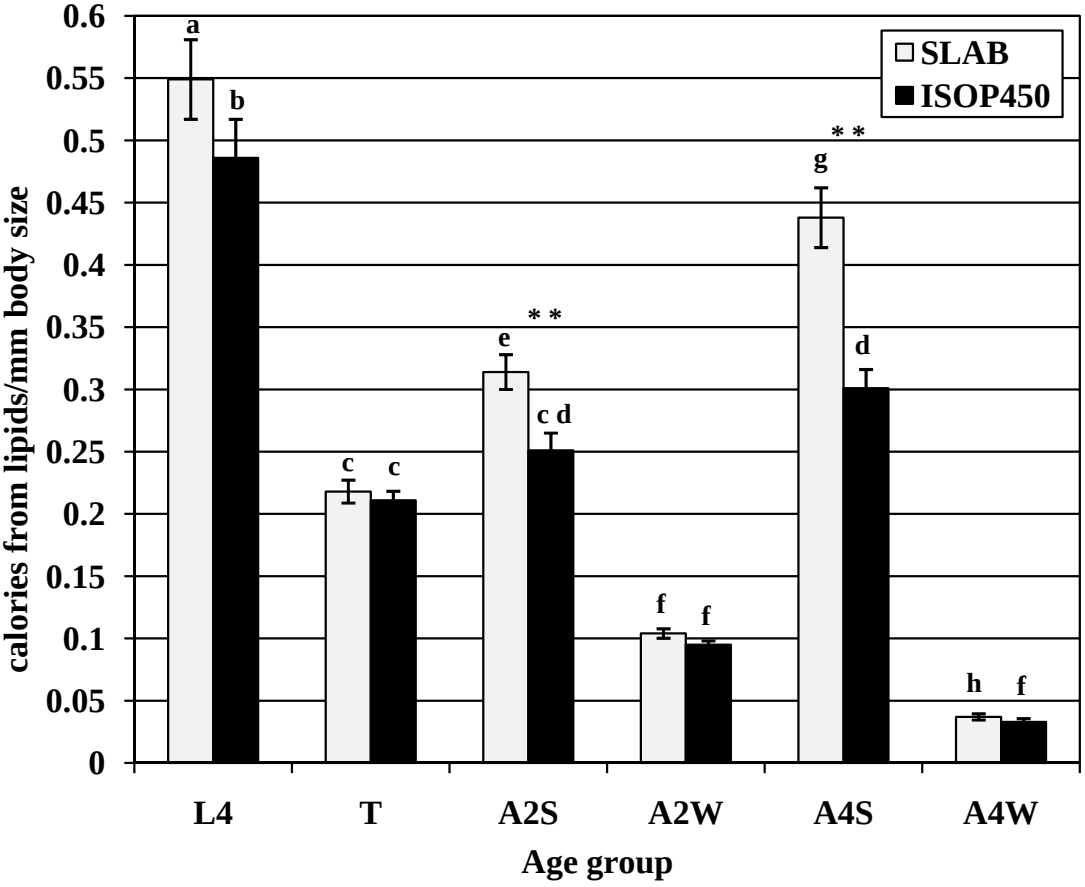


Figure 6.4: Lipid energetic resources of SLAB and ISOP450 strains at different age groups. Values are mean ± S.E. (n=75 samples per age group). Letters which differ indicate statistical differences (p<0.05) and ** indicates significant differences at p<0.01 level. L4: 4th instar larvae, T: teneral female adults, A2S: 2 dyo female adults provided 20% sugar water, A2W: 2 dyo female adults provided distilled water, A4S: 4 dyo female adults provided 20% sugar water, A4W: 4 dyo female adults provided distilled water.

increases with age with access to sugar water and decrease with age when only provided distilled water (Figure 6.4). From teneral adults to 4 dyo adults provided sugar water, lipid levels increased by 200% and 143% for SLAB and ISOP450, respectively, while sugar deprivation decreased the lipid level from the higher level in teneral adults to the lower level in 4 dyo adults provided distilled water by 84% for SLAB and 84.4% for ISOP450.

6.4 Discussion

Results from this study provide data which confirm that the P450 detoxification R allele (present in ISOP450) appears to be slightly less fit than the S allele (present in SLAB) as previously reported (Chapter 5) [476]. Given that the P450-mediated resistance in ISOP450 was larval-specific [476], it was expected that biological differences might be most evident at this life stage. No larval or pupal developmental parameter differences between SLAB and ISOP450 were observed, but SLAB females were smaller and developed faster than ISOP450 female adults. Female body size of ISOP450 reared in a group was smaller than SLAB reared in a group versus when both strains were reared as individuals, suggesting a developmental deficiency associated with ISOP450 when placed in competition for food. Energy resources from glycogen were higher in SLAB adult females of the teneral, 2 dyo provided distilled water and 4 dyo provided sugar water life stages compared to ISOP450. Energy provided by lipids were higher in fourth instar larvae, 2 dyo adults provided distilled water and 4 dyo adults provided sugar water of SLAB as compared to ISOP450. The energy levels measured in this study are consistent with previous reports [494,495]. Thus, the estimates of fitness measured in this experiment show potential costs associated with P450-mediated resistance in ISOP450 relative to SLAB in a smaller female body size when grouped as

larvae, a longer egg-to-adult female eclosion time and lower relative energetic resources provided from glycogen and lipid nutrients.

Body size of adult females in SLAB and ISOP450 strain were dependent on the number of other larvae present during development. A stronger negative developmental effect of group living was present in the ISOP450 strain such that SLAB females reared in groups were larger in size than the equivalently treated ISOP450 females. Size differences within a sex were only observed between the two strains when larvae were reared in groups suggesting a possible decreased ability for ISOP450 larvae to obtain food when competing with con-specifics. It is well known in mosquitoes that a positive correlation exists between body size as measured by adult wing length and egg production [6,496]. Therefore, it could be possible for SLAB to have a higher reproductive potential than ISOP450 in the laboratory and in the wild based on these observations, though future experimentation is necessary to confirm this hypothesis.

Despite provision of sugar water, ISOP450 adult females survived approximately 4 days longer than SLAB adult females. In multiple mosquito species it has been observed that female fecundity is inversely correlated to age [497,498], though positive correlations of egg laying with age have also been reported [499]. Thus, it is unclear if the longevity of ISOP450 females is a reproductive benefit especially since wild females may not survive as long as females kept under laboratory conditions.

Emergence time is an important factor to consider for fitness. SLAB females emerged earlier than females of the ISOP450 strain by almost 7 hrs. Males of both strains did not significantly differ in emergence time such that females of SLAB emerge 9 and 9.5 hrs and ISOP450 females emerge 16 and 16.5

hrs after SLAB and ISOP450 males, respectively. In small laboratory mating cage experiments, Williams and Patterson (1969) observed that at 82±1°F (28±1°C) male *Cx. p. quinquefasciatus* did not actively seek females to mate with until males were 72 hrs old and no females were found to be inseminated by males less than 24 hrs old [500] since inversion of the male genitalia occurs in a temperature dependent manner during this period (ranging from 6 hrs to >72 hrs post-emergence to complete depending on the temperature) [7]. Observations from this study provide a scenario whereby males from both strains have an equal opportunity to mate with the earlier emerging SLAB females, allowing S alleles to be increased in the population given that female *Culex* mosquitoes are thought to be monogamous [501–503]. Since field populations of mosquitoes have overlapping generations, it is unclear whether this pattern would drive the P450 R allele out of a resistant wild population. This observation could be what provided the S allele an advantage over the R allele in the insecticide-free population cage experiment since the experimental design included discrete generations [476].

Glycogen energy storage of ISOP450 and SLAB differed between teneral female adults, 2 dyo adults provided distilled water and 4 dyo adults provided sugar water. No differences were found at the fourth instar larval stage indicative of the ability for both strains and store equal amounts of glycogen from the diet provided. Intriguingly, a significant difference was observed at the teneral female adult stage where ISOP450 levels of calories obtained from glycogen were lower than SLAB levels (Figure 6.2). This difference may be due to ISOP450 utilizing more of the glycogen resources for metamorphosis or the inability of ISOP450 to store energy as readily as SLAB. ISOP450 does not have as much energy from glycogen for various biological processes as compared to

SLAB. The biological significance of the differences observed between the 2 dyo adults provided distilled water and 4 dyo adults provided sugar water of ISOP450 and SLAB are unknown since no differences in glycogen levels were measured between 2 dyo adults provided sugar water and 4 dyo adults provided distilled water. Both strains were able to equally gain glycogen resources when provided sugar water as adults, they also both equally depleted glycogen resources when starved [504].

No significant differences in calories from total sugars were found between SLAB and ISOP450. Both strains were equally able to store sugars over time when provided a food source (larval food or sugar water) and sugars accounted for the least amount of energy of the three nutrient types measured.

Lipid reserves differed significantly between SLAB and ISOP450 with SLAB larvae containing the greatest level of reserves. For a number of insect species, the lipid content corrected for dry weight is higher in larvae compared to adults [505] as was observed for both SLAB and ISOP450. At the teneral adult stage, the levels became equal between the strains and when adults were provided sugar water, ISOP450 were less efficient at storing lipids or utilized more lipids for survival. Under conditions of adult starvation, both strains exhausted the long-term lipid stores in order to survive [483,505]. Previous reports on insecticide resistance (mostly resistance to DDT) and lipid content have shown that they are not correlated [505–510]. Therefore, it appears that lipid levels are not associated with the resistance status of a population (in which case ISOP450 would be expected to have higher amounts), but are developmentally important suggesting that ISOP450 has inferior development versus SLAB. Previously it was shown that *Ae. aegypti* which were fed radioactive sugar water before taking a blood meal, had appreciable amounts

of radioactivity in the fat body and oocytes [511]. This suggests that the higher level of lipids in SLAB might result in increased fecundity especially when provided sugar water [512] as compared to ISOP450 which had lower calorie levels from lipids.

Laboratory analysis of fitness associated with insecticide resistance using highly related strains, such as SLAB and ISOP450, can provide valuable information since the genetic background is more uniform than using unrelated strains. While developmental and energetic indices suggest that a minor fitness cost under laboratory conditions is associated with P450-mediated resistance in ISOP450, other fitness parameters (mating competition, sperm competition, predator avoidance, egg viability, fitness/metabolic levels under field conditions, etc.) not studied here could also be informative. Therefore, further investigations into reproductive output or life table construction of SLAB and ISOP450 are necessary. Development of allele specific markers to differentiate individuals that are homozygous or heterozygous for P450 resistance will open the door for studies of the population genetics (and fitness) of this allele under both laboratory and field conditions.

ISOP450 has lower estimates of fitness (using measurements of development and energetic resources) when compared alongside SLAB in the laboratory. Most notably, larger body size when reared in groups, faster development time and greater calories (energy) from nutrients [513] provide SLAB with greater measurements of fitness indices when compared to ISOP450. The developmental comparisons between the P450-mediated permethrin resistant ISOP450 strain and susceptible SLAB strain of *Cx. p. quinquefasciatus* did not show a larval-specific fitness cost as expected. Minor

adult stage costs were associated with the P450 detoxification R allele. Determining whether the laboratory results observed here are relevant for resistant populations in the field will require future experimentation such as determining fitness of SLAB and ISOP450 provided carbohydrates from various natural sources.

CHAPTER 7

EXPRESSION PATTERNS OF *CYP4H34* AND *CYP9M10* IN SUSCEPTIBLE AND PERMETHRIN RESISTANT STRAINS OF *CULEX PIPIENS QUINQUEFASCIATUS*

7.1 Introduction

Cytochrome P450 monooxygenases are one of the primary enzymatic systems involved in conferring resistance. P450-mediated resistance occurs due to the over-expression of P450s principally due to increased transcription [264] of the P450 gene as opposed to gene amplification (which is common for esterase-based resistance to OPs [279, 298, 304, 344]) or mRNA stabilization [264]. Changes in a P450 gene that result in changes in the P450 amino acid sequence (leading to a P450 with greater metabolism of the insecticide) may also be involved in resistance [253, 514].

Many P450s that have been shown to be involved in insecticide resistance belong to the *CYP6* family. One of the most completely studied systems is the over-production of CYP6D1 in permethrin resistant *M. domestica* [345] due to transcription up-regulation. The up-regulation of *Cyp6g1* has been proposed as the P450 which is responsible for world-wide DDT resistance in *D. melanogaster* [348], though its role has been questioned [278]. Members of the *CYP4* family have also been found to be up-regulated in deltamethrin resistant *Cx. p. pallens* [282].

There are few previous reports on the gene expression of P450s which confer resistance to insecticides in mosquitoes. In many of the previous reports, P450s are cloned but then found to be equally expressed in both the resistant and susceptible strains [288] or the expression levels are not analyzed [269]. In a permethrin-resistant strain of *An. gambiae*, an adult-specific P450, *CYP6Z1*,

was expressed 11-fold higher in males and 4.5-fold higher in females when compared to a susceptible strain collected from the same geographical region of Kenya [279]. In the permethrin-resistant strain JPAL (*Cx. p. quinquefasciatus*) expression of *CYP6F1* was assessed by Northern blotting and found to be over-expressed as compared to the susceptible strain [288]. *CYP6F1* and *CYP6E1* were subsequently cloned from JPAL, but further analysis did not implicate them as candidate genes conferring pyrethroid resistance [288].

In the present study, two candidate P450 genes (provided by T. Tomita) that are reportedly over-expressed in JPAL were analyzed by quantitative real-time PCR to determine if they were over-expressed in the resistant strain, ISOP450, as compared to the related susceptible strain, SLAB. Previous research indicated that P450-mediated resistance to permethrin in ISOP450 was larval-specific (Chapter 3, [455]). Herein, results of the qRT-PCR analysis implicate *CYP9M10* as the gene which confers permethrin resistance. In addition, polymorphic sites to differentiate the S and R alleles of *CYP9M10* are detailed and provide a potential molecular tool to assess allele frequencies of *CYP9M10* in resistant populations.

7.2 Materials and methods

7.2.1 Mosquito strains

Three strains of *Cx. p. quinquefasciatus* Say were used and reared as previously described (Chapter 3). SLAB is a laboratory susceptible strain [420]. JPAL is a permethrin resistant strain containing *kdr* and P450 detoxification. ISOP450 is a permethrin resistant strain (1,300-fold) related to SLAB containing the P450 resistance mechanism present in JPAL, but lacking *kdr*. Bioassay data (Chapter 3) implicated the resistance mechanism present in ISOP450 as highly PBO-suppressible and larval-specific [455]. Sequences of two P450 genes that

may confer resistance to permethrin in the *Cx. p. quinquefasciatus* strain JPAL were obtained by Dr. T. Tomita from the University of Tsukuba, Japan. These two P450s are over-expressed by 18-fold (*CYP4H34*) and 365-fold (*CYP9M10*) in JPAL relative to a susceptible strain (Ogasawara) (T. Tomita, personal communication).

7.2.2 Isolation of gDNA

Individual 3 dyo virgin female adults (A3) and individual fourth instar larvae (L4) were used to prepare gDNA as previously described in Chapter 3.

7.2.3 mRNA extraction and cDNA synthesis

Each mRNA sample consisted of 15 three dyo virgin female adults fed on 20% sugar water or 15 fourth instar larvae. Samples were processed using the illustra QuickPrep *Micro* mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK) and treated with DNA-*free* (Ambion, Austin, TX) according to manufacturer's instructions. First strand cDNA was synthesized from 5 μg of mRNA with SuperScript III First Strand Synthesis System for RT PCR kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Concentrations of mRNA were determined on a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA).

7.2.4 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed in a 10 μl volume with the Fast SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA) and a 7900 HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA). Primers used for qRT-PCR are listed in Table 7.1. Preliminary PCR to test primer pairs used GoTaq DNA polymerase (Promega, Madison, WI) and thermal cycler conditions as follows: 95°C for 2 min, followed by 35 cycles of

95°C for 30 sec, 57°C for 30 sec and 72°C for 1 min with a final extension of 72°C for 10 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. For each of the cDNA samples, measurements of gene expression were obtained in triplicate. The real-time PCR reaction cycle consisted of a melting step of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Lack of gDNA contamination of cDNA samples was confirmed by running a negative control (no reverse transcriptase during cDNA synthesis) and checking for the presence of a PCR product directly after real-time PCR completed. Relative expression levels for the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method which normalizes Ct values of the target gene to an endogenous control (actin) (primers are given in Table 7.1) and sample calibrator, using SDS software [515]. SLAB fourth instar larval cDNA preparation #1 and SLAB 3 dyo adult cDNA preparation #1 were present on both plates and these samples were used as inter-plate calibrators for calculations. Therefore, intra-plate expression levels were determined and then averaged across the plates for a total of 5 biological replicates (individually prepared cDNA samples) per strain per life stage [516]. Relative gene expression levels within life stages between the two strains were statistically compared using one-way ANOVA.

7.2.5 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed in 15 μl reaction volumes with GoTaq polymerase using 10-fold dilutions of cDNA. Primers used (listed in Table 7.1) resulted in a 451 bp product for *CYP4H34*, a 419 bp product for *CYP9M10* and a 410 bp product for actin. All samples and primer pairs were run simultaneously under thermal cycler conditions of 95°C for 2 min,

Table 7.1: Primer pairs used in quantitative real-time RT-PCR and semi-quantitative RT-PCR.

Gene	Primer name	Function	Primer sequence
CYP4H34	4HRTF1	qRT-PCR	5′ CATCCAGCTGGCAAAGCACC 3′
	4HRTR1	qRT-PCR	5′ GACTTCTGCGCCGAGTACG 3′
	4HRTF2	preliminary results	5′ GGATTTGCGCTTCAAGATGG 3′
	4HRTR2	preliminary results	5′ AATCGAGCCTCACTTCCTTGC 3′
	4HF	semi-quantitative RT-PCR	5′ CCGACGTGCTGGTGAAC 3′
	4HR	semi-quantitative RT-PCR	5′ ATGTCGGAATCGGAGAGG 3′
CYP9M10	9MRTF	qRT-PCR	5′ GCCTAACCAACCGAGCCTG 3′
	9MRTR	qRT-PCR	5′ GCCGACCTGGATGGAGACT 3′
	9MF	semi-quantitative RT-PCR	5′ GAGGCGGATCCAGTGTTAG 3′
	9MR	semi-quantitative RT-PCR	5′ TCAGTAGCTTCTTTAGGGATTATG 3′
actin (accession #: XM_001866844)	actRTF	qRT-PCR	5′ GGCGTCGAGGATCTGGACTT 3′
	actRTR	qRT-PCR	5′ AGTCCTCGACCAGCCCG 3′
	act2F	semi-quantitative RT-PCR	5′ CATCAACGCCATCACGAA 3′
	act2R	semi-quantitative RT-PCR	5′ TGGCACACCTGGTAGAACTC 3′

followed by 35 cycles of 95°C for 30 sec, 53°C for 30 sec and 72°C for 1 min with a final extension of 72°C for 10 min.

7.3 Results

7.3.1 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR revealed that the expression of *CYP9M10* was increased by 2,200-fold in fourth instar larvae and 12-fold in 3 dyo female adults of ISOP450 compared with SLAB. As such, *CYP9M10* is strongly implicated as the P450 gene which confers permethrin resistance in *Cx. p. quinquefasciatus*. In addition, multiple polymorphic sites were found in the coding region of this gene between the S and R strains. No significant differences in expression of *CYP4H34* between SLAB and ISOP450 strains in fourth instar larvae (F=0.053, p=0.82) or 3 dyo adult (F=0.591, p=0.46) were observed. Therefore, *CYP4H34* is not the P450 conferring permethrin resistance in *Cx. p. quinquefasciatus*.

Preliminary results confirmed that the primers designed for real-time PCR (Table 7.1) were producing expected size products (*CYP4H34*#1: 76 bp; *CYP4H34*#2: 85 bp; *CYP9M10*: 77 bp; actin: 97 bp). These results provided a method to determine if the cDNA samples were contaminated with gDNA by assessing the size of the actin band (Figure 7.1), because gDNA resulted in a band of approximately 200 bp in size due the presence of an intron, while the cDNA template provides the expected band of 97 bp. In Figure 7.1 no bands are seen in lanes 6 and 14 where the gDNA template of JPAL larvae and adults are amplified with 9MRTF and 9MRTR primers indicating that one of the primers is spanning an exon-exon junction such that only the cDNA template is amplified.

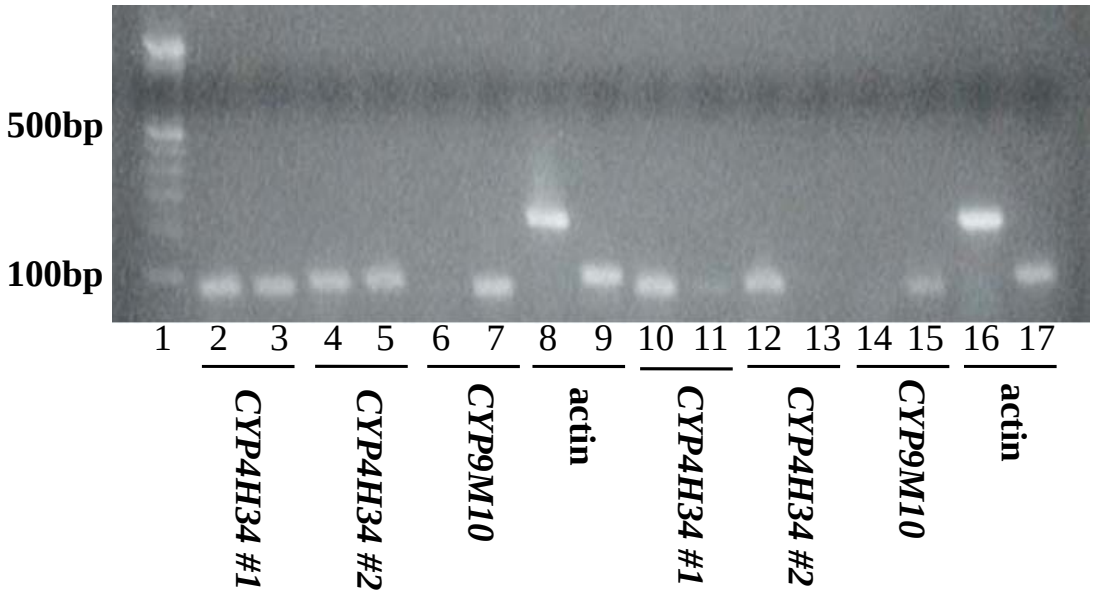


Figure 7.1: Results of primer pairs designed for real-time PCR. Listed below the lane numbers are the primer pairs used. Lane 1: 100 bp ladder, Lanes 2, 4, 6, 8: gDNA from JPAL fourth instar larvae, Lanes 3, 5, 7, 9: cDNA from JPAL fourth instar larvae, Lanes 10, 12, 14, 16: gDNA from JPAL 3 dyo virgin female adults, Lanes 11, 13, 15, 17: cDNA from JPAL 3 dyo virgin female adults.

The PCR products which resulted from qRT-PCR on cDNA obtained from fourth instar larvae of SLAB and ISOP450 were not contaminated with gDNA since actin bands are less than 100 bp and the no reverse transcriptase negative controls have no product. The same results occurred with PCR products obtained after qRT-PCR on cDNA derived from 3 dyo adult cDNA samples (data not shown).

Results of the qRT-PCR approach showed that *CYP9M10* was significantly over-expressed by 2,200-fold (±240) in ISOP450 fourth instar larvae compared to SLAB fourth instar larvae (1.5±0.38-fold) (F=82.56,

p<0.0001). ISOP450 3 dyo adults had slightly, but significantly (F=7.27, p=0.02), higher expression of *CYP9M10* (12±3.7-fold) versus SLAB 3 dyo adults (1.4±0.59-fold). Expression of *CYP4H34* was not statistically different between ISOP450 and SLAB for either life stage. Relative *CYP4H34* expression levels for SLAB fourth instar larvae, ISOP450 fourth instar larvae, SLAB 3 dyo adults and ISOP450 3 dyo adults were 0.90±0.23-fold, 0.97±0.19-fold, 1.3±0.20-fold and 1.6±0.32-fold, respectively. Table 7.2 lists Ct values and Figure 7.2 summarizes the results.

7.3.2 Semi-quantitative RT-PCR

To confirm the 2,200-fold expression of *CYP9M10* in ISOP450 fourth instar larvae relative to SLAB, semi-quantitative RT-PCR was conducted independently on 3 biological samples (cDNA) of each strain and life stage which were used in the qRT-PCR approach. Different primer pairs were used (see Table 7.1) from those used in the qRT-PCR experiment. PCR with cDNA showed that the primers used for semi-quantitative RT-PCR amplified the target genes of interest. A bright band at the 10^{-3} dilution of ISOP450 fourth instar larvae cDNA for *CYP9M10* was present while no band was present at that dilution for SLAB confirming the >1,000-fold over-expression of *CYP9M10* in ISOP450 relative to SLAB. The 10^{-3} ISOP450 fourth instar larvae *CYP9M10* band was also brighter than the same cDNA dilution amplifying actin. The adult bands agree with with the qRT-PCR results. A band is present at the 10^{-1} dilution of ISOP450 3 dyo adult cDNA for *CYP9M10* whereas a fainter band is present at that dilution for the SLAB 3 dyo adult cDNA. Results from the 3 replicates were identical (Figure 7.3). These results confirm the level of over-expression of *CYP9M10* in ISOP450 larvae and adults measured in the qRT-PCR method (Figure 7.3).

Table 7.2: Ct values of target genes *CYP4H34* and *CYP9M10* and endogenous control actin from quantitative real-time RT-PCR. Plate #1 and plate #2 are technical replicates.

Sample[a]	Rep.[b]	Ct values			Sample[a]	Rep.[b]	Ct values		
		CYP4H34	*CYP9M10*	actin			*CYP4H34*	*CYP9M10*	actin
PLATE #1									
SLAB L4 #1	#1	24.8856	31.4468	24.6966	SLAB A3 #1	#1	26.7632	30.2587	24.3666
	#2	24.8548	31.0221	24.3775		#2	26.4857	30.3258	24.3073
	#3	24.9951	31.6624	24.9585		#3	26.6030	30.1574	24.6312
SLAB L4 #2	#1	24.2828	30.5775	24.7698	SLAB A3 #2	#1	25.9467	29.7723	23.6229
	#2	24.7641	30.4864	24.8406		#2	25.8089	29.6679	23.5688
	#3	24.4833	30.5044	24.8259		#3	25.7171	29.5418	23.3458
SLAB L4 #3	#1	24.2600	30.0312	23.8472	SLAB A3 #3	#1	25.9424	30.0484	23.5638
	#2	24.2241	29.8168	23.8934		#2	25.8772	29.7102	23.5218
	#3	24.3343	29.9568	24.3097		#3	25.7319	29.6679	23.9454
ISOP450 L4 #1	#1	23.9006	19.1078	23.7827	ISOP450 A3 #1	#1	25.0147	25.7531	22.7714
	#2	23.8310	19.1347	23.7012		#2	24.9476	25.7138	22.7422
	#3	23.8311	19.1297	23.6699		#3	24.8276	25.7477	22.6795
ISOP450 L4 #2	#1	22.9962	18.2154	23.2714	ISOP450 A3 #2	#1	24.5907	25.5287	23.6286
	#2	22.9579	18.2464	23.1539		#2	24.4383	25.7028	22.8118

Table 7.2 (Continued):

	#3	22.9712	18.2484	23.1508		#3	24.2861	25.6872	22.7743
ISOP450 L4 #3	#1	23.4268	18.2712	23.4395	ISOP450 A3 #3	#1	25.9284	25.7099	23.69753
	#2	23.3779	18.2793	23.2859		#2	25.7316	25.7006	23.8417
	#3	23.3777	18.2996	23.2185		#3	25.7073	25.7148	23.9651
				PLATE #2					
SLAB L4 #1	#1	24.3443	29.9723	23.8007	SLAB A3 #1	#1	24.0822	28.4324	22.0244
	#2	24.3955	29.9853	23.8037		#2	24.0851	29.3645	22.1548
	#3	24.1414	29.8787	23.9056		#3	24.5752	29.2041	22.2205
SLAB L4 #4	#1	24.1472	28.1218	22.4916	SLAB A3 #4	#1	24.4563	27.7955	22.8160
	#2	24.8355	28.0293	22.9271		#2	24.7901	27.7193	23.0164
	#3	24.3625	28.3644	22.5896		#3	24.6707	27.7587	22.9833
SLAB L4 #5	#1	24.2651	28.0718	22.8514	SLAB A3 #5	#1	24.7736	27.6939	22.8655
	#2	24.2657	27.9349	22.9034		#2	24.5529	27.6774	22.8788
	#3	24.0534	28.3668	22.9913		#3	24.3629	27.7121	22.9377
ISOP450 L4 #1	#1	23.6157	18.9854	23.3964	ISOP450 A3 #1	#1	24.6528	25.1963	22.7182
	#2	23.5038	18.8821	23.1057		#2	24.9378	25.2702	22.9139

Table 7.2 (Continued):

	#3	23.7840	18.9840	23.4623		#3	24.7270	25.1085	22.6641
ISOP450 L4 #4	#1	24.0309	18.4350	22.7298	ISOP450 A3 #4	#1	25.1145	26.6649	23.3818
	#2	23.9720	18.3873	n/a		#2	25.0108	26.7763	23.5243
	#3	23.8299	18.8867	22.6699		#3	24.8659	26.7074	23.3479
ISOP450 L4 #5	#1	24.0283	18.3771	n/a	ISOP450 A3 #5	#1	25.2328	26.9020	23.7927
	#2	24.0174	18.4166	22.9700		#2	25.2414	26.8820	23.7777
	#3	23.9341	18.9180	22.5542		#3	24.9761	26.8150	23.8106

[a] L4: cDNA template from fourth instar larvae; A3: cDNA template from 3 dyo adult females.

The number refers to the biological replicate (independent isolation of cDNA).

[b] Three determinations were made for each biological replicate.

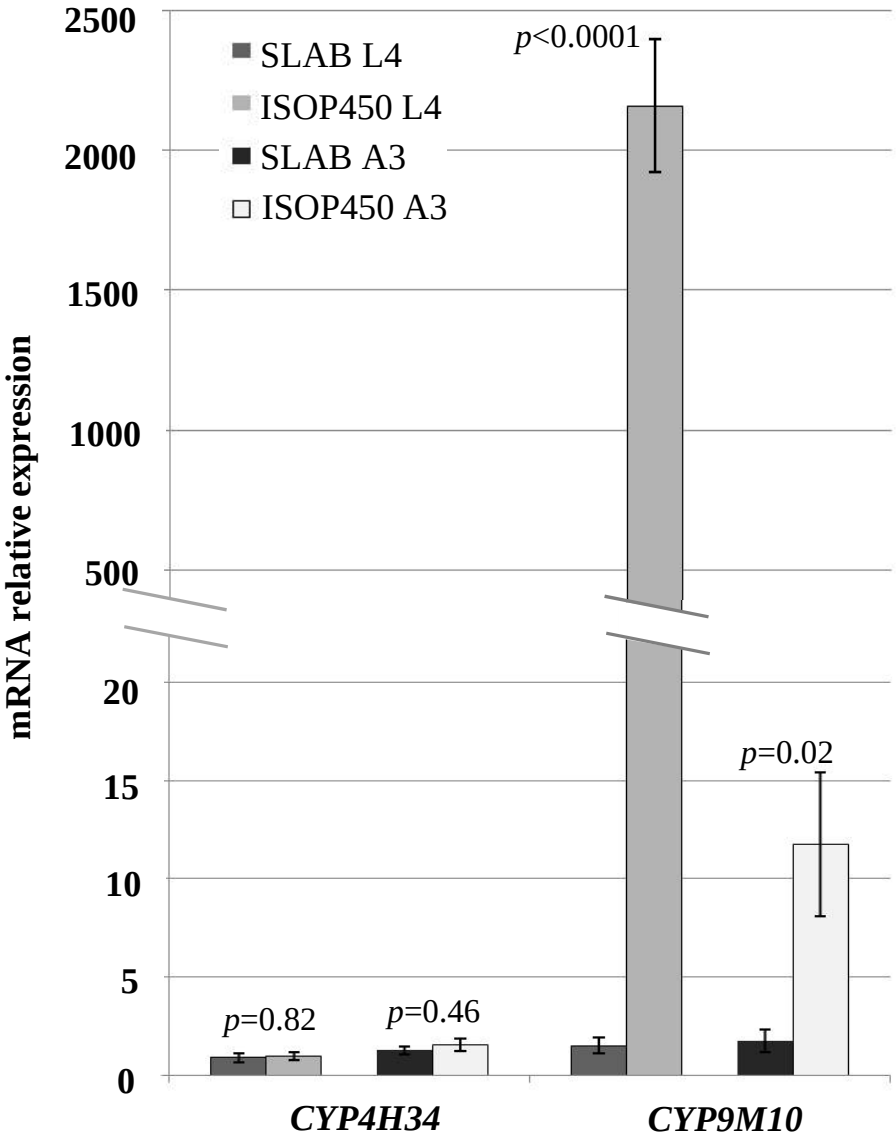


Figure 7.2: Quantitative real-time RT-PCR summary of target genes *CYP4H34* and *CYP9M10* of SLAB and ISOP450 fourth instar larvae (L4) and 3 dyo female adults (A3). Data are the mean ± S.E. of 5 biological samples expressed as expression level relative to calibrator samples. ANOVA was used for statistical comparisons between strains within life stages (*p*-values indicated).

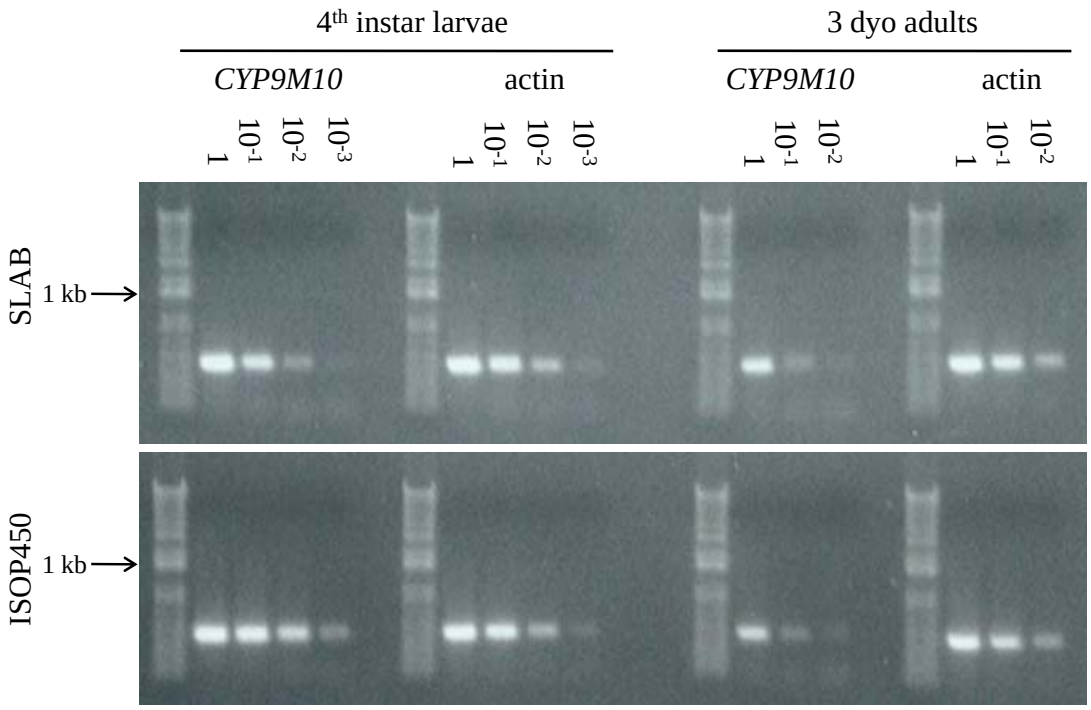


Figure 7.3: Semi-quantitative RT-PCR of *CYP9M10* expression in ISOP450 and SLAB fourth instar larvae and 3 dyo female adults. 10-fold dilutions of cDNA were amplified with primers for *CYP9M10* and actin. The same cDNA was used for both genes within a life stage.

7.3.3 Sequences of *CYP4H34* and *CYP9M10*

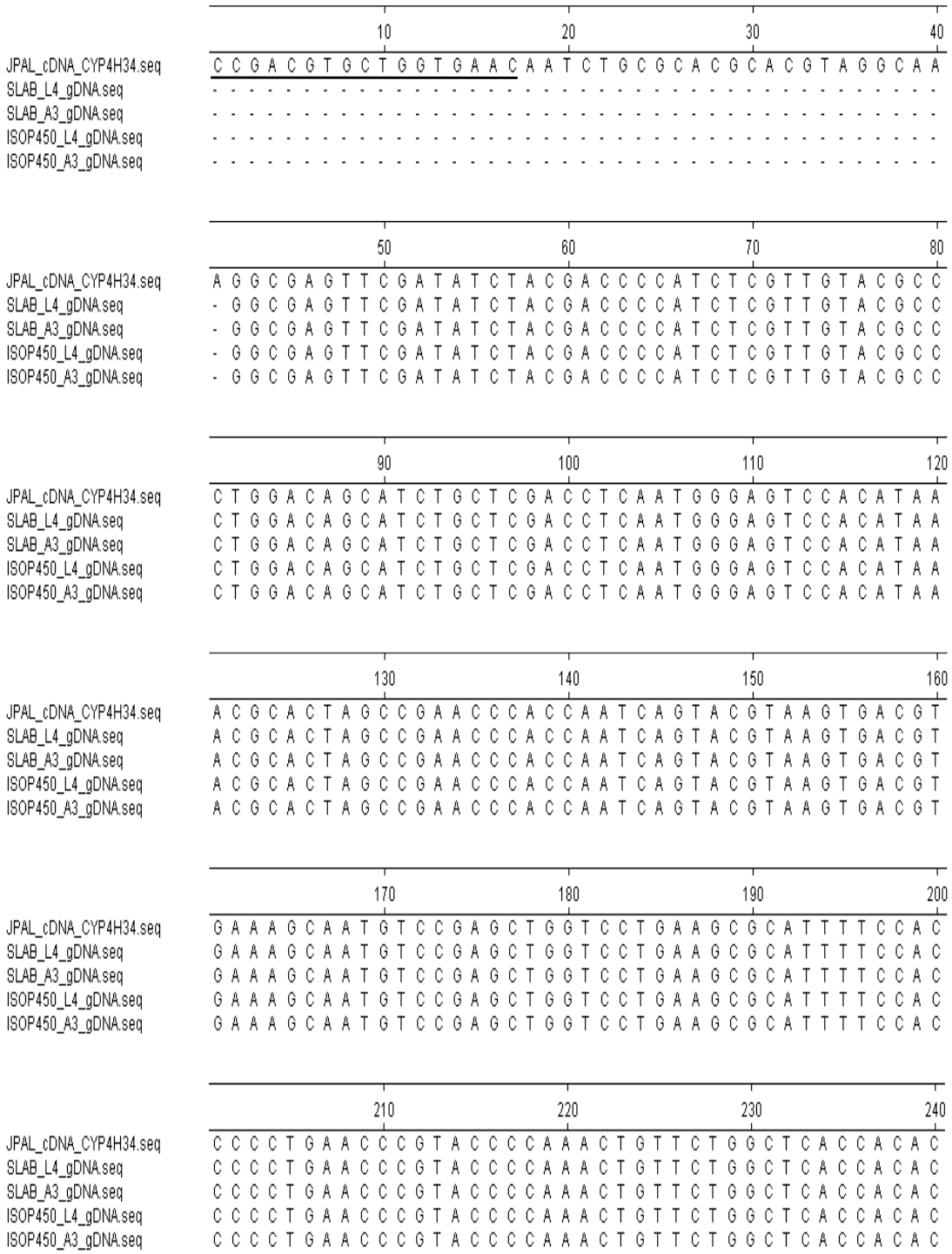
Figures 7.4 and 7.5 show the gDNA sequences of the products amplified using semi-quantitative RT-PCR primer pairs in relation to the reference cDNA sequence obtained from JPAL (provided by T. Tomita). Sequences of *CYP4H34* were not different between susceptible (SLAB 3 dyo adult and fourth instar larvae) gDNA and resistant (ISOP450 3 dyo adult and fourth instar larvae) gDNA when compared to the sequence of the resistant JPAL strain (Figure 7.4). This further affirms that *CYP4H34* is likely not the P450 conferring permethrin resistance in JPAL and ISOP450.

The partial sequences of *CYP9M10* differed at 8 sites between the susceptible (SLAB) and resistant (ISOP450 and JPAL) strains (Figure 7.5). Single nucleotide polymorphisms (SNPs) were present between SLAB and ISOP450 within the intron at positions 226, 241 and 262. SNPs within the exons at positions 33 (T to C), 96 (T to A), 134 (C to T), 288 (C to T) and 390 (A to G) code for non-synonymous substitutions. This fragment of the gene is likely to be indicative of the divergence present in the whole gene displaying the importance of sequencing the entire gene. It is possible that non-synonymous mutations could alter how the protein folds in the resistant strain, conferring resistance. It is also possible that mutations in the non-coding regions (particularly the promoter region) could lead to the differences in expression level between susceptible and resistant strains. Further investigations into these molecular mechanisms of resistance will need to be pursued. Since 8 polymorphic sites are present in the fragment of *CYP9M10* sequenced in this study, it might be possible to use this fragment as a molecular tool for determining resistance status in an individual mosquito.

7.4 Discussion and Future Experiments

This study showed that *CYP9M10* is 2,200-fold over-expressed in ISOP450 fourth instar larvae and is likely the P450 which confers permethrin resistance. *CYP9M10* was also significantly over-expressed in the 3 dyo female adults of ISO450 as compared to the susceptible strain. Genomic sequence information also revealed polymorphisms that differentiate the R and S allele of *CYP9M10* such that the fragment analyzed could be used as a molecular tool to determine allele frequencies in wild resistant populations. It is not likely that *CYP4H34* is involved in permethrin resistance.

Figure 7.4: Alignment of a partial *CYP4H34* gDNA sequence from resistant (ISOP450) and susceptible (SLAB) strains compared to the JPAL reference cDNA sequence (provided by T. Tomita). No differences in sequence were found between the resistant and susceptible strains. Primer sequences are underlined. L4: gDNA template from fourth instar larvae; A3: gDNA template from 3 dyo adult females.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SLAB_A3_gDNA.seq -
ISOP450_L4_gDNA.seq -
ISOP450_A3_gDNA.seq -
50 60 70 80
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SLAB_L4_gDNA.seq - G G C G A G T T C G A T A T C T A C G A C C C C A T C T C G T T G T A C G C C
SLAB_A3_gDNA.seq - G G C G A G T T C G A T A T C T A C G A C C C C A T C T C G T T G T A C G C C
ISOP450_L4_gDNA.seq - G G C G A G T T C G A T A T C T A C G A C C C C A T C T C G T T G T A C G C C
ISOP450_A3_gDNA.seq - G G C G A G T T C G A T A T C T A C G A C C C C A T C T C G T T G T A C G C C
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SLAB_L4_gDNA.seq C T G G A C A G C A T C T G C T C G A C C T C A A T G G G A G T C C A C A T A A
SLAB_A3_gDNA.seq C T G G A C A G C A T C T G C T C G A C C T C A A T G G G A G T C C A C A T A A
ISOP450_L4_gDNA.seq C T G G A C A G C A T C T G C T C G A C C T C A A T G G G A G T C C A C A T A A
ISOP450_A3_gDNA.seq C T G G A C A G C A T C T G C T C G A C C T C A A T G G G A G T C C A C A T A A
130 140 150 160
JPAL_cDNA_CYP4H34.seq A C G C A C T A G C C G A A C C C A C C A A T C A G T A C G T A A G T G A C G T
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SLAB_A3_gDNA.seq A C G C A C T A G C C G A A C C C A C C A A T C A G T A C G T A A G T G A C G T
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SLAB_A3_gDNA.seq G A A A G C A A T G T C C G A G C T G G T C C T G A A G C G C A T T T T C C A C
ISOP450_L4_gDNA.seq G A A A G C A A T G T C C G A G C T G G T C C T G A A G C G C A T T T T C C A C
ISOP450_A3_gDNA.seq G A A A G C A A T G T C C G A G C T G G T C C T G A A G C G C A T T T T C C A C
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SLAB_L4_gDNA.seq C C C C T G A A C C C G T A C C C C A A A C T G T T C T G G C T C A C C A C A C
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Figure 7.4 (Continued):

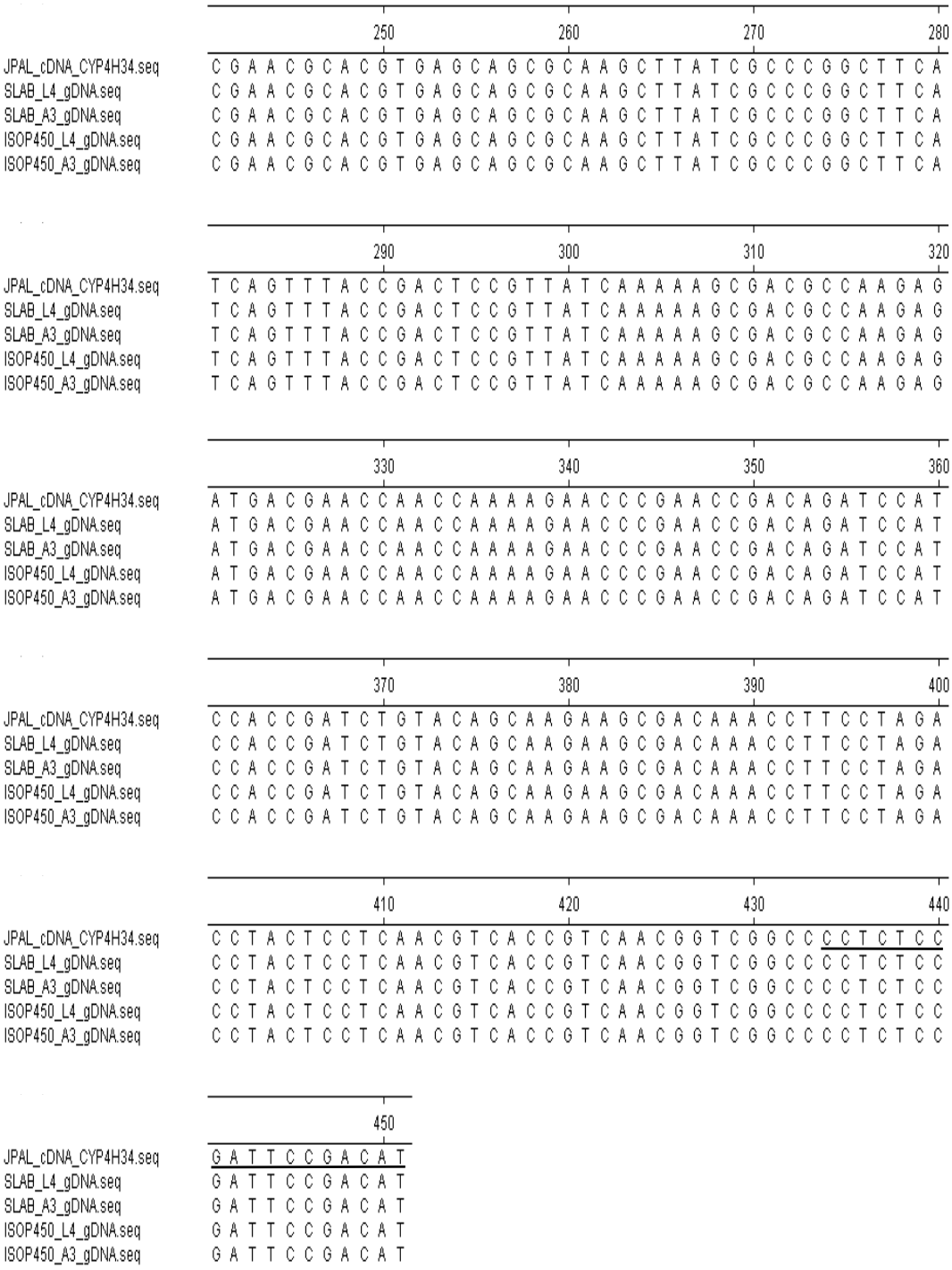
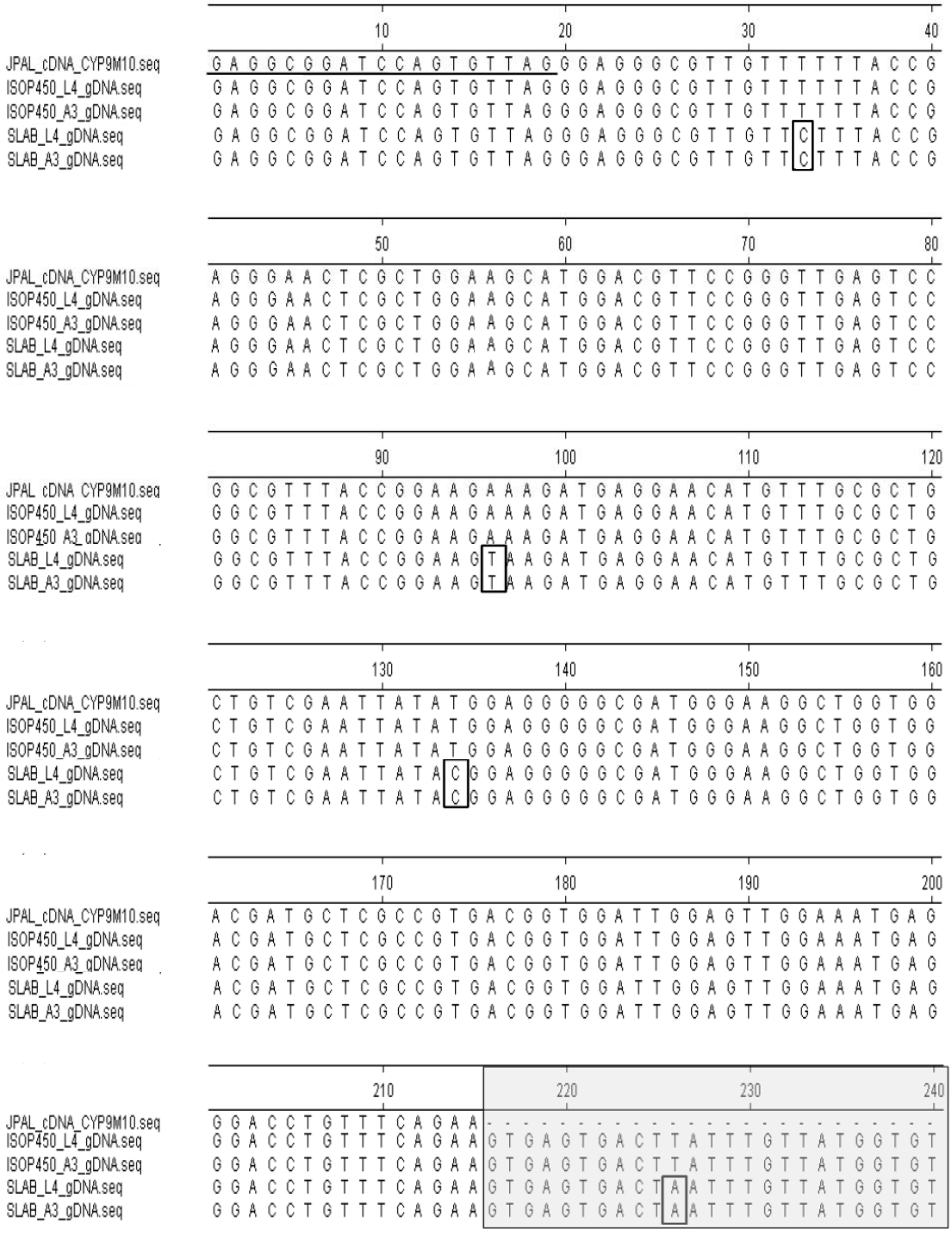


Figure 7.5: Alignment of a partial *CYP9M10* gDNA sequence from ISOP450 and SLAB compared to the JPAL reference cDNA sequence (provided by T. Tomita). Primers are underlined, boxes indicate polymorphisms between the susceptible and resistant alleles, and shaded region indicates an intron. L4: gDNA template from fourth instar larvae; A3: gDNA template from 3 dyo adult females.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ISOP450_A3_gDNA.seq G A G G C G G A T C C A G T G T T A G G G A G G G C G T T G T T T T T T A C C G
SLAB_L4_gDNA.seq G A G G C G G A T C C A G T G T T A G G G A G G G C G T T G T T C T T T A C C G
SLAB_A3_gDNA.seq G A G G C G G A T C C A G T G T T A G G G A G G G C G T T G T T C T T T A C C G
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ISOP450_L4_gDNA.seq A G G G A A C T C G C T G G A A G C A T G G A C G T T C C G G G T T G A G T C C
ISOP450_A3_gDNA.seq A G G G A A C T C G C T G G A A G C A T G G A C G T T C C G G G T T G A G T C C
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ISOP450_A3_gDNA.seq G G C G T T T A C C G G A A G A A A G A T G A G G A A C A T G T T T G C G C T G
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SLAB_A3_gDNA.seq G G C G T T T A C C G G A A G T A A G A T G A G G A A C A T G T T T G C G C T G
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ISOP450_A3_gDNA.seq C T G T C G A A T T A T A T G G A G G G G G C G A T G G G A A G G C T G G T G G
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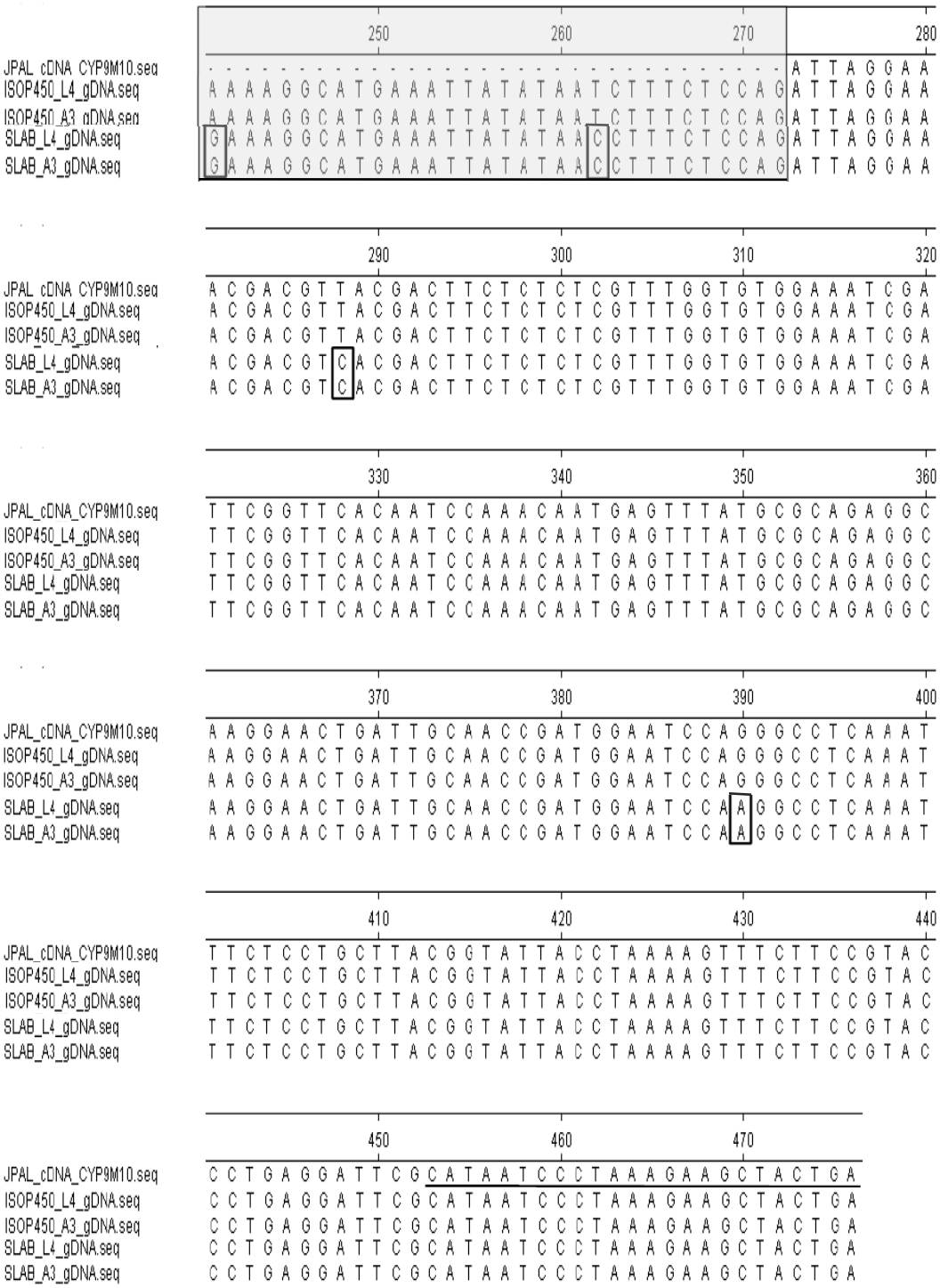
ISOP450_L4_gDNA.seq G G A C C T G T T T C A G A A G T G A G T G A C T T A T T T G T T A T G G T G T
ISOP450_A3_gDNA.seq G G A C C T G T T T C A G A A G T G A G T G A C T T A T T T G T T A T G G T G T
SLAB_L4_gDNA.seq G G A C C T G T T T C A G A A G T G A G T G A C T A A T T T G T T A T G G T G T
SLAB_A3_gDNA.seq G G A C C T G T T T C A G A A G T G A G T G A C T A A T T T G T T A T G G T G T

Figure 7.5 (Continued):



Quantitative real-time PCR analysis indicates that the extreme over-expression (2,200-fold) of *CYP9M10* in ISOP450 larvae is consistent between biological replicates and technical replicates. This level of over-expression is much larger than previously published values associated with insecticide resistance [267, 279, 280, 287, 288, 343, 345, 348, 517–530] which range from 2 to 50-fold. However, the confirmation of the qRT-PCR results by semi-quantitative RT-PCR gives me great confidence in my results.

Few previous reports have found levels of mRNA over-expression to be as large as those observed in this study. One study examining the permethrin resistant ALHF strain of house fly measured over-expression of *CYP6A5v2* by approximately 1,000-fold as compared to two susceptible strains by qRT-PCR [531]. Tissue specific qRT-PCR analysis of *CYP6A5v2* in ALHF showed that head+thorax and abdomen tissues were 500-fold and >2,000-fold, respectively, higher than the susceptible CS strain [531]. A previous study evaluating P450s in *D. melanogaster* by transgenic over-expression showed that a *Cyp6a19* transgene was over-expressed by 755-fold compared to the transgene control [526]. A study conducted on procarcinogen-activating P450s in human livers found that expression of CYP1A2 mRNA was approximately 1,000-fold higher in an ex-smoker than in the control nonsmokers [532]. Wang et al. [533] determined that CYP26 in rats treated with all-*trans*-retinoic acid (RA) was approximately 10-fold greater after 8 hrs and approximately 2,000-fold higher after 10 hrs post-treatment compared to the no-RA control rats [533]. Lowe and colleagues analyzed mouse P-19 stem cells for expression of neural and stem cell genes after induction with retinoic acid [534]. They determined that during the cell differentiation period, target genes GABA (γ-aminobutyric acid receptor B1a) and GLUR1 (glutamate receptor 1)

expression levels increased 1,000-fold compared to the 0-time point control [534]. Induction by β-naphthoflavone in salmonid species resulted in 100-1,000-fold increases in CYP1A transcript levels [535].

The extraordinarily high level of *CYP9M10* present in ISOP450 larvae could be due to a multitude of mechanisms which include, but are not limited to, gene amplification or duplication, mRNA stabilization, promoter region mutations, and transcription factor binding. It is unknown which of these or other mechanisms might be the cause of the observed over-expression, therefore further experimentation is necessary. It is not likely that I am amplifying an unintended target in the qRT-PCR method since the semi-quantitative RT-PCR method confirmed the real-time results using a different primer pair and the real-time PCR products were the expected sequence (results not shown). Future experiments include conducting qRT-PCR on another strain (ISOJPAL, see Appendix E), which is isogenic to SLAB, permethrin resistant and is homozygous resistant for *kdr* and *CYP9M10*.

CHAPTER 8

RESEARCH SUMMARY AND FUTURE DIRECTIONS

8.1 Research summary

The objectives of this study were to investigate cytochrome P450 monooxygenase-mediated permethrin resistance in the southern house mosquito, *Cx. p. quinquefasciatus*. Genetics, cross-resistance patterns, interactions with *kdr*, fitness in the laboratory, and identification of a potential gene conferring resistance were determined.

This is the first study to examine resistance exclusively conferred by P450s via the isolation of the ISOP450 resistant strain. P450-mediated detoxification of permethrin was determined to be expressed in the larvae as an incompletely dominant and monofactorially inherited trait. Cross-resistance patterns revealed that the P450 isoform is highly substrate specific detoxifying only permethrin (1,300-fold relative resistance to the susceptible SLAB strain). The alteration of the insecticide compound by the addition of an α-cyano group or modification of the alcohol into a non-phenoxybenzyl moiety decreased resistance levels to <12-fold. Significant, but small, levels of cross-resistance to the OPs diazinon (RR=1.7) and fenitrothion (RR=8.1) were observed. Bioactivation of the OPs temephos (RR=0.73) and malathion (RR=0.84) were small, but significant. Results from this study provided base-line information about the P450 mechanism and practical information that vector management groups can use for controlling mosquito populations with this resistance mechanism.

The interaction of multiple genes which confer resistance was examined by looking at the relative contribution to permethrin resistance of P450-mediated detoxification and *kdr*. Observed resistance ratios obtained

from genotype combinations of the two independent loci were compared with linear-based and logarithmic-based null models of additivity. Each of the genotype combinations for P450 detoxification and *kdr* resulted in non-additive interactions and were found to conform to the multiplicative null model. Implications of these findings suggest that populations which have both P450 detoxification and *kdr* are capable of evolving extraordinarily (for example, JPAL RR=29,000) high levels of permethrin resistance and controlling these populations will be difficult. Fitness associated with the P450 mechanism was examined since fitness can alter and impact the trajectory of resistance evolution in the field. In laboratory-based experiments the P450 resistance allele was tracked through time in sub-optimal environments.

Fitness associated with P450 resistance varied with environment where standard laboratory conditions were only minorly costly (with a steady, but not significant, decrease in R allele frequency). Conversely, temephos exposure increased the cost of having an R allele since this insecticide was known to be bioactivated by the P450 present in the ISOP450 strain. Additionally, colder temperatures were not a stressor for the R allele and an excess of heterozygotes resulted from this environment. Significance of these results suggests that using temephos could eliminate the R allele from a resistant population with this mechanism. There is the potential for the R allele to spread from populations of *Cx. p. quinquefasciatus* into the hybrid zones and finally into populations of *Cx. p. pipiens* potentially compromising control of both mosquito species. In direct comparisons between SLAB and ISOP450, fitness indices that differed between the strains included early emergence of SLAB females, increased longevity of ISOP450 females, and larger levels of energy obtained from glycogen and lipids in SLAB. In conclusion, these results suggest

that in an insecticide-free environment, only a minor cost is associated with the P450 detoxification mechanism and the costs manifest more evidently in the adult stages as compared to the expectation that larvae would exhibit a cost.

Using sequences provided by collaborators (T. Tomita, University of Tsukuba, Japan) based on analysis of P450-mediated permethrin resistance in JPAL, expression patterns of *CYP4H34* and *CYP9M10* were determined in ISOP450. The strength of using ISOP450 was that monofactorial inheritance had been identified and the level of genetic differentiation when analyzed with SLAB was minimal. No differences in expression level of *CYP4H34* were found between SLAB and ISOP450 fourth instar larvae or 3 day old female adults indicating that over-expression of this gene is not responsible for resistance. Conversely, *CYP9M10* expression in ISOP450 larvae was 2,200-fold higher than SLAB larvae and 12-fold higher in ISOP450 adults versus SLAB adults. Additionally, multiple polymorphic sites differentiated the resistant and susceptible alleles in a 476 bp fragment of the 1,613 bp gene sequence. These results strongly implicate *CYP9M10* as the P450 which is responsible for the P450-mediated permethrin resistance observed in JPAL and ISOP450.

In summary, further investigations of cytochrome P450-mediated permethrin resistance are certainly required in order to understand how this mechanism works and evolves, particularly in field settings where control failures can lead to disease outbreaks. Since monofactorial inheritance of P450-mediated permethrin resistance was determined in the ISOP450 strain, it is extremely probable that *CYP9M10* is responsible for resistance. If *CYP9M10* is the P450 isoform conferring resistance it was unexpected to measure low fitness costs in relation to the >2,000-fold over-expression of *CYP9M10* in ISOP450 larvae. Additionally, due to the low fitness costs and incomplete

dominance of this mechanism, merely decreasing the application frequency/concentration of permethrin or eliminating insecticides from the environment will not attain control of resistant populations. Alternatively a strategic shift to using non-phenoxybenzyl pyrethroids, α-cyano pyrethroids or temephos will provide varied levels of control. Of these alternatives, using temephos is the best option because this compound creates a costly environment for the P450 resistance mechanism (due to the bioactivation of temephos), which results not only in a decrease of the P450 R allele frequency but also kills mosquitoes providing the necessary decrease in densities in order to attain control of vector-borne diseases.

In order to enhance our ability to delay the onset of permethrin resistance in *Cx. p. quinquefasciatus* and other insect taxa, it is imperative to know about the resistance mechanisms responsible. Investigating the underlying biology and molecular mechanisms can provide more precise tools which can be used by vector control teams. While tools used in the field (such as the WHO susceptibility test) can detect if resistance is present in a population, they will not necessarily provide detailed information about the level of resistance (especially when the R allele is at a low frequency), if other resistance mechanisms are present in a population, nor provide insight into alternative insecticides that might be effective in controlling the population of interest [536]. Hence, gaining a wide-range of information (biochemical, molecular, etc.) on a resistance mechanism can provide vector control teams with useful data that can inform their future decisions.

The particular complexity of the cytochrome P450-mediated detoxification leaves many new avenues of research to be explored. The following are some questions in order of how I would approach them which

would be useful to answer to gain a more complete picture of P450-mediated permethrin resistance in *Cx. p. quinquefasciatus*.

8.2 Future directions

8.2.1 Is *CYP9M10* over-expressed in ISOJPAL (Appendix E)?

Additional experimentation on ISOJPAL would provide results leading to a more complete picture of the cytochrome P450 detoxification mechanism. Analysis of P450 content in ISOJPAL microsomes will add to the bioassay and genotype data already collected for this strain (Appendix E). Conducting quantitative real-time PCR for *CYP9M10* in ISOJPAL can serve as a method for determining if the 2,200-fold over-expression of *CYP9M10* in ISOP450 larvae is a steady result or an anomaly.

8.2.2 Does CYP9M10 metabolize permethrin?

Conduct metabolism studies *in vitro* to determine the specificity of the catalytic pocket of CYP9M10. Recombinant P450s have been used to examine deltamethrin metabolism by CYP6AA3 in *An. minimus* [537,538]. In order to determine specifically what CYP9M10 can metabolize, it would be necessary to create a recombinant CYP9M10 [539] and then screen the construct in a reconstituted reaction against pyrethroid (especially permethrin), organophosphate and carbamate insecticides as well as other insecticides commonly used in vector management programs. This will confirm that CYP9M10 present in ISOP450 is metabolizing permethrin and will also inform us of which compounds should not be used for controlling mosquito populations which have this particular P450 isoform.

8.2.3 What is the fitness associated with cytochrome P450-mediated resistance when a life table analysis approach is used?

Fitness is measured as the success of an individual to produce offspring and contribute genes to the next generation. As such, it would be important to conduct a life table analysis of SLAB and ISOP450 in an insecticide-free environment in order to obtain the net replacement rate, R_0, and the instantaneous growth rate, r, of these two strains. This level of measurement would be needed to quantify the fitness of the strains and conclude if a fitness cost is truly associated with P450-mediated detoxification (and verify the minor cost measured in an insecticide-free environment as detailed in Chapters 5 and 6). These more direct fitness measurements could be conducted in varied environmental conditions to determine how fitness of individuals with P450-mediated resistance changes with environment. It might also be possible to incorporate the fitness associated with P450 detoxification (and *kdr* by using the ISOJPAL strain (see Appendix E)) into the model of how the phenotypes of the mechanisms interact to see how the fitness might alter the resistance phenotype (see Chapter 4).

8.2.4 What is the molecular mechanism of *CYP9M10* over-expression in ISOP450 larvae?

Previous research on the molecular mechanisms of up-regulation of P450s have been found to be due to mutations in *trans*-regulatory loci, indels or *cis*-acting elements, and coding sequence differences [540]. There could also be changes in the catalytic activity of the protein that confer resistance [540]. Previous studies investigating the molecular mechanisms responsible for P450 over-expression have primarily examined the promoter sequence to identify any differences between the susceptible and resistant strains. Cloning the

promoter region of *CYP9M10* from ISOP450 and SLAB *Cx. p. quinquefasciatus* strains, might provide information on how *CYP9M10* is over-expressed. Does the sequence suggest that specific transcriptional regulators can bind to the promoter region? If so, how do they act, as enhancers or suppressors? It would also be of interest to determine if the non-synonymous polymorphisms which differ between SLAB and ISOP450 alter the active site and therefore the catalytic properties of the resistant enzyme. An additional curiousity would be to determine the mechanism by which *CYP9M10* expression is larval-specific. Could it be that JH induces a transcription factor which binds specifically to some part of the sequence in the resistant strain? Methods to approach this question might be: 1. shutting off JH in ISOP450 larvae by conducting RNAi (injecting dsRNA for JH and then measuring *CYP9M10* expression after treatment); 2. turning on JH in ISOP450 adults by using a JH mimic such as methoprene; or 3. performing gel-shift assay to determine if a transcription factor is preferentially binding to the DNA of ISOP450 larvae versus SLAB larvae.

8.2.5 What factors increase heterozygote fitness in cold temperatures?

Cold temperatures are found in the northern boundary of *Cx. p. quinquefasciatus* range which overlaps with the range of the northern house mosquito, *Cx. p. pipiens* where a hybrid zone is present. In Chapter 5, a heterozygote advantage was observed in the cold temperature environment. It is unknown why this occurred; therefore, determining the specific factors associated with the increased fitness of the heterozygote genotype in colder temperatures would be important for restricting gene flow of this resistance mechanism into *Cx. p. pipiens* populations.

8.2.6 What is the fitness associated with P450 detoxification under field conditions?

Analyzing if fitness costs are present in resistant strains of insects is important and can provide necessary information on the possible evolutionary trajectory of a resistance allele. It is necessary to determine if the P450 detoxification R allele is costly when placed in competition with the S allele under field conditions. Are the results determined in the laboratory more or less pronounced under field conditions? This could be conducted in small cages placed in multiple locations and examining various biological parameters such as survival/longevity and reproduction.

8.2.7 What is the distribution and population genetics of *CYP9M10*?

Diagnostic polymorphic sites of *CYP9M10* which correlate with permethrin resistance in JPAL and ISOP450 and not SLAB can be used as a molecular tool to monitor and track allele frequencies for this mechanism of resistance in fitness experiments or in field collected mosquito populations. Seven diagnostic polymorphic sites were determined in Chapter 7. It would be of interest to determine if *CYP9M10* resistance allele associates with permethrin resistance in *Cx. p. quinquefasciatus* worldwide or is it restricted to certain areas or populations. Is this the gene conferring P450-mediated detoxification in all resistant *Culex* populations found to have a P450 mechanism, or is it one P450 of many? Also, since multiple susceptible alleles were found in SLAB (Appendix E), determining the total number of alleles present in wild populations would be a necessary follow-up to comprehensively assess the geographic distribution of *CYP9M10*.

Since the consequences of failed vector control result in human and animal morbidity and mortality, better knowledge of insecticide resistance

mechanisms are clearly necessary and alternative control measures will need to be incorporated into the management approaches. Additionally, because many of the insecticides used in vector control have originally been developed and used by the agricultural sector, resistance in mosquito vectors evolves quickly. Therefore, as vector biologists, toxicologists and geneticists, we should strive to implement an integrative vector control system, which ought to include an emphasis on gaining knowledge about mechanisms of insecticide resistance.

APPENDIX A

TOXICITIES OF PERMETHRIN ISOMERS TO ISOP450

Table A.1: Toxicities of permethrin isomers to ISOP450 and SLAB fourth instar larvae.

Cis:*trans* ratio	Company	LC_{50}[a] (95% CI)		Resistance ratio
		SLAB	ISOP450	of ISOP450
100:0	FMC	0.0014 (0.0011-0.0018)	0.32 (0.22-0.45)	229
100:0	Zeneca	0.0012 (0.0011-0.0013)	0.22 (0.19-0.26)	183
100:0	ChemService	0.0016 (0.0012-0.0021)	0.45 (0.38-0.53)	281
50:50	ChemService	0.0017 (0.0015-0.0019)	2.1 (1.9-2.5)	1,300
0:100	ChemService	0.0035 (0.0032-0.0038)	4.0 (2.4-6.9)	1,100

[a] Units: μg/ml.

Table A.1 lists the various permethrin isomers screened against the susceptible (SLAB) and resistant (ISOP450) strains. *Cis*-permethrin was more toxic to ISOP450 than 50:50 (*cis*:*trans*) permethrin, with the *trans*-permethrin isomer being the least toxic. All isomers were approximately equally toxic to SLAB with the exception of the *trans*-permethrin which was the least toxic as well. The *trans*-permethrin isomer is degraded more rapidly than the *cis* isomer due to ester cleavage and oxidation [541] and therefore tends to have lower toxicity. It is unclear why the 50:50 permethrin affects SLAB in a fashion that is similar to the *cis*-permethrin and ISOP450 in a fashion that is like *trans*-permethrin.

APPENDIX B

PLOTS OF PHENOTYPE VERSUS P450-MEDIATED DETOXIFICATION AND *KDR* TWO-LOCUS GENOTYPE *

To determine if epistasis is present between two independent loci that contribute to a single phenotype, deviations from additivity can be mathematically calculated (see Chapter 4) or visualized on plots of phenotype values in relation to the two-locus genotypes. The graphical plots can be constructed either by conducting a linear regression ("linear additive model") or by following the "multiplicative model" whereby the phenotypic values are logarithmically transformed. The "linear additive model" follows the underlying mathematics of phenotype value = $\alpha + \beta$, where α represent the phenotype given a genotype of locus A and β represent the phenotype given a genotype of locus B. When the lines on the plot are parallel then additivity is implied and if the lines are not parallel departures from additivity are concluded (indicating epistasis). For the "multiplicative model" the underlying mathematics are based on probabilistic properties such that phenotype value = $\alpha\beta$ and $\log_{10}$phenotype value = $\log_{10}\alpha + \log_{10}\beta$. In this null hypothesis, parallel lines imply the two loci are interacting multiplicatively (which is considered epistasis to those who follow the "linear additive model") and non-parallel lines indicate that epistasis is acting [365,366].

In the case of the interaction between P450-mediated detoxification and *kdr* in *Cx. p. quinquefasciatus*, the plot models of phenotype values in relation to the two-locus genotype showed conflicting results. In the "linear additive

* Presented with minor modifications from the originally published article Hardstone, M. C., C. A. Leichter and J. G. Scott. (2009). "Multiplicative interaction between the two major mechanisms of permethrin resistance, cytochrome P450- monooxygenase detoxification and *kdr*, in mosquitoes." J. Evol. Biol. 22:416-423.

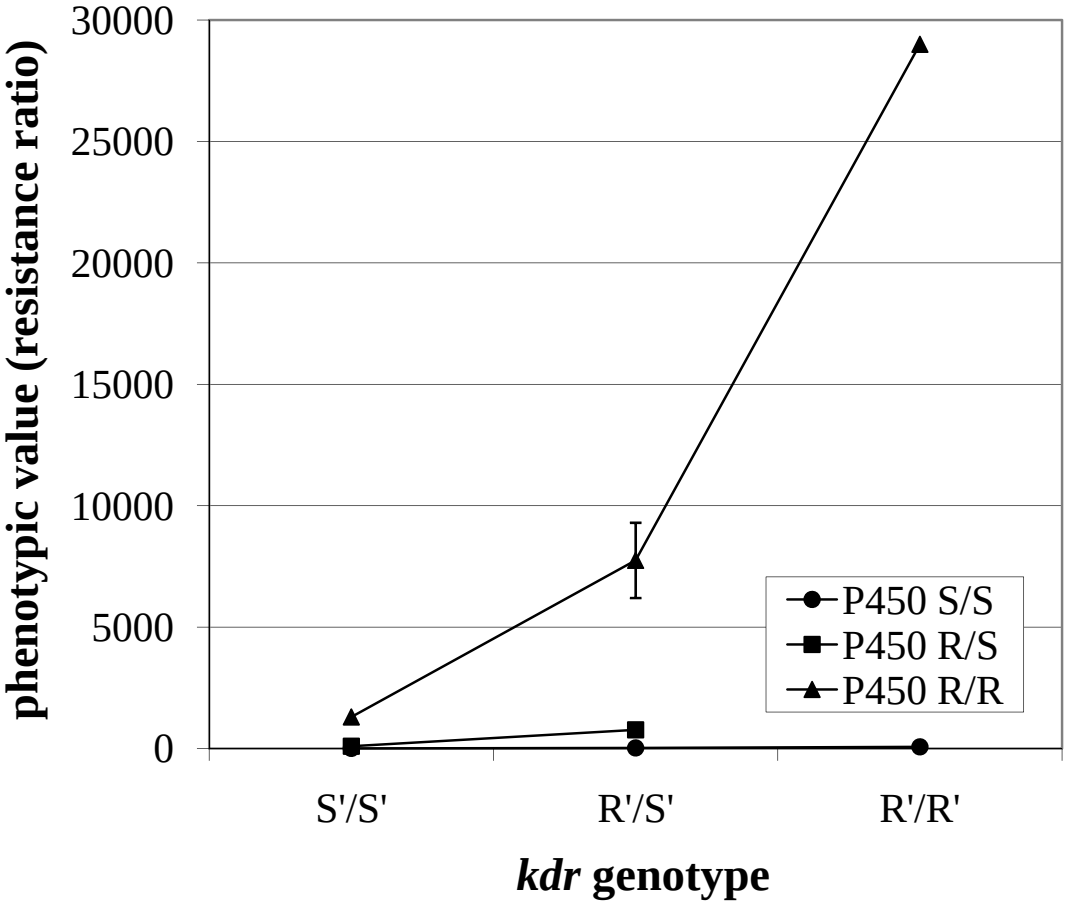


Figure B.1: "Linear additive model" plot of phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of P450 detoxification and *kdr*.

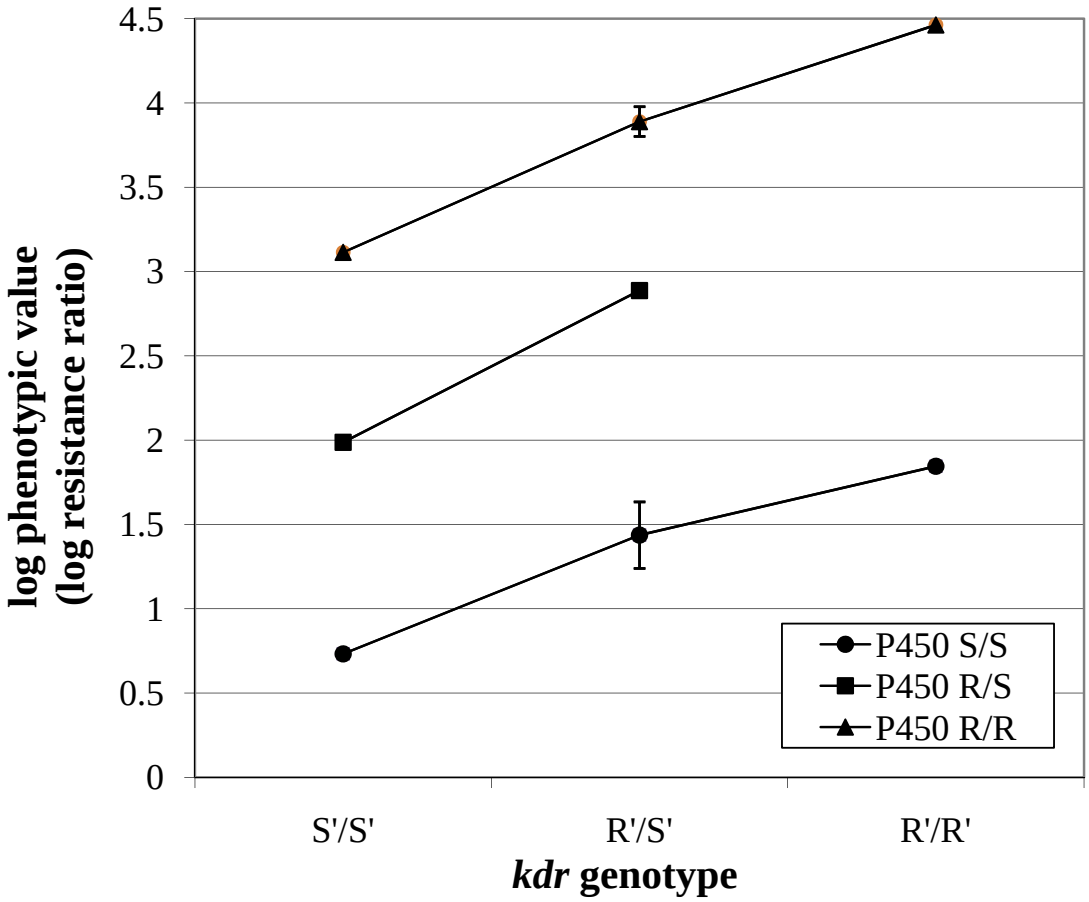


Figure B.2: "Multiplicative model" plot of logarithmically-transformed phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of P450 detoxification and *kdr*.

model" plot, the lines were not parallel (Figure B.1) however, when the phenotype values were logarithmically transformed for the "multiplicative model," the lines were parallel (Figure B.2).

Thus, according to the two analytical approaches, the results from Chapter 4 could be variously classified. However, these results could be uniformly labeled as having a multiplicative type interaction between P450 detoxification and *kdr*.

APPENDIX C

COMPUTER SIMULATION CODE TO CALCULATE GENETIC DRIFT

I created the following program (in R) to simulate multiple generations of genetic drift where allele frequencies are not known at each generation. The output of this function is the probability (*p*-value) of seeing a change in allele frequency from a known start frequency to a known end frequency where two or more generations have elapsed. If the *p*-value is large then drift is likely to be occurring between the generations of interest. Conversely, if the *p*-value is small that means there is a low probability of seeing a change in frequency as large as what was observed and therefore drift is not likely to be driving that change (rather if the generation is out of HWE, selection may be occurring). Comments after a "pound sign" (#) indicate the function of that particular command within the program. This function was developed and can be run in the R environment (http://www.cran.r-project.org/).

Variables of the function include: SIMS (number of simulations), gens (number of generations elapsed), size_of_pop (population size, can be fixed or designated by a vector with differing values), R_start_freq (frequency of R allele at the start of the interval of interest), and R_end_freq (frequency of R allele at the end of the interval of interest).

Function:

```
MultiGenDrift<-function(SIMS, gens, size_of_pop, R_start_freq, R_end_freq) {
result<-NULL; #results vector
for (i in 1:SIMS) #number of simulations
{
        drift_result<-NULL; #vector to track allele frequencies each generation
        gen<-gens; #number of generations
```

```
        pop_size<-size_of_pop; #starting population size
        R_alleles<-R_start_freq; #starting R allele frequency
        pop_alleles<-c(rep("R",2*pop_size*R_alleles),rep("S",2*pop_size*(1-R_alleles)));
        #makes initial population of alleles
        pop_alleles<-sample(pop_alleles,2*pop_size,replace=TRUE);
        #takes random sample from population to make g+1 generation
        R_alleles<-(length(pop_alleles[pop_alleles=="R"]))/(2*pop_size);
        #freq of g+1 R alleles
        drift_result<-c(drift_result, R_alleles);
        #vector for R allele frequency from each generation
        for(i in 2:gen) #runs the same procedure as above
        {
                R_alleles<-R_alleles; #uses new R frequency
                pop_alleles<-c(rep("R",2*pop_size*R_alleles,rep("S",2*pop_size*(1-R_alleles)));
                pop_alleles<-sample(pop_alleles,2*pop_size,replace=TRUE);
                R_alleles<-(length(pop_alleles[pop_alleles=="R"))/(2*pop_size);
                #R allele frequency
                drift_result<-c(drift_result, R_alleles);
        }
        #within the for loop: the R frequency when loop is completed is used as the
        start R frequency for the next loop (i.e., the next generation)
        drift_result<-as.vector(drift_result); #vector for drift calculation results
        res<-drift_result[gen]; #R frequency at generation of interest
        result<-c(result, res); #vector of above over all simulations
}
result<-as.vector(result);
#new vector of R frequency values at generation of interest for all simulations
hist(result) #creates histogram of the R frequency distribution
#need to count number of times R frequency at generation of interest is equal to or more
extreme than observed frequency (designated by R_end_freq)
#need to designate which direction (more than or less than) the extreme values is wanted
if(R_end_freq < R_start_freq) #declares less than direction
```

```
{
        result<-result[result <= R_end_freq] #takes all R frequency values from
        the given generation of interest that are less than or equal to the R_end_freq
        result<-length(result); #counts the number from above
        result<-result/SIMS; #divides by the total number of simulations
        }else{ #does the same as above but for the more than direction
        result<-result[result >= R_end_freq];
        result<-length(result);
        result<-result/SIMS;
} }
```

APPENDIX D

LEVELS OF CYTOCHROME P450 IN RESISTANT (ISOP450 AND JPAL) AND SUSCEPTIBLE (SLAB) STRAINS OF *CULEX PIPENS QUINQUEFASCIATUS*

D.1 Introduction

Cytochrome P450 monooxygenases in insects are involved in multiple processes such as the metabolism of hormones, toxins from plants and natural and synthetic insecticides [242,258,413,542]. As such, identifying insect P450s responsible for insecticide resistance has direct implications for control of pest species.

In an attempt to determine specific P450 isoforms that were conferring the high levels of permethrin resistance observed in the JPAL and ISOP450 strains of *Cx. p. quinquefasciatus*, I isolated microsomes from resistant and susceptible strains and visualized the proteins on a 1-D SDS-PAGE gel. Microsomes are part of the endoplasmic reticulum and contain cytochrome P450s.

D.2 Materials and methods

D.2.1 Mosquito strains

Three strains of *Cx. p. quinquefasciatus* were used and reared as previously described (Chapter 3). SLAB is a standard susceptible strain [420]. JPAL is a strain originating from Saudi Arabia [295] with high levels of permethrin resistance due to *kdr* and cytochrome P450 detoxification [147]. ISOP450 is a 1,300-fold permethrin resistant strain highly related to SLAB having the P450 resistance mechanism present in JPAL, but lacking *kdr* [455].

D.2.2 Reagents and buffers

All reagents were obtained from Sigma Chemical Company (St. Louis, MO). Homogenization buffer was made of 10% glycerol, 1 mM EDTA (ethylenediaminetetracetic acid), 0.1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM PTU (pheylthiourea) in 100 mM sodium phosphate buffer (pH 7.5). Resuspension buffer was prepared the same as the homogenization buffer except glycerol was increased to 20% and PTU was omitted [543].

D.2.3 Microsome preparation

Microsome samples of the three strains were prepared on the same day by dissecting and washing midguts of 200 fourth instar larvae kept on ice and following the protocols of Kasai and colleagues [147] and Lee and Scott [543]. Midguts plus 2 ml homogenization buffer were placed in a teflon pestle homogenizer. A motorized homogenizer (Caframo Wiarton, ON) set at 125 RPM was used to completely pulverize the tissue. The tissue extract was rinsed with homogenization buffer and filtered through cheesecloth into a chilled centrifuge tube. The extract was centrifuged at 10,000 x g for 20 min at 4°C in a JA-20 rotor fitted to a Beckman Coulter Avanti J-E centrifuge (Palo Alto, CA). Supernatant was centrifuged at 100,000 x g for 1 hr at 4°C in a 70TI rotor fitted to a Beckman L8-70M ultracentrifuge (Palo Alto, CA). The resulting pellet was suspended in 2.4 ml resuspension buffer. Microsomes were measured on the same day of preparation and stored at −80°C.

D.2.4 Biochemical determinations

Total protein content was determined according to the method of Bradford [544] with bovine serum albumin (BSA) used as a standard. Total

cytochrome P450 levels were determined by the method described by Omura and Sato [243] using sodium dithionite as a reducing agent and saturating the sample with carbon monoxide. One-dimensional SDS-PAGE was performed using a 10% gel loaded with 20 μg protein into each lane and was stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Invitrogen, Eugene, OR) according to manufacturer's instructions. SDS-PAGE gels were visualized on a Typhoon 9410 Laser Scanner (Amersham Biosciences, Piscataway, NJ) at the Cornell University Biotechnology Resource Center.

D.3 Results and Discussion

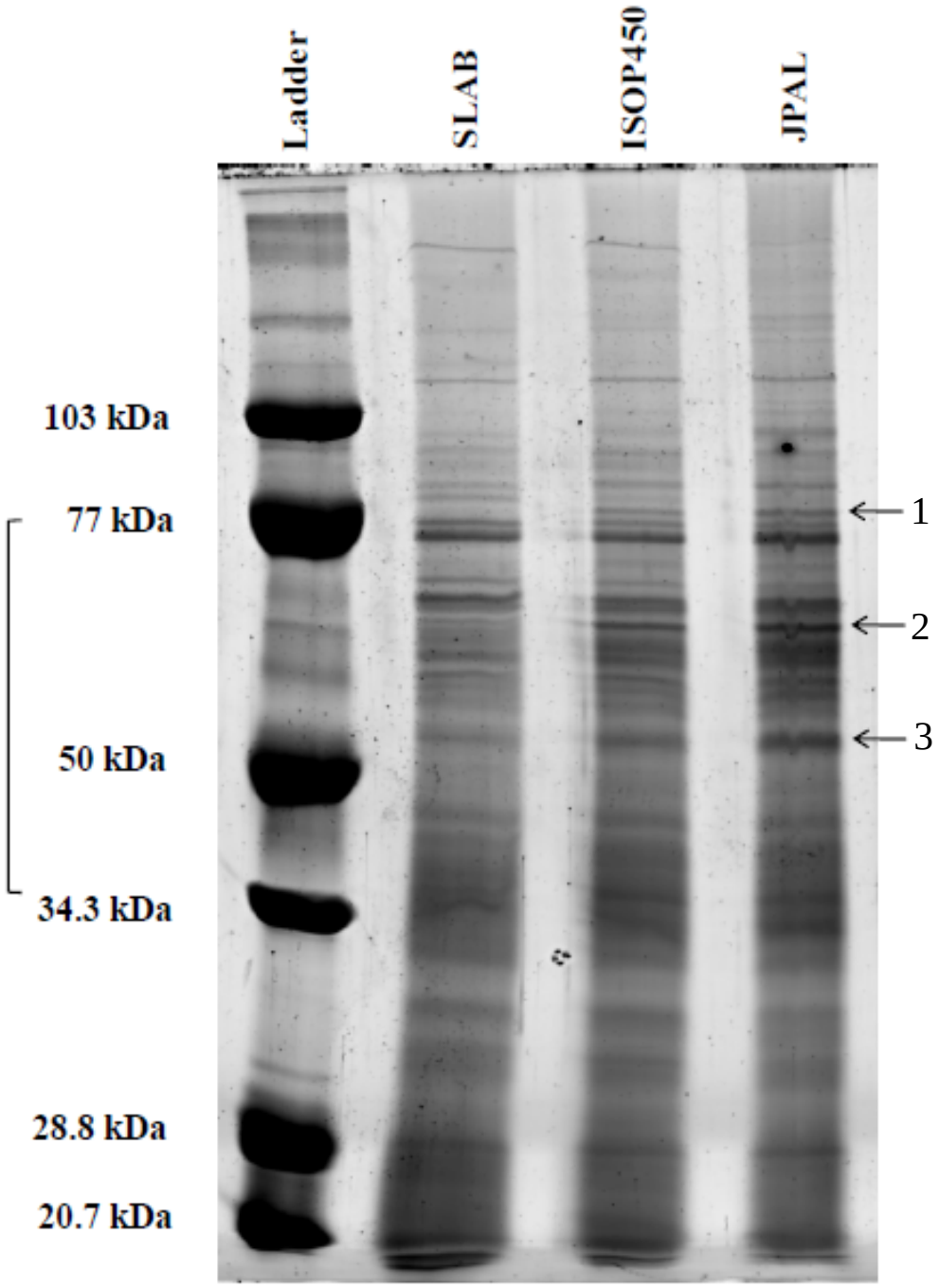
Cytochrome P450 levels were higher in JPAL and ISOP450 as compared to SLAB (Table D.1). Both resistant strains, JPAL and ISOP450, had equivalent levels of total P450s. This is expected since the P450 present in ISOP450 originated from JPAL and was selected to homozygosity. Since *CYP9M10* transcript over-expression in ISOP450 larvae was 2,200-fold and total P450 protein expression was only 1.5-fold higher when compared to SLAB, investigations in to possible post-transcriptional modifications (for example, alternative splicing leading to non-functional protein expression) should be pursued.

The 1-D SDS-PAGE gel (Figure D.1) provided 3 possible candidate protein bands for further investigation. *CYP9M10* contains a 1,613 bp open reading frame (ORF) and a deduced amino acid sequence of the ORF of 537 amino acids. This gives a mass of 61,492 Da (61 kDa) which likely aligns with band #3 (Figure D.1). Identification and characterization of these bands were not pursued, but it would be interesting to see if in fact band #3 is CYP9M10. This could be performed by purifying CYP9M10 and running it with microsome samples on a 1-D SDS-PAGE gel. Or, by using 2-DIGE SDS-PAGE

Table D.1: Protein, cytochrome b_5 and cytochrome P450 levels in susceptible (SLAB) and resistant (JPAL and ISOP450) strains of *Cx. p. quinquefasciatus*.

Strain	Replicate	Protein (mg/ml)	Cytochrome b_5 (nmol/mg protein)	Cytochrome P450 (nmol/mg protein)	Ratio of P450
SLAB	#1	0.232	0.350	0.408	
	#2	0.367	0.372	0.541	
	#3	0.219	0.414	0.552	
	average (± S.E.)	0.273 (0.027)	0.378 (0.011)	0.500 (0.027)	1.0
ISOP450	#1	0.272	0.375	0.907	
	#2	0.334	0.294	0.715	
	#3	0.358	0.277	0.634	
	average (± S.E.)	0.321 (0.015)	0.315 (0.017)	0.752 (0.047)	1.50
JPAL	#1	0.286	0.341	0.757	
	#2	0.164	0.735	0.744	
	#3	0.159	0.254	0.712	
	average (± S.E.)	0.203 (0.024)	0.443 (0.085)	0.738 (0.008)	1.47

Figure D.1: 1-D SDS-PAGE gel of *Cx. p. quinquefasciatus* microsomes. The bracket indicates expected size region of P450 proteins. Arrows indicate possible P450 protein bands which are more abundant in ISOP450 and JPAL versus SLAB. Band #1 is approximately 85 kDa, band #2 is approximately 73 kDa and band #3 is approximately 62 kDa.


Ladder
SLAB
ISOP450
JPAL
103 kDa
77 kDa
50 kDa
34.3 kDa
28.8 kDa
20.7 kDa
1
2
3

approach, comparisons could be made between SLAB, ISOP450 and JPAL, and candidate spots could be picked and analyzed by mass spectrometry.

Combining the information provided by the P450 assay and the 1-D SDS-PAGE gel, I would pursue investigation of band #3 on Figure D.1. Since this band is in the middle of the expected size for a P450 protein and it is equivalent in intensity between JPAL and ISOP450, it correlates well with the microsome data and the expected size of CYP9M10.

APPENDIX E

ISOLATION OF A PERMETHRIN RESISTANT STRAIN OF *CULEX PIPIENS QUINQUEFASCIATUS* ISOGENIC TO A SUSCEPTIBLE STRAIN (SLAB) WITH RESISTANCE MECHANISMS, *KDR* AND CYTOCHROME P450-MEDIATED DETOXIFICATION

E.1 Introduction

Production of highly related (*i.e.*, isogenic) strains is usually accomplished through repeated backcrossing together with selection for the trait of interest in order to isolate the locus of interest in a genetic background shared with another strain. By repeatedly backcrossing one strain into another over multiple generations, the level of genetic relatedness increases, while selection on backcross progeny will purge individuals that do not possess the trait of interest. Isogenic strains have been produced with >8 generations of backcrosses [378,379,389,394,398]. This is the primary method by which to attain isogenic diploid insect strains, though caution must be taken as it is not always successful [545]. Therefore, additional generations of selection for the trait of interest should take place to assure homozygosity, as well as comparisons at multiple loci to assure complete introgression of the genetic background of interest.

The goal of this experiment was to prove that a strain of *Cx. p. quinquefasciatus* which was isogenic to SLAB but contained both *kdr* and P450 detoxification could be isolated. In order to obtain this strain, I crossed males of JPAL, which have P450-mediated resistance and *kdr* with females of SLAB. Since I previously determined (Chapter 3) that the P450 locus was more easily maintained through the backcrossing scheme than *kdr*, I hypothesized that the P450 mechanism would be maintained in the strain. Therefore, during the

backcrossing scheme I monitored the presence/frequency of *kdr* to insure that it remained in the population. To determine if the P450 detoxification mechanism was still present, I conducted bioassays with permethrin and permethrin + PBO and genotyped for the presence/frequency of the *CYP9M10* R allele on the final strain (named ISOJPAL). One way ISOJPAL can be used in future experimentation is to examine fitness associated with resistant individuals with both *kdr* and P450 detoxification and meaningfully compare them to ISOP450 and SLAB to determine the effects/implications of having *kdr*.

E.2 Materials and methods

E.2.1 Mosquito strains

Two strains of *Cx. p. quinquefasciatus* were used and reared as previously described (Chapter 3). SLAB is a laboratory susceptible strain [420]. JPAL is a permethrin resistant strain that contains *kdr* and P450 detoxification resistance mechanisms.

E.2.2 Backcrossing scheme

The backcross protocol was similar to that outlined in Chapter 3, except in this instance I wanted to produce a strain isogenic to SLAB with both *kdr* and P450-detoxification. To construct this isogenic strain, SLAB females were crossed with JPAL males *en masse* and F_1 males were backcrossed to SLAB females. Fourth instar larvae were treated with permethrin (Chem Service, Westchester, PA) at 0.02 μg/ml (the LC_{25} for F_1 progeny) to select for heterozygotes. Surviving males were then backcrossed to SLAB females and this backcrossing procedure was repeated for 5 generations. At generation 5, the population was then selected with permethrin for 4 consecutive generations to produce the final strain (named ISOJPAL).

E.2.3 Genotyping for *kdr* and *CYP9M10* resistance alleles

Genomic DNA (gDNA) was extracted from single adult male mosquitoes by completely homogenizing the whole body as previously described (Chapter 3). Briefly, an individual mosquito was homogenized in 200 μl Buffer A and was incubated at 65°C for 30 min. Four hundred μl of LiCl/KAc solution was added and the sample was incubated on ice for at least 10 min. The sample was then centrifuged for 15 min at room temperature and 0.5 ml of supernatant was transferred to a new tube. Genomic DNA was precipitated by adding 300 μl isopropanol, followed by centrifugation for 15 min. The gDNA pellet was washed with 70% ethanol and the pellet was dried for approximately 30 min. The gDNA pellet was dissolved in 30 μl ddH_2O and concentration of samples was determined on a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE). GoTaq DNA polymerase (Promega, Madison, WI) was used in all PCR reactions. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions and were sequenced at the Cornell University Biotechnology Resource Center.

A partial genomic sequence of the voltage sensitive sodium channel (*VSSC*), including the *kdr* mutation site and adjacent intron were sequenced every backcross and subsequent selection generation. Primers used were those outlined in Chapter 3: forward primer CulexkdrF (5' GGAACTTCACCGACTTCATGC 3') and reverse primer CulexkdrR (5' CGCCGACAGACTTGAGGAACC 3'). Thermal cycler conditions were: 94°C for 5 min, 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 min.

A partial genomic sequence of *CYP9M10* was determined by sequencing a 419 bp fragment of the 1.6 kb gene which contains 8 polymorphic sites differentiating R and S alleles. The following primers were used: forward primer 9MF (5′ GAGGCGGATCCAGTGTTAG 3′) and reverse primer 9MR (5′ TCAGTAGCTTCTTTAGGGATTATG 3′). Thermal cycler conditions were: 95°C for 2 min, 35 cycles of 95°C for 30 sec, 53°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 10 min.

E.2.4 Bioassays for P450 detoxification determination

To test for the presence of the P450 detoxification mechanism in ISOJPAL, larval bioassays using permethrin and permethrin + PBO (a P450 inhibitor) were conducted. For each larval bioassay, 20 fourth instars were placed in 4 oz. waxed paper cups (Sweetheart Cup Co., Owings Mills, MD) with 99 ml of distilled water and 1 ml of either permethrin or permethrin + PBO solution. Experiments from Chapter 3 indicated that 0.001 mg/ml (final concentration) was the maximum sublethal concentration of PBO. Mortality was assessed after 24 hrs, and larvae were considered dead if they failed to move or resurface after being probed.

E.3 Results and Discussion

Five generations of backcrossing were conducted with insecticide selection at each generation making ISOJPAL 98.4% similar (Table E.1) to SLAB. I was able to keep both the *kdr* and *CYP9M10* loci conferring permethrin resistance within the strain. In the backcross selection generations, the frequency of susceptible alleles was higher than expected (Table E.1).

This could be explained by a selection concentration that was too low to kill all of the susceptible homozygotes. This could be why *kdr* did not remain

Table E.1: Construction of ISOJPAL strain including *kdr* and *CYP9M10* frequencies.

Cross (female x male)	Generation	% introgression	BC selection		*kdr* [b]	*CYP9M10* [c]
			% mortality	n [a]		
SLAB x JPAL	F_1	50.0%				
SLAB x F_1	BC1	75.0%				
SLAB x BC1	BC2	87.5%	78%	3,240	TTT: 0/9	R/R: 10/10
					TTT/A: 7/9	R/S: 0/10
					TTA: 2/9	S/S: 0/10
SLAB x BC2	BC3	93.8%	50%	2,560	TTT: 0/10	R/R: 1/10
					TTT/A: 5/8	R/S: 6/10
					TTA: 3/8	S/S: 3/10
SLAB x BC3	BC4	96.9%	57%	639	TTT: 0/10	R/R: 10/10
					TTT/A: 2/10	R/S: 0/10
					TTA: 8/10	R/S: 0/10
SLAB x BC4	BC5	98.4%	50%	2,780	TTT: 0/10	R/R: 8/10
					TTT/A: 3/10	R/S: 0/10
					TTA: 7/10	S/S: 2/10

[a] Number of fourth instar larvae treated with permethrin at 0.02 μg/ml.

[b] *kdr* genotypes; TTT: homozygous resistant, TTT/A: heterozygous, TTA: homozygous susceptible.

[c] *CYP9M10* genotypes; R/R: homozygous resistant, R/S: heterozygous, S/S: homozygous susceptible.

Table E.2: Selection of the ISOJPAL strain with frequencies of *kdr* and *CYP9M10* through the 4 additional generations of permethrin selection.

Generation	Concentration [a]	n [b]	Mortality (%)	*kdr* [c]	*CYP9M10* [d]
BC5selection#1	1.0	1,480	94%	TTT: 0/10	R/R: 8/10
	0.1	3,960	52%	TTT/A: 2/10	R/S: 0/10
				TTA: 8/10	S/S: 2/10
BC5selection#2	1.0	1,780	59%	TTT: 3/8	R/R: 10/10
				TTT/A: 2/8	R/S: 0/10
				TTA: 3/8	S/S: 0/10
BC5selection#3	17	2,453	85%	TTT: 10/10	R/R: 10/10
				TTT/A: 0/10	R/S: 0/10
				TTA: 0/10	S/S: 0/10
BC5selection#4	30	3,980	68%	TTT: 10/10	R/R: 10/10
				TTT/A: 0/10	R/S: 0/10
				TTA: 0/10	S/S: 0/10

[a] Final concentration in cup in μg/ml.

[b] Number of fourth instar larvae treated.

[c] *kdr* genotypes; TTT: homozygous resistant, TTT/A: heterozygous, TTA: homozygous susceptible.

[d] *CYP9M10* genotypes; R/R: homozygous resistant, R/S: heterozygous, S/S: homozygous susceptible.

throughout the backcrossing protocol when ISOP450 (Chapter 3) was isolated. An additional complication to this observation was that *kdr* seemed to present a higher cost than P450 detoxification mechanism such that dilutions (through backcrossing with SLAB) made it difficult to keep the *kdr* allele in the population despite undergoing insecticide selective pressures each backcross generation.

Table E.3: Toxicity of permethrin and permethrin + PBO to ISOJPAL versus other laboratory strains previously studied (SLAB, JPAL and ISOP450).

Strain	LC_{50} [a](95% CI)	slope (SD)	n [b]	RR [c]
Permethrin				
SLAB [d]	0.0017 (0.0015-0.0019)	4.5 (0.6)	320	
JPAL [d]	49 (45-54)	4.6 (0.4)	480	29,000
ISOP450 [d]	2.1 (1.9-2.5)	1.8 (0.1)	860	1,300
ISOJPAL	22 (11-41)	2.4 (0.8)	720	13,000
Permethrin + PBO				
SLAB [d]	0.0011 (0.0009-0.0014)	8.0 (2.8)	480	
JPAL [d]	0.077 (0.049-0.12)	3.5 (1.4)	240	70
ISOP450 [d]	0.0059 (0.0053-0.0065)	3.0 (0.2)	960	5.4
ISOJPAL	0.046 (0.036-0.064)	1.3 (0.2)	340	42

[a] Units: μg/ml.

[b] Number of fourth instar larvae treated.

[c] Resistance ratio = LC_{50} resistant strain/ LC_{50} SLAB.

[d] Data collected originally for Chapter 3 and provided here for reference.

Selection following the backcross scheme showed the expected result of an increase in the *kdr* allele and an increase in the *kdr* homozygous genotype (Table E.2). An interesting observation of the *CYP9M10* genotypes was that two susceptible alleles are present in the ISOJPAL population. One that contains the intron (described in Chapter 7) with the polymorphisms associated with SLAB and the other allele is missing the intron. All heterozygotes contained the susceptible allele missing the intron and all homozygous resistant

genotypes contained the intron with the polymorphisms associated with ISOP450. This would be an interesting observation to investigate in detail.

After the final generation of permethrin selection was completed (BC5selection#4) the bioassays of larvae were performed to confirm that the P450 mechanism was also still present in the population. The P450 mechanism was not expected to leave the population (even through the backcrossing) because previously (Chapter 3) it was the only mechanism that remained through >12 generations of backcrossing with subsequent selection. Tables E.1, E.2 and E.3 provide data verifying that the P450 detoxification mechanism remained in the strain.

I was able to successfully isolate a permethrin resistant strain isogenic to SLAB homozygous for *kdr* and *CYP9M10*. The LC_{50} of ISOJPAL was 22 μg/ml (11-41) rendering it 13,000-fold resistant to permethrin as compared to SLAB. This is slightly, but statistically, lower than expected from the level observed in JPAL (Table E.3). ISOJPAL as a strain genetically related to SLAB and ISOP450 which will certainly be useful in future research on insecticide resistance.

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