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MONITORING INSECTICIDE RESISTANCE MECHANISMS IN CULEX TARSALIS FROM SUTTER COUNTY, CALIFORNIA

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MONITORING INSECTICIDE RESISTANCE MECHANISMS IN *CULEX TARSALIS* FROM SUTTER COUNTY, CALIFORNIA

by

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Monitoring insecticide resistance mechanisms in *Culex tarsalis* from Sutter County, California

Abstract

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Culex mosquitoes are known for carrying several harmful viruses in the United States. *Culex tarsalis* is found in rural as well as some residential areas in the Western United States, so they are under insecticide pressure from both agricultural spraying and vector control. In response to insecticide pressure, mosquitoes can evolve two primary resistance mechanisms: target site insensitivity, as a result of DNA mutation, and elevated levels of detoxifying enzymes (GST, alpha and beta esterases, and P450 oxidases). The two types of target site insensitivity studied here in *Cx. tarsalis* are *kdr*, which is a mutation in the para-type voltage gated sodium channel and *ace-1,* which is a mutation in acetylcholinesterase gene. This study focused on a population of *Cx. tarsalis* in Sutter County, where insecticide use shifted from sumithrin to Naled over the course of the summer. The goal of this study was to determine if there was resistance to insecticides and characterize the mechanisms of resistance. Mosquitoes were separated into resistance levels based on CDC bottle bioassay results using Naled, sumithrin, and permethrin insecticides. Mosquitoes were used to test for elevated levels of detoxifying

enzymes and genetic qPCR testing for either *kdr* and *ace-1* mutations. Bottle bioassay results suggest *Cx. tarsalis* populations from Sutter County are mostly resistant to pyrethroids while not being resistant to organophosphates. Enzymatic assays suggest high concentrations or activities of detoxifying enzymes are commonly seen in resistant individuals, occasionally elevated levels of multiple enzymes within an individual. The *ace-1* mutation was seen in a single susceptible individual (0.036%). Either one or two *kdr* alleles were present in every single semi-resistant or resistant mosquito tested.

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Chapter 1: Background

Mosquitoes as Vectors

Vectors are living, blood-feeding organisms that transmit infectious diseases through their blood meals. Vectors transmit 17% percent of all infectious diseases (CDC 2017a). When these arthropods take their blood meals, they are ingesting disease-producing microorganisms from an infected host, such as a human or animal. If these microorganisms replicate at a high enough abundance within the vector, the vector will become infected and later will inject these microorganisms through saliva into a new host during a subsequent blood meal. Biting flies, ticks, and fleas can all serve as vectors, but mosquitoes are responsible for transmitting the most pathogens. Female mosquitoes take blood meals in order to complete oogenesis, giving high nutrient content for developing eggs (Clements 1992). Female mosquitoes are attracted to hosts, including humans, by carbon dioxide expelled from breathing. Once a blood source has been located, the mosquito starts the blood-feeding process. The proboscis, which contains six needlelike structures, is inserted into the skin. The proboscis sheath rolls up and stays outside the skin, while two of the needles, maxillae saw through the skin. Another set of two needles, called the mandibles, holds the human tissue while the mosquito's labrum pierces the blood vessel (Lee 1974). There are numerous receptors on the tip of the labrum allowing easy detection of blood vessels. The last needle that is injected into the blood vessel is the hypopharynx; which injects mosquito saliva into the blood stream of the victim (Lee 1974). The saliva contains an anticoagulant substance; therefore, blood continues to flow without clotting, causing the blood vessels to dilate and block host's immune response (Lee 1974).

Mosquito-borne Disease

Some mosquito-borne diseases include malaria, dengue, yellow fever, Western equine encephalitis, Zika, chikungunya and West Nile virus. Malaria, just one of many devastating diseases; is caused by a protozoan parasite from the genus *Plasmodium* and transmitted by *Anopheles* species. According to the Center for Disease Control and Prevention (CDC), in 2015 there were more than 212 million malaria cases worldwide with approximately 438,000 deaths. While most of the cases are in Sub-Saharan Africa, there are roughly 1500 cases brought into United States every year from travelers (CDC 2017a). Mosquito vectors also transmit Dengue, which is the fastest emerging pandemic viral disease affecting between 50 and 100 million people in over 100 countries in many parts of the world (WHO 2017). Incidences have increased 30 fold in the last 50 years, affecting both young children and adults (WHO 2017). The *Aedes* mosquito genus is the primary vector that bites during the day and can potentially transmits dengue.

Fortunately, malaria and dengue are not currently a significant problem in the United States. However, West Nile virus is seen throughout the United States and is transmitted by the *Culex* genus (AMCA 2017). Between 1999 and 2015 there were a total 43,937 confirmed cases of West Nile virus in the United States. Of those cases 20,265 have been classified as the more serious neuroinvasive disease, which affect the nervous system causing meningitis, encephalitis, and other long lasting, flu-like symptoms. Of those individuals who developed the neuroinvasive disease, between 8-16% of the cases resulted in death. In 2016, there were 2,038 confirmed cases of West Nile virus in the United States. Of those, 56% or 1,140 developed into meningitis or encephalitis (CDC 2017b).

Mosquito-borne diseases are serious health issues that are the focus of significant efforts to alleviate. Presently, all we can do is offer assistance with the symptoms from some vector borne diseases since many do not have vaccines or cures readily available. For example, there is no cure or vaccine for West Nile virus in humans, so we can only treat the symptoms of the disease. Therefore, targeting the vector with insecticides seems to be the most effective way to reduce the incidence of these vector-borne diseases that kill over one million people annually.

Disease Transmission in *Culex tarsalis*

The mosquito genus *Culex* is known for carrying St. Louis encephalitis, Western equine encephalomyelitis, Eastern equine encephalitis viruses, lymphatic filariasis, and West Nile virus, in the United States. *Culex tarsalis* is found in rural, as well as residential areas, meaning it is under insecticide pressure from both agricultural spraying and vector control. *Culex tarsalis* is believed to aid in the West Nile Virus outbreak in New York and helped spread it to the Western United States in the 1990's. These mosquitoes are found commonly west of the Mississippi River to the West Coast, from Northern Mexico to Southern Canada and up to 3000 meters altitude (Pahk and Roles 2004).

Insecticide use

Pesticides encompass most aspects of pests control including: insecticides, bactericides, fungicides, herbicides and rodenticides. Worldwide there are 5.6 billion pounds of pesticides sprayed every year and over one billion pounds sprayed in the United States alone (Alavanja 2009). Pesticides contain an active ingredient which differs depending on the type if pesticide, and then "other ingredients" that aid in the

efficacy of the active ingredient (Alavanja 2009). Pesticides are sprayed over a large geographical area with small amounts of active ingredients so minimal harm is done to vertebrates. A small amount of pure insecticide is used per acre in order to avoid harm to humans and animals. Some insecticides target the egg, larva, and pupa life stages of the mosquito, but since they live in water it is harder to get lethal doses to these stages. Mass spraying of insecticides is the best way to target the adult mosquito as it flies around. This approach of spraying insecticides to kill the vector does help in the eradication of these vectors, but due to evolution and mutations, vectors can become resistant to insecticides. For example, in places where malaria is prevalent the use of insecticide treated nets is very common (WHO 2015). These treated nets are nets that surround your bed when you sleep that have been sprayed with insecticide, which will hopefully kill any mosquito that touches the net. In fact, between 2008 and 2010 there were 294 million insecticide treated nets delivered to sub-Saharan Africa, where malaria is most prevalent (Malaria [CDC], 2015). Consequently, recent studies have shown that insecticide resistance is undermining the effectiveness of the insecticide treated nets in Malaria (Malaria [CDC], 2015). Knowledge of resistance and its underlying mechanisms aid in the decision making process for selection of appropriate and effective insecticides (Brogdon and McAllister 1998).

Insecticides and Resistance

Presently, there are four different classes of insecticides used on adult mosquitoes with only two different modes of action. These neurotoxin insecticides include: Organochlorines and pyrethroids, targeting voltage gated sodium channels, while organophosphates and carbamates target and inhibit acetylcholinesterase. Insecticide

resistance is known as the ability of an individual to withstand toxic substances that would be lethal for other individuals of that specific population (Hagstrum et al. 1996). In 1914, A.L. Melander had asked the question, "Can insects become resistant to sprays?" and since then, published cases of resistance increased through the 1970's and 1980's (Melander 1914, Whalon et al. 2008). The first case of resistance in the United States was in *Aedes nigromaculis* in 1950 to DDT (Bohart and Murray 1950). Resistance has now been observed in more than 500 insect species around the world. Interestingly, more than 50 of those 500 species are *Anopheline* mosquitoes, perhaps due to this group being a major target for malarial control. One of these species, *Anopheles gambiae,* is the main species that transmits malaria (Hemingway and Ranson 2000).

There are two different forms of known insecticide resistance: target site insensitivity and elevated detoxifying enzymes. Target-site insensitivity is due to a single nucleotide polymorphism resulting in an overall change in the protein being produced. Over-expression of detoxifying enzymes such as: α-esterases, β-esterases, P450 oxidases, or Glutathione-S-Transferases also confers resistance to insecticides (Hemingway and Ranson 2000).

Insecticide mechanism of action: organochlorines and pyrethroids.

Organochlorines and pyrethroids both target the voltage gated sodium channels; which are transmembrane proteins with four homologous domains, each of which composed of six helices connected by loops (Chandre et al. 1999, Frank and Catterall 2003). These channels are involved in neuronal action potentials. Action potentials are short-lived electrical charged signals of equal strength that travel from the trigger zone of a neuron down the axon. Sodium is very abundant outside of the cell, while potassium is very

abundant within the neuronal cell. As the membrane potential changes and reaches a set threshold, and the charge is too low, the voltage gated sodium channels open allowing sodium into the axon, which diffuses. The sodium influx causes the charge within the immediate area to rise, which in turn triggers the opening of the voltage-gated potassium channels, releasing potassium outside of the neuronal cell. This depolarization and repolarization occurs over and over again traveling down the axon in a chain reaction until the neurotransmitter acetylcholine is released into the synapse (Silverthorn and Johnson, 2013).

Organochlorines and pyrethroids disrupt action potentials by binding to the voltage gated sodium channel and keeping the channel in an open state (Narahashi 2002). This permanent open state in the sodium channel causes the knockdown effect, meaning body spasms and tremors in the mosquito, ultimately results in paralysis and death (Schleier III and Peterson 2011).

The most common type of Organochlorines used is DDT (Dichlorodiphenyl-trichloroethane) and has been used heavily throughout the 1950's and 1960's until it was banned in the United States in 1970's (Weill et al. 2004). Pyrethroids, such as permethrin, sumithrin, and deltamethrin are now commonly used worldwide for not only vector control but also agricultural spraying for insects. Pyrethroids are also used in indoor residual spraying (IRS) and insecticide treated nets (ITN), especially in sub-Saharan Africa where malaria death rates are the highest in the world (Hougard et al., 2003, Malaria [WHO], 2015). These pyrethroids are used so commonly because of their high toxicity to insects and very low toxicity to mammals (Palchick et al. 1996). Due to high

use of these insecticides, mosquitoes have evolved resistance to both Organochlorines and pyrethroids.

Mechanism of resistance: target site insensitivity (*kdr*). One type of resistance to these insecticides is caused by a single nucleotide polymorphism in the voltage-gated sodium channel gene that results in the inability of the insecticides to bind to the channel. This type of resistance is commonly referred to as *kdr*, or knockdown resistance, shown in Figure 1. There are multiple single nucleotide polymorphisms that are considered *kdr* mutations that can potentially cause resistance at various locations in the sodium channel gene, yet there is one position in the gene that is most commonly seen, codon 1014. This mutation, which results in a phenylalanine instead of the wild type leucine at position 1014 has been seen previously in *Anopheles gambiae* populations from Africa and other mosquito species (Martinez‐Torres et al. 1998, Koou et al. 2014). Other point mutations at this position can result in the leucine being replaced by a histidine or a serine, which all result in conformational changes in the voltage-gated sodium channel (Zhou et al. 2009). Although there are different possible mutations within the sodium channel gene, leucine to phenylalanine substitution at position 1014 is the most common because it confers a higher level of resistance to insecticides compared to other point mutations at this location (Martinez‐Torres et al. 1998, Ranson et al. 2000, Zhou et al. 2009, Chen et al. 2010). It has been noted that in some heterozygotic individual *Culex quinquefasciatus* mosquitoes they have both *kdr* mutations phenylalanine and serine instead of leucine at the same position 1014 on separate alleles (Zhou et al. 2009).

Insecticide mechanism of action: Organophosphates and carbamates. In addition to becoming resistant to organochlorines and pyrethroids, mosquitoes have also

become resistant to organophosphates and carbamates, both targeting the acetylcholinesterase by binding and inhibiting function (O'Brien 1976, Weill et al. 2004). More specifically, organophosphates and carbamates inhibit the enzymatic activity of acetylcholinesterase by phosphorylating the serine residue in the active site of the enzyme, inhibit binding of the enzyme's intended substrate (Corbett 1974, O'Brien 1976). Acetylcholinesterase is a natural enzyme found inside the body that catalyzes the breakdown of acetylcholine into acetate and choline. Acetylcholine functions as a neurotransmitter in the neuromuscular junctions that bind to a receptor on the post-synaptic membrane, which relays the signal from the nerve (Silverthorn et al. 2009). Therefore, when the nerve sends a signal from the axon across the synaptic cleft, the acetylcholinesterase breaks it down before it reaches its receptor stopping stimulation of the muscle fibers (Silverthorn et al. 2009)

Mechanism of resistance: target site insensitivity (*ace-1*). The mutation in the acetylcholinesterase gene commonly referred to as *ace-1*, is another single nucleotide polymorphism but this time in the *ace-1* gene (Weill et al. 2004). In *Anopheles* this mutation results from a glycine to serine at position 119 (Weill et al. 2004). There are two different types of ace genes, *ace-1* and *ace-2*, but thus far *ace-1* is linked to insecticide resistance while the function of *ace-2* is still unknown (Weill et al. 2004). The amino acid substitution affects steric hindrance, which does not allow the organophosphates and carbamates to bind to the acetylcholinesterase (Weill et al. 2004). Insecticide and insecticide resistance mechanisms are summarized in Figure 1.

Mechanisms of resistance: metabolic enzymes. Metabolic resistance occurs when there are either one or multiple enzymes that are detoxifying the insecticide before

it actually reaches its target (Ranson et al. 2011). There are three different superfamilies of enzymes: esterases, oxidases such as cytochrome P450's, and Glutathione S-Transferases (GST) that all aid in insecticide resistance (Hemingway and Ranson 2000). Numerous studies have linked all three of these detoxifying enzymes with pyrethroids resistance in mosquitoes (Shi et al. 2015). Elevated levels of enzyme production could be due to gene regulation and gene amplification (Hemingway 2000).

A common mechanism for which insects are resistant to organophosphates and carbamates are the overproduction of detoxifying esterase enzymes, caused by amplification of two closely linked esterase loci Esterase α and Esterase β (Georghiou and Pasteur 1978). According to Ferrari, the elevated activity of α-esterase is due to gene regulation while elevated activity of β-esterase is due to gene amplification of the number of gene copies they carry (Ferrari 2015). Esterases can hydrolyze amine, phosphate or ester linkages on phosphates and carboxylate esters through the addition of water. Esterases hydrolyze organophosphates by cleaving the aromatic esters within the organophosphate and can easily sequester the insecticide. β-esterases can be further classified into cholinesterases and carboxylesterases. Both α-esterase and β-esterase have been seen in *Culex* species (Georghiou and Pasteur 1978). Even though esterases are seen as a resistance mechanism to organophosphates and carbamates, previous work in *Culex quinquefasciatus* has found elevated levels of esterases were observed in resistant mosquito strains (Gordon and Ottea 2012).

Elevated levels of P450 oxidases have been seen in many pyrethroid-resistant malaria vectors in Africa (Vulule et al. 1999, Brooke et al. 2001, Etang et al. 2004). P450 Oxidases have been seen as a mechanism of resistance against pyrethroids and

DDT in Culex species through constitutive expression (Kasai et al. 1998, Kasai et al. 2000). Oxidases are the largest gene superfamily and are involved in detoxifying exogenous compounds (Scott 1999). Oxidases metabolize substances by binding to the substrate and with the aid of NADPH acting as a cofactor donating an electron, renders the molecule less toxic and more excretable by adding a hydroxyl group into the toxic substance (Liu et al. 2015).

Glutathione-S-transferase, GST, is another detoxifying enzyme that has been linked to pyrethroids, organophosphates, carbamates, and organochlorine resistance (Low et al. 2013, Zhong et al. 2013). Glutathione-S-Transferases are a family of isozymes that catalyze the conjugation of the reduced form of Glutathione to electrophilic centers on a wide variety of toxic substrates in order to make them more soluble and prevents their interaction with cellular proteins and nucleic acids (Habig et al. 1974). The thiol group on the Glutathione structure acts as a reducing agent; which reduces the disulfide bonds formed within the cytoplasmic proteins to cysteines by serving as an electron donor (Habig et al. 1974).

Under insecticide pressures over time mosquitoes have developed mechanisms of resistance to insecticides. It is important to understand these mechanisms of resistance and monitor changes in order to be able to properly apply insecticides. In addition, understanding these mechanisms can help aid in the ability to develop a new class of insecticides with a different mode of action.

Chapter 2: Introduction

Mosquito-borne infectious diseases are responsible for killing millions of people every year. The genus *Culex* is known for carrying St. Louis encephalitis virus, Western equine encephalomyelitis and West Nile virus in the United States (Center for disease Control and Prevention [CDC], 2016). *Culex tarsalis* is the primary vector of West Nile virus in the Western United States. This species of mosquito is found in rural as well as residential areas in the Western United States, meaning it is under insecticide pressure from both agricultural spraying and vector control. Since there is no vaccine for these diseases, the only way to protect against them is through control of vectors. Insecticide spraying helps to eradicate these vectors; however, long-term application can lead to the evolution of resistance to insecticides.

Presently, there are four major classes of insecticides used on adult mosquitoes with only two different modes of action (David et al. 2013). Of these, pyrethroids and organophosphates are the most commonly used insecticides by vector control districts. Pyrethroids inhibit the function of the voltage-gated sodium channel by binding and keeping the channel permanently open causing a huge influx of sodium into the cell, which causes the mosquito to seizure (Narahashi 2002). Organophosphates bind to and inhibit the enzymatic function of acetylcholinesterase, which normally degrades signals in the neuromuscular junction (Silverthorn et al. 2009). When acetylcholinesterase does not terminate the synaptic signal, muscular paralysis and death occur (Toutant 1989).

In response to insecticide pressure, mosquitoes have evolved two primary resistance mechanisms: target site insensitivity and elevated levels of detoxifying enzymes. Target site insensitivity is due to a single nucleotide polymorphism in either

the para-type voltage-gated sodium channel gene or the acetylcholinesterase gene (*ace-1*) (Soderlund and Knipple 2003, Weill et al. 2004). A leucine to phenylalanine (L1014F) substitution or a Leucine to Serine substitution (L1014S) on segment two domain six of the voltage gated sodium channel gene confers resistance, known as knockdown resistance (*kdr*) (Martinez‐Torres et al. 1998). This results in the insecticides not being able to bind to the voltage gated sodium channel rendering resistance. The mutation *ace-1* resulting in resistance is an amino acid substitution from glycine to serine in the acetylcholinesterase gene (G119S) (Weill et al. 2004). This amino acid substitution results in steric hindrance, which does not allow the organophosphates and carbamates to bind to acetylcholinesterase.

Detoxifying enzymes, such as, Glutathione-S-Transferase, P450 oxidases, α-and β- esterases are able to break down the insecticide within the mosquito rendering them ineffective. Elevated levels of these enzymes as well as acetylcholinesterase can confer resistance in mosquitoes (Whyard et al. 1995b, Small 1996, Brogdon et al. 1999a, b, Karunaratne et al. 1999, Vulule et al. 1999, Zayed et al. 2006).

Insecticide resistance, including target site insensitivity and enzymatic activity, is well studied in *Anopheles gambiae* and the *Culex pipiens* complex, but very little has been studied in *Culex tarsalis*. *Culex tarsalis* has shown to enhance enzymatic metabolism in the past, yet to our knowledge, no target site insensitivities have been characterized (Apperson and Georghiou 1975). The objective of this study is to monitor a population of *Cx. tarsalis* from Sutter County California to determine the prevalence of resistance, the mechanisms that cause resistance, and if these mechanisms change over the course of the 2016 mosquito season.

Chapter 3: Materials and Methods

Mosquito Collection

Mixed aged female mosquitoes were collected from Sutter County, California using CO_2 baited traps (Sudia and Chamberlain 1962). Five collections were completed in total over the course of the 2016 summer. The first collection taken on 14 June 2016 was before any aerial insecticide spraying started in the area. The second collection, taken on 11 July 2016, was also before any aerial spraying had occurred in the trap areas. The reason for the second collection was because of the need of basal resistance data for an additional insecticide not tested for in the first collection. The third collection, taken on 25 July 2016, occurred after sumithrin had been sprayed aerially once a week for two weeks. The fourth and fifth collections, 11 August 2016 and 12 September 2016 respectively, were collected after Sutter-Yuba Mosquito and Vector Control District switched to spraying Naled (Figure 2). Collected mosquitoes were brought to the lab at University of the Pacific in Stockton and fed 10% sucrose solution until the next day when CDC bottle bioassay was performed.

A known susceptible *Cx. tarsalis* colony originally collected in 2002 from Kern National Wildlife Refuge (35.7458 N, 119.6179 W), kept at UC Davis and Sutter-Yuba Mosquito and Vector Control District, was used in CDC bottle bioassays and biochemical tests. Mixed aged females were brought back to the lab at University of the Pacific, Stockton, CA and fed 10% sucrose solution until needed.

CDC Bottle Bioassay

The CDC bottle bioassay protocol (Brogdon and McAllister 1998) was followed with modifications. Glass Wheaton bottles (250ml) were coated with Permethrin (43

μg/bottle), Sumithrin (22 μg/bottle), or Naled (25 μg/bottle) dissolved in 2ml of acetone. The Sac-Yolo mosquito control district determined the LD50 dosages used per bottle previously for *Culex tarsalis*. These insecticides are commonly used in vector control programs and represent pyrethroids and organophosphates. Once the bottles were evenly coated, they were allowed to dry 2-3 hours giving enough time for the acetone to completely evaporate. Between 20-25 mosquitoes were transferred into each bottle. For each insecticide a total of 12 bottles were used; two bottles contained colony mosquitoes; eight bottles were used for wild caught mosquitoes; two bottles coated with acetone only and no insecticide, one with colony and one with wild mosquitoes were used as controls. Mosquitoes on their backs that could no longer right themselves were classified as dead. Every 15 minutes the number of dead mosquitoes were counted until the end of the three hours. Once colony mosquitoes reached one hundred percent mortality, the wild population that had died at this time were separated out and classified as susceptible (S). At the end of the three hours the wild mosquitoes that had died were classified as semi-resistant (SR) and the ones that were still alive were classified as resistant (R) (Figure 3).

Enzyme Assays

Assays originally described by Brogdon and Dickerson, (1983) were used to determine levels of detoxifying enzymes: α-esterases and β-esterases, P450 Oxidases, Glutathione-S-Transferases, acetylcholinesterases (Brogdon and Dickinson 1983). Protocols were followed using the Mosquito Pesticide Resistance Monitoring Working Group (Macedo, Su et al., 2015). The legs of the mosquitoes were separated from the body. The legs were used in molecular assay while the body of the mosquito was used in the enzyme assay. The total amount of protein in each mosquito was used to normalize

for mosquito size. Individual mosquitoes were homogenized in 100µl KPO_4 potassium phosphate buffer (adjusted to pH 7.2 with hydrochloric acid) then diluted up to 2ml in potassium phosphate buffer.

To test for α-esterases, diluted mosquito homogenates were distributed into every well of the microplate, then 100µl of α–naphthyl acetate solution (56mg α- naphthyl acetate, 20ml acetone, 80ml KPO_4 buffer) was added and incubated for 20 minutes, O-dianisidine solution (100µl) (50mg O-dianisidine tetrazotized dissolved in 50ml deionized water) was added, incubated four minutes then read as an absorbance using BioTek Synergy microplate reader with Gen5 2.0 reader software at 540nm. The same procedure was completed for β-esterases with the exception of using β-naphthyl acetate solution (56mg β-naphthyl acetate dissolved in 20ml acetone and 80ml KPO_4 buffer). Standard curves were created for both α-esterases and β-esterases to calculate amount of enzyme based on absorbance. Standard curve ranges for α-esterase were well concentrations from 0-210µg/ml. Standard curve ranges for β-esterase were generated to account for 0-160µg/ml of esterase per mosquito.

Amount of Oxidase present was measured using the substrate TMBZ (50mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride dissolved in 12.5ml methanol and 37.5ml 0.25M Sodium acetate buffer at pH 5.0, adjusted with glacial acetic acid). Each well contained 100µl mosquito homogenate, 200 µl TMBZ, and 25 µl 3% hydrogen peroxide then was incubated 10 minutes and read absorbance at 620nm. A standard curve was generated for oxidase concentrations form 0-1.8µg/ml per mosquito.

Activity of Glutathione-S-Transferase (GST) was measured using the substrate 1-chloro-2,4-dinitrobenezene CDNB (100µl) (20mg 1-chloro-2,4-dinitrobenezene dissolved

in 10ml acetone and 90ml KPO4 buffer) and 100µl reduced glutathione (61mg reduced glutathione dissolved in 100ml KPO4 buffer) was added to the mosquito homogenate in each microplate well. Spectrophotometric readings were conducted at 340nm immediately after the addition of substrate and again after a ten-minute incubation.

Activity of acetylcholinesterase was measured by adding 100µl of ATCH (75mg Acetlythiocholine iodine dissolved in 10ml acetone and 90ml KPO4) and 100µl DTNB (13mg Dithio-bis-2-nitrobenzoic acid dissolved in 100ml KPO4 buffer) to 100µl mosquito homogenate. Spectrophotometric readings were conducted at 414nm immediately after addition of substrate to the mosquito homogenate, and again after a twenty-minute incubation.

Enzymatic results are shown as box plots showing all outliers (1.5 standard deviations away from the mean) and extreme values (3 standard deviations away from the mean) after the raw data had been normalized by the amount of protein present in each individual mosquito. The outliers and extreme values were set by SPSS as per standard criteria.

Molecular Assay

A large majority (n=266) of the mosquitoes treated with Naled during the CDC bottle bioassay were sequenced after a genomic DNA extraction on only their legs using a Qiagen DNeasy Blood and Tissue kit. The genomic DNA was used in a polymerase chain reaction (PCR) to amplify the *ace-1* gene. Specific *ace-1* primers were designed, based on Weill et. al, (2004) to amplify the sequence of interest. Samples were sequenced by Quintara Biosciences (Berkeley, CA) and processed in Geneious R9.0.5 (Newark, New Jersey).

A subset (n=514) of the mosquitoes treated with permethrin and sumithrin during the bottle bioassays had their legs removed and genomic DNA extracted with a Qiagen DNeasy Blood and Tissue kit. Next, allele specific primers were designed, each of which containing a 3' nucleotide corresponding to either wild type or one of the most common *kdr* alleles (Table 1). GC rich tails were generated and added to the 5' end of the allele specific primers, originally described by Germer and Higuchi (1999), to separate the amplification products in a melt curve after PCR has been completed (Germer and Higuchi 1999, Tripet et al. 2006, Saavedra‐Rodriguez et al. 2007, García et al. 2009). PCR was performed in 20µl volume consisting of 5µl template DNA, 10µl SYBR Select Master Mix by Applied Biosystems (Foster City, CA), 0.2µL reverse primer CxTkdrMC_R5, 0.2µL Leucine forward primer CxTkdrMC_LeuF3, 0.2µL Serine forward primer CxTkdrMC_SerF4, 0.25µL Phenylalanine forward primer CxTkdrMC_PheF2 and brought up to total volume using nuclease-free water. Thermocycler conditions were (1) 50°C for 2 min, (2) 95°C initial denaturation for 2 min, (3) 95°C denaturation for 15 seconds, (4) 61°C annealing and elongation for 1 minute, repeated steps 3-4 35 times, (5) melt curve starts with 15 seconds at 95°C, (6) 60°C for one minute then temperature starts increasing 0.3°C every 10 seconds until complete denaturation of amplicons. To confirm the presence of Leu/Ser heterozygotes or Serine homozygotes a secondary test was ran using CxTkdrMC_LeuF4 and CxTkdrMC_SerF4 forward primers. The conformation test followed the same protocol as the primary qPCR run except only using, 0.2µL CxTkdrMC_LeuF4 forward primer, 0.2µL CxTkdrMC_SerF4 forward primer, 0.2µL reverse primer CxTkdrMC_R5 and no phenylalanine primer present. Individual samples treated with either permethrin or

sumithrin where definitive genotypes could not be made were either ran in PCR and sent to Quintara Biosciences (Berkeley, CA) for sequencing or were TOPO TA cloned using Invitrogen TOPO cloning kit (Carlsbad, CA) and sent for sequencing in a the TOPO vector. A small percentage (10%) was sequenced by Quintara Biosciences as a check to ensure our primers were binding correctly.

Statistics

Statistical analysis was completed using linear models in R Studio Version 1.0.143 (RStudio, Inc). Linear models were used to compare each resistant group across time to the colony. The t-values used in linear models represent the comparison between slopes. Univariant analysis of variance (ANOVA) with a Tukey HSD post-hoc was also used in R Studio to identify differences between resistance levels in the enzyme assays. All statistical analysis for enzyme assay data was completed using log-transformed data. Chi-squared analysis was used in comparisons of alleles and genotypes for molecular assay data (Tables 2-3). P-values lower than 0.05 were considered significant.

Chapter 4: Results

CDC Bottle Bioassay

Naled. In total, 734 female *Culex tarsalis* were treated with Naled in the CDC bottle bioassay showing very little resistance (Figure 4). In the first two collections, (Figure 4a & Figure 4b) a majority of these individuals did not live longer than the KNWR colony; and none had lived longer than the three-hour duration of the test. In the third collection there was only one single mosquito that was resistant to Naled (Figure 4c). The fourth collection, taken after Naled had been sprayed aerially in Sutter County for a couple weeks, showed 20% of the females collected and tested were resistant to Naled (Figure 4d). Interestingly this trend did not continue, in the fifth and final collection there were no resistant mosquitoes seen, with all mosquitoes dead by 120 minutes (Figure 4e). A reason for this decline in resistance could be the collection was taken too late in the mosquito season when sample numbers were low.

Sumithrin. There were 710 female *Cx. tarsalis* in total treated with sumithrin over the course of five collections. There was already resistance to sumithrin in the population before aerial spraying began in 2016 (Figure 5). In the first collection, 77% of the collected population that was completely resistant to sumithrin (Figure 5a). However, in the second collection, there is a major increase in percent mortality (Figure 5b). Resistance in the population again increased with collection 3, making it likely there was an error in the testing of collection 2 (see Discussion). Overall resistance to sumithrin increased over the course of the 2016 summer, with some variation. Excluding collection two, resistance started high and continued to increase throughout the third collection where 80% of collected females were resistant to sumithrin. The variation came in the

fourth collection where only 60% were completely resistant, but still the total number showing some level (SR or R) of resistance to the assay was 95% (Figure 5d). Lastly, by the fifth and final collection more than 99% of the female *Culex tarsalis* tested were completely resistant to sumithrin (Figure 5e).

Permethrin. The last insecticide testing for resistance was permethrin, which is a similar pyrethroid with the same mechanism of action as sumithrin. During the first collection, only sumithrin and Naled were used in the CDC bottle bioassay (Figures 4&5). In total there were 645 female *Cx. tarsalis* treated with permethrin. Resistance to permethrin was low, relative to sumithrin, in the beginning of the summer (Figure 6). Starting out in the second collection only 3% of the females were completely resistant to permethrin, but 91% showed some level of resistance (Figure 6b). This trend continued throughout the next few collections. In the third collection 89% of the collected individuals were completely resistant (R) or showed some resistance (SR) to permethrin (Figure 6c). Similar to sumithrin results, there is a decrease in resistance in the fourth collection with only 68% of the collected individuals showing either moderate or complete resistance to permethrin (Figure 6d). Also similar to sumithrin, in the fifth collection there was an increase in the number of resistant individuals. In the final collection, 95% showed some level of resistance while 43% of the collected female *Cx. tarsalis* being completely resistant to permethrin (Figure 56).

Enzyme Assay

Naled. Results of enzyme assays for mosquitoes treated with Naled during the CDC bottle bioassay are presented in Figure 7. Throughout the summer, the absorbance

of acetylcholinesterase, seen in Figure 7a, in the groups of susceptible mosquitoes decreased significantly over time ($t=-4.005;p<0.001$). The resistant female *Culex tarsalis* seen in collection four had significantly higher acetylcholinesterase activity (Figure 7a) compared to susceptible group from collection four ($p<0.001$). The amount of α-esterase and β-esterases present in mosquitoes treated with Naled remains consistent across collection numbers in all of the resistance levels, except for semi-resistant group (Figure 7b, 7c). Mosquitoes classified as semi-resistant had increasing concentrations of α-esterase and β-esterases as the summer progressed ($t=3.89$ and 6.69 respectively; $p<0.001$) (Figure 7b, 7c). In the beginning of the summer β-esterase concentrations in semi-resistant mosquitoes treated with Naled were lower than susceptible mosquitoes. However, β-esterases increased over time and by the final collection semi-resistant groups had a higher esterase concentration than susceptible ($p<0.001$) (Figure 7c). Within that same semi-resistant group, the concentrations of oxidases increased throughout the summer ($p=0.002$). Similar to levels of other detoxifying enzymes, the presence of P450 Oxidases remained consistent across collections within each resistance level with the exception of semi-resistant groups (Figure 7d). There was no correlation in GST activity between resistance level and collection number (Figure 7e). There is a large range of enzyme concentrations and activity. Having high enzyme activity or concentrations does not necessarily guarantee resistance since these individuals are still susceptible in the CDC bioassays.

Sumithrin. Results from the enzyme assay in mosquitoes treated with sumithrin during the bottle bioassay are shown in Figure 8. Acetylcholinesterase absorbance levels in mosquitoes treated with sumithrin were roughly five times as high than in the

mosquitoes treated with Naled, which was expected because pyrethroids do not directly affect acetylcholinesterase activity (Figure 8a). The susceptible group's acetylcholinesterase activity increased significantly over the course of the mosquito season (p=0.007). In these groups of *Cx. tarsalis* treated with sumithrin, there was no significant relationship between resistance level and acetylcholinesterase level at each time point (Figure 8a). Previous studies have shown non-specific esterases are a mechanism of resistance to pyrethroids as well as organophosphates in many mosquito species (Brogdon & Barber, 1998). In this study there was a slight increase in α-esterase concentration over the course of the summer in susceptible and a slight decrease in resistant groups (t=3.061, p=0.009 & t=2.65, p=0.002, respectively; Figure 8b). This decrease was likely driven by an extreme value from collection one (with this individual having >1500ug α-esterase/mg protein). Overall, there is significantly more α-esterase present in semi-resistant groups compared to susceptible groups treated with sumithrin; but this is primarily driven by individuals from first collection (p=0.03, Figure 8b). Oxidases are also commonly known to lead to resistance in many species to pyrethroids. Here, concentrations of oxidases increased as the course of the summer progressed in both susceptible and semi-resistant populations (t=3.24, p=0.001 & t=2.54, p=0.01 respectively). Overall the resistant groups had more Oxidase present than the semi-resistant groups, and resistant groups had significantly more oxidase present when compared to susceptible groups (p=0.02). β-esterases and GSTs, on the other hand, showed no significant changes over the course of the summer with regards to resistance level (Figure 8d, 8e). Although β-esterase appear to contain more outliers and extremes in Collections 3-5, the trends were not significant.

Permethrin. Permethrin was not tested until the second collection. Permethrin treated individuals that were classified as resistant showed a negative correlation over time, meaning there was less acetylcholinesterase (Figure 9a) activity in these groups as time progressed ($t=-2.98$, $p=0.003$). There was a difference in acetylcholinesterase activity between resistance levels at each collection (Figure 9a). In collection 2, the resistant group had significantly more acetylcholinesterase activity than the semi-resistant group ($p=0.04$). In collection 3, the semi-resistant groups have less acetylcholinesterase activity than both susceptible and resistant groups ($p=0.003$ & 0.005, respectively). By collection four, (Figure 9a) semi-resistant group has more acetylcholinesterase than susceptible group ($p=0.001$). Regarding α-esterase, concentrations in the resistant group decreased over time ($t=-4.58$, $p<0.001$;Figure 9b). Besides this decrease, there are no other significant trends in α-esterase concentrations. However, it is interesting to note the large number of extreme values, particularly in the later collections. Thus meaning, more individuals have elevated levels of esterases, compared to other insecticide treatments. For β-esterase concentrations (Figure 9c) the semi-resistant groups showed a positive linear correlation over time ($t=0.53$ $p<0.001$). Overall there is slightly more β-esterase present in the resistant groups compared to the susceptible groups. Over the course of the mosquito season, there were increasing concentrations of oxidases present in susceptible groups, though, not enough to be significant (Figure 9d). Additionally, there was a positive correlation between semi-resistant groups and collection number, meaning more oxidase present as time progressed ($t=5.49$, $p<0.001$). Oxidase levels in the resistant group appear to increase from collection 2 to 4, but then activity lowers in collection 5 (Figure 9d). For GSTs, values remained consistent across resistance levels and across

collection numbers, except for collection 4 (Figure 9e) where both semi-resistant and resistant groups are significantly higher than the susceptible group ($p<0.001$ & $p=0.048$, respectively).

Molecular Assay

***ace-1* mutation.** The DNA from a subset of female *Culex tarsalis* treated with Naled from each of the collections was sequenced (n=275). Sequencing results concluded the glycine to serine substitution corresponding to position 199, in *Anopheles gambiae*, was only present on one allele in a single mosquito, during the fifth collection. Unexpectedly, this mosquito was susceptible to Naled in the bottle bioassay.

***kdr* mutation.** In total there were 514 mosquitoes treated with pyrethroids and tested for the presence of the *kdr,* both the leucine to phenylalanine and leucine to serine substitutions. Roughly 10% of the ones tested were sent out for sequencing to confirm efficacy of qPCR, all came back with the same results. Of the 514 tested mosquitoes, 99.2% had one or more alleles with a kdr mutation. In fact, in the *Cx. tarsalis* classified as resistant there was not a single individual that had the wild type leucine allele present. Of the 90 *Cx. tarsalis* classified as susceptible and tested for the presence of *kdr*, roughly 92% of them had either one or two of the kdr mutated alleles (Table 2). Of the mosquitoes classified as semi-resistant and tested for kdr, roughly 97% (n=204) had either one or two mutated alleles. Of the mosquitoes classified as resistant, 100% of the 220 were either homozygous for one of the *kdr* mutations (L1014S/F) or heterozygous, meaning they have both kdr mutations present, shown in Table 2. The *kdr* genotype distribution in semi-resistant and resistant groups is skewed toward F/F and F/S. Even though, the kdr genotype for susceptible mosquitoes was also skewed towards F/F and

F/S, the wild type leucine alleles were only found in the susceptible groups (Table 2). In the first two collections, roughly 97% of the alleles from collected individuals had the *kdr* mutation in this population (Table 3). The *kdr* allele distribution does not change over the course of the summer ($p>0.05$) (Table 3). Therefore, the *kdr* mutation was common in *Culex tarsalis* even before increasing insecticide pressure over the course of the summer 2016.

Correlation Between Metabolic Enzyme and Molecular Data

Since a subset of the individuals used in the enzyme assay were also used in the genetic testing it is interesting to see what the genotypes of some of the outliers and extreme values are during the enzyme assay. The one and only mosquito out of 363 sequenced that had the *ace-1* mutation from the fifth collection also showed it had elevated levels of α- and β-esterases. Interestingly, 13.8% of all the mosquitoes treated with Naled (n=478) showed elevated levels of at least one detoxifying enzymes meaning they were at least 1.5 standard deviations outside the mean for each enzyme. Of those, only two of them had elevated levels of either four or five detoxifying enzymes (0.42%).

As for mosquitoes treated with sumithrin 19.4% of the collected population had elevated levels of detoxifying enzymes. Eight mosquitoes (2%) had elevated levels of either 4 or 5 enzymes. Of those eight individuals genetic testing was completed for six of them. There were five individuals treated with sumithrin that were classified as either semi-resistant or resistant that also had elevated levels of all five detoxifying enzymes tested. Four of the five were resistant, and all four were homozygous for the leucine to phenylalanine mutation. While the remaining individual was classified as semi-resistant, she was heterozygous for the leucine to phenylalanine substitution and the leucine to

serine substitution. Three more individuals treated with sumithrin showed elevated levels of four out of the five detoxifying enzymes tested. Two of the three were semi-resistant and one was resistant, but all three were homozygous for the leucine to phenylalanine substitution. The remaining outliers from the enzyme assay that also have genotype data showed some have elevated levels of a up to three enzymes which could be aiding in their resistance to sumithrin. The remaining outliers' kdr genotypes were all homozygous for the phenylalanine substitution; except for one; which was heterozygous for leucine and phenylalanine. This is the only individual in the subset of outlier genotypes looked at that had one wild type allele.

There are numerous outliers and extremes seen in every enzyme tested regardless of insecticide treatment. This means in this population there are individuals that have elevated levels of detoxifying enzymes before undergoing CDC bottle bioassay (Supplemental Figure 1). Mosquitoes treated with permethrin have less slightly lower number of individuals that showed elevated levels of multiple detoxifying enzymes. Of the collected population treated with permethrin, there were 13.4% of them that had elevated levels of detoxifying enzymes. In fact, 15 (3%) of all the mosquitoes tested had elevated levels of either 4 or 5 detoxifying enzymes. Genetic testing was completed on only three of these individuals. There was one single mosquito, with a homozygous phenylalanine *kdr* genotype, from the third collection that had elevated levels of all five enzymes tested. Two more mosquitoes, both with homozygous phenylalanine kdr genotypes, showed elevated levels of enzyme activity and concentration to four out of the five enzymes tested. All of the individuals tested for both enzyme and molecular data were either semi- resistant or susceptible and homozygous phenylalanine with the

exception of three individuals. One of those individuals was a susceptible, with the rare homozygous serine *kdr* mutation. While the remaining two were both semi-resistant and heterozygous for both *kdr* mutations.

Chapter 5: Discussion

Bioassay

Resistance to insecticides is commonly found in insects such as mosquitoes. It is becoming a very well documented evolutionary adaptation to environmental pressures and continued insecticide use (Liu and Yue 2000, Soderlund and Knipple 2003). Within this population of *Culex tarsalis* from Sutter County, California there was very low levels of resistance to organophosphates. Within the same population there was high levels of resistance to pyrethroids, which only increased as the mosquito season progressed, from mid-June through mid-September under continued insecticide in that area. When undergoing CDC bottle bioassay (Figure 4 & 5), many mosquitoes in this population were completely resistant to both sumithrin and permethrin, with the exception of collection 2. Possible reasons for this increase in mortality in collection 2 could be due to the fact that many of the mosquitoes treated with sumithrin were missing legs. Perhaps the bottles used in the assay were not completely dry before females were transferred; resulting in high mortality. This increase in mortality could also be due to the glass Wheaton bottles being overdosed with too much insecticide. The amount of active ingredient given in technical grade insecticide stock is actually 10% more than what it is labeled. When the lab stock was made the extra 10% was included. Regardless of collection 2, resistance only increased as the summer progressed, by mid-September, where 43% were completely resistant to permethrin while <99% were completely resistant to sumithrin. Resistance to pyrethroids is very prominent in this population.

Naled

Metabolic enzymes. There was very low resistance to Naled within this *Culex tarsalis* population. Of those resistant individuals they had more acetylcholinesterase activity than semi-resistant, susceptible or colony groups. This might suggest having higher acetylcholinesterase activity might lead to resistance to organophosphates such as Naled, but more studies need to be conducted to conclude. Semi-resistant groups show a slight increase in non-specific α-and β-esterases under continued insecticide pressure. Esterases could be aiding in organophosphate resistance, but not at high enough elevated level to confer phenotypic resistance to the CDC bottle bioassay. Few studies have been conducted looking at enzymatic resistance in *Culex tarsalis*, but, Whyard (1995) conducted a study on *Cx. tarsalis* and found that unlike other species of mosquitoes that show an over production of esterases to organophosphates, *Culex tarsalis* did not (Whyard et al. 1995a). He concluded that if enzymes alone are resulting in resistance it must be from a qualitatively different enzyme and not the quantitative increase of esterases (Whyard et al. 1995a). Contradictory, this study found increased levels of acetylcholinesterase, which is in the β-esterase family, showed elevated levels in resistant individuals. Previous studies have concluded mixed function oxidases are involved in the ability of an individual mosquito to degrade organophosphates (Dauterman 1971). Oxidase concentrations do not differ between resistance levels, but semi-resistant individuals have higher oxidase concentrations as the summer progresses. This could aid in resistance, but will not cause resistance alone. There are reports demonstrating elevated oxidase activity in insecticide resistant mosquitoes, but its usually in conjunction with another mechanism of resistance, like elevated activities of other enzymes (Vulule et

al. 1999, Hemingway et al. 2004). Resistance due to the quantitative increase in GST activity was first noted in organophosphate resistance in many different insect species (Hayes and Wolf 1988, Hayes and Wolf 1990). GSTs have been the major enzyme that have the ability to catalyze DDT and the increased activity of GST conferred resistance in *Ae. egypti* (Grant et al. 1991). There is no correlation between levels of GSTs and resistance in this study, so they are an unlikely mechanism of resistance for this mosquito population.

ace-1. The glycine to serine substitution, which results in the creation of insensitive acetylcholinesterase, has been previously seen in *Culex pipiens pipiens, Culex pipiens quinquefasciatus, Culex tritaeniorhynchus, An. gambiae, Anopheles nigerimus, Anopheles atroparvus, and Ae. aegypti* (Hemingway 1982a, b, Hemingway et al. 1985, Hemingway et al. 1986, Villani and Hemingway 1987, Bisset et al. 1990, N'guessan et al. 2003, Corbel et al. 2007). The wild type glycine at this position in *An. gambiae* is encoded by GGC with a one base pair change in the first position creating AGC would then encode for serine. In *Culex tarsalis* the wild type at this position is encoded by GGA and in order for this glycine to be changed to a serine, there needs to be two single nucleotide polymorphisms, one in the first position and one in the third position of the codon (Weill et al. 2003). Previous studies have concluded insensitive acetylcholinesterase which is the new enzyme created if this ace-1 mutation occurs comes at a high fitness cost seen in various mosquito species (Georghiou et al. 1980, Roush and McKenzie 1987, Lenormand et al. 1999). The fact that this mutation needs two simultaneous base changes and comes at a high fitness cost could be the reason it was so rare (0.036%) in this study.

Sumithrin

Metabolic enzymes. Previously, non-specific esterases have been shown to be a mechanism of resistance against pyrethroids in other mosquito genera (Brogdon and Barber 1990). Although there were no overall differences between resistance level and amount of esterases present in mosquitoes treated with sumithrin, there were more individuals with extremely high esterase concentrations in the resistant groups (60% of all outliers were classified as resistant) when compared to other resistance levels (Figure 5). Thus meaning, these resistant individuals were producing more esterases than the rest of the population, supporting the idea that esterases aid in resistance in this population of *Culex tarsalis.* Oxidase concentrations in *Cx. tarsalis* treated with sumithrin increased over time while under continued insecticide pressure. Previous studies have shown increasing oxidase concentrations play a role in insecticide resistance (Low et al. 2013). There is no gathered evidence in this study to support the idea of GSTs confering resistance to pyrethroids. Previously GST's have been commonly known to play a role in DDT resistance, but recent studies have found altered GST activity was not identified in *Aedes aegypti* samples when treated with a pyrethroid (Francis et al. 2017).

Permethrin.

Metabolic enzymes. Previously, Vulule et al. (1999) found elevated oxidases and esterases in permethrin-resistant *An gambiae,* but that does not seem to be the case in this population of *Culex tarsalis.* Non-specific α-and β-esterases are likely not a mechanism of resistance in *Cx. tarsalis* treated with permethrin due to the fact that there was no correlation between concentrations and resistance levels. Acetylcholinesterase activity, however, did correlate to resistance level, but not until the fourth collection.

This could be due to the fact that two weeks before collection four, the vector control district started spraying Naled, an organophosphate that inhibits acetylcholinesterase. As previously discussed, resistant mosquitoes treated with Naled in the bioassay showed elevated levels of acetylcholinesterase in collection four as well. This could mean in the natural population acetylcholinesterase increased under insecticide pressures. Oxidase concentrations increased as the summer progressed with more insecticide pressure indicating oxidases could be aiding in resistance within this population. Previous studies have shown increasing oxidase concentrations play a role in pyrethroid resistance (Hemingway et al. 2004, Strong et al. 2008, Low et al. 2013, Francis et al. 2017). In this study there was more GST activity in semi-resistant and resistant mosquitoes when compared to susceptible. Upregulation of GST can cause resistance to organophosphates, DDT and pyrethroids (Hemingway et al. 2004). A previous study saw more GST activity in permethrin resistant Culex tarsalis in California (Strong et al. 2008). This could mean GST activity is aiding in permethrin resistance along with presence of kdr mutations.

kdr

In total there have been over 20 unique sodium channel polymorphisms found in domain two of the sodium channel gene and all have been linked to conferring resistance to pyrethroids (Soderlund and Knipple 2003). The most common and well studied is the L1014F or the L1014S from *An. gambiae* (Williamson et al. 1996, Martinez‐Torres et al. 1998, Ranson et al. 2000). As seen in this study, the phenylalanine and serine substitutions are seen commonly in every resistance level classification. Most of the collected individual females were resistant to pyrethroids and carrying two copies of the mutated L1014F allele to pass on to future generations. Every single individual resistant

to a pyrethroid had two copies of a *kdr* mutation at all time points over the summer. Some completely susceptible individuals were homozygous for phenylalanine at the same position; however, there were no resistant mosquitoes with a wild type leucine allele. Even though the few mosquitoes classified as susceptible during the bioassay died, other circumstances could have aided in their death such as handling previous to the bioassay. This population of *Culex tarsalis*, regardless of which type of insecticide was used in the field, continued to show resistance to pyrethroids even when pyrethroids were not used in the field. We would have expected resistance to pyrethroids decrease in the field population, but we did not. Possibly, the large proportion of mosquitoes with the kdr mutation could explain this trend. Having two copies of the kdr allele could confer resistance in this population in the field, but it may not be enough for some mosquitoes when exposed to the high pyrethroid dosages in the CDC bottle bioassay. In *Culex quinquefasciatus* it has been seen that having kdr genotype does not always confer phenotypic resistance (Xu et al. 2006). Other factors such as elevated levels of detoxifying enzymes could be partially playing a role in the mosquito's ability to be resistant to insecticides. This population of *Culex tarsalis*, regardless of which type of insecticide was used in the field, continued to show resistance to pyrethroids even when pyrethroids were not used in the field. We would have expected resistance to pyrethroids decrease in the field population, but we did not. Possibly, the large proportion of mosquitoes with the kdr mutation could explain this trend. Having the *kdr* mutation can confer resistance to insecticides, but the presence of elevated levels of detoxifying enzymes aids in resistance (Shi et al. 2015) as seen in this study as well.

Conclusion

Throughout this study we found there is very little resistance to Naled within this field population of *Cx. tarsalis* and the likely mechanism causing resistance is an overproduction of acetylcholinesterase. Resistance to sumithrin within the same population is incredibly high. The likely mechanism of resistance is either the leucine to phenylalanine or leucine to serine substitution resulting in *kdr*. Aiding in this resistance is the overproduction of both esterases and oxidases. Similar to sumithrin, permethrin resistance was prominent within this field population from Sutter County California. Resistance to permethrin was most likely due to kdr mutation and increased levels of detoxifying enzyme P450 Oxidase.

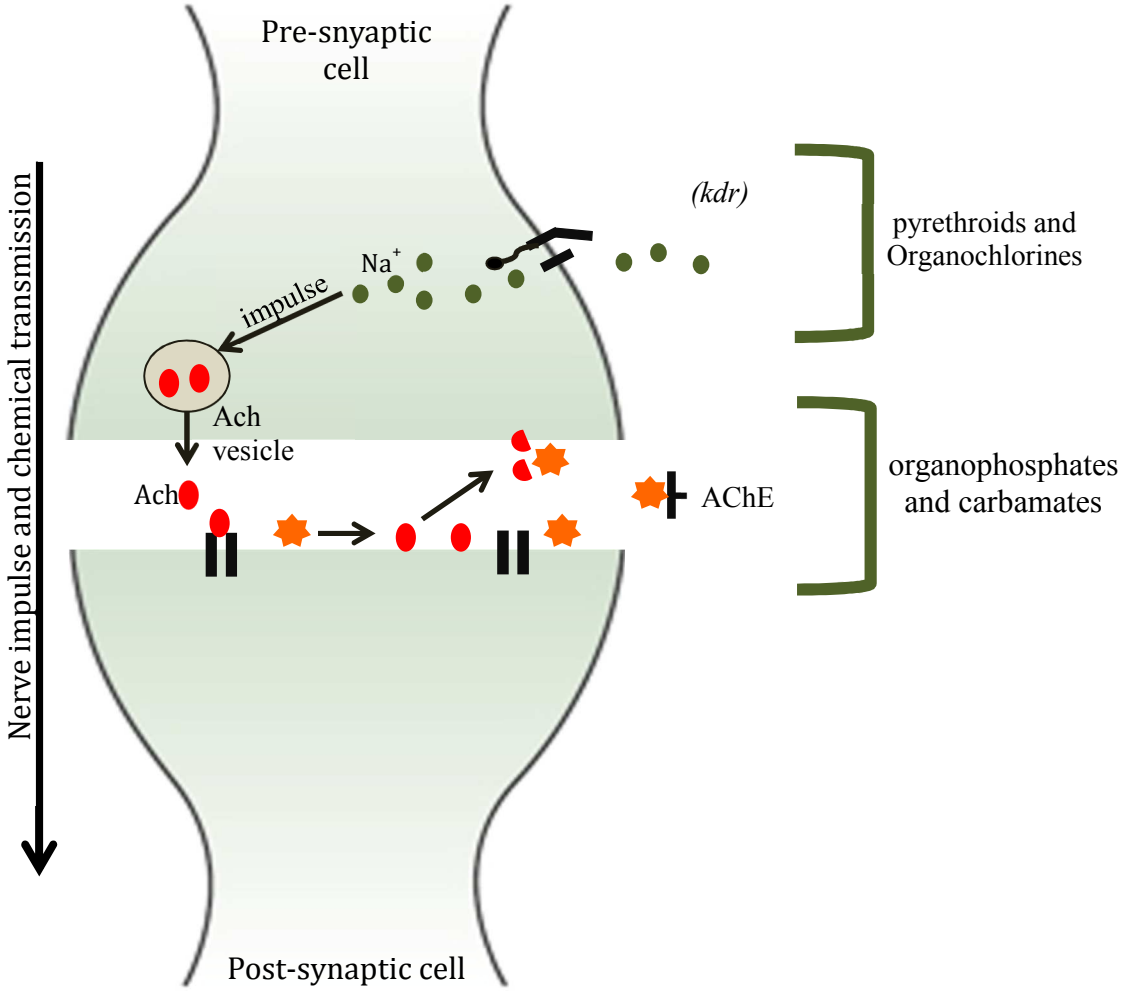


Figure 1. Insecticides action on neuronal cells. Pyrethroids and DDT inhibit the voltage gated sodium channels from closing. Knock down resistance (kdr) mutation does not allow for insecticide binding to this voltage gated sodium channel. This is shown on the left side of the figure. Organophosphates and carbamates inhibit acetylcholinesterase (AChE); which normally plays an important role in degrading nerve impulses sent from the presynaptic cell to the postsynaptic cell while in the synapse.

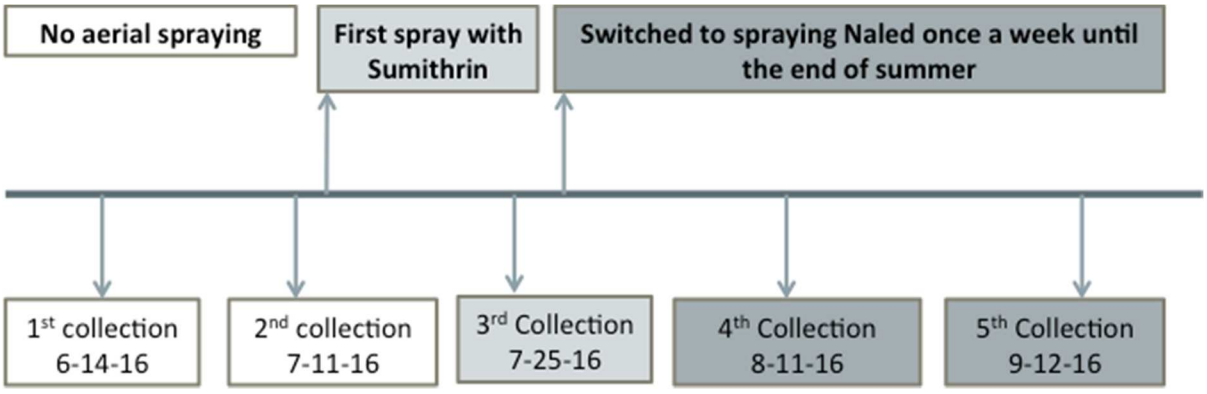


Figure 2. Timeline of mosquito collections with dates and mosquito control district spray schedule.

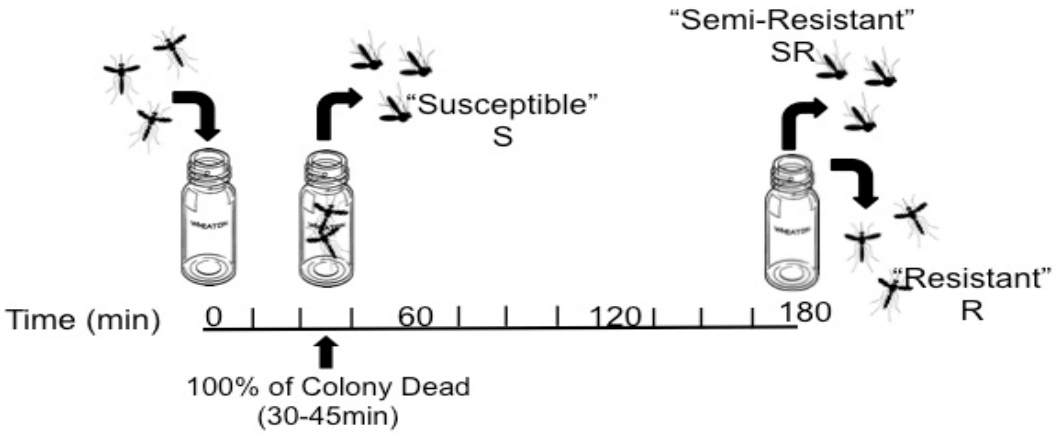


Figure 3. Depiction of CDC bottle bioassay and classification of resistance levels.

Table 1. Primer information for *kdr* melt curve assay. The bolded portions of the primer sequence are the GC rich added 5' tails. Other nucleotides were added in the Serine primer to prevent self-complementarity and hairpin formation. The underlined portion of the primer on the 3' end signifies the *kdr* codon where the single nucleotide polymorphism occurs.

	Primer Sequence	**Amplicon Length**	**Range of melt temps**
Leucine CxTkdrMC LeuF3	**GGGGCGG**GGCCACCGTAGTGATAGGAAACTTA	87 bp	79.1-80.8 ° C
Phenylalanine CxTkdrMC PheF2	GGCCACCGTAGTGATAGGAAACTTTT	76 bp	77.37-77.82°C
Serine CxTkdrMC SerF4	**GGGCGAGGCGGGCGGGGGGGCGCGG GCGAGGG**CACCGTAGTGATAGGAAACTC	112 bp	83.1-83.79°C
Reverse CxTkdrMC R5	TACAGACTCCTACCTCCGGA		
Leucine CxTkdrMC LeuF4	**GAGGGCGG**GGCCACCGTAGTGATAGGAAACTT	87 bp	79-79.95°C

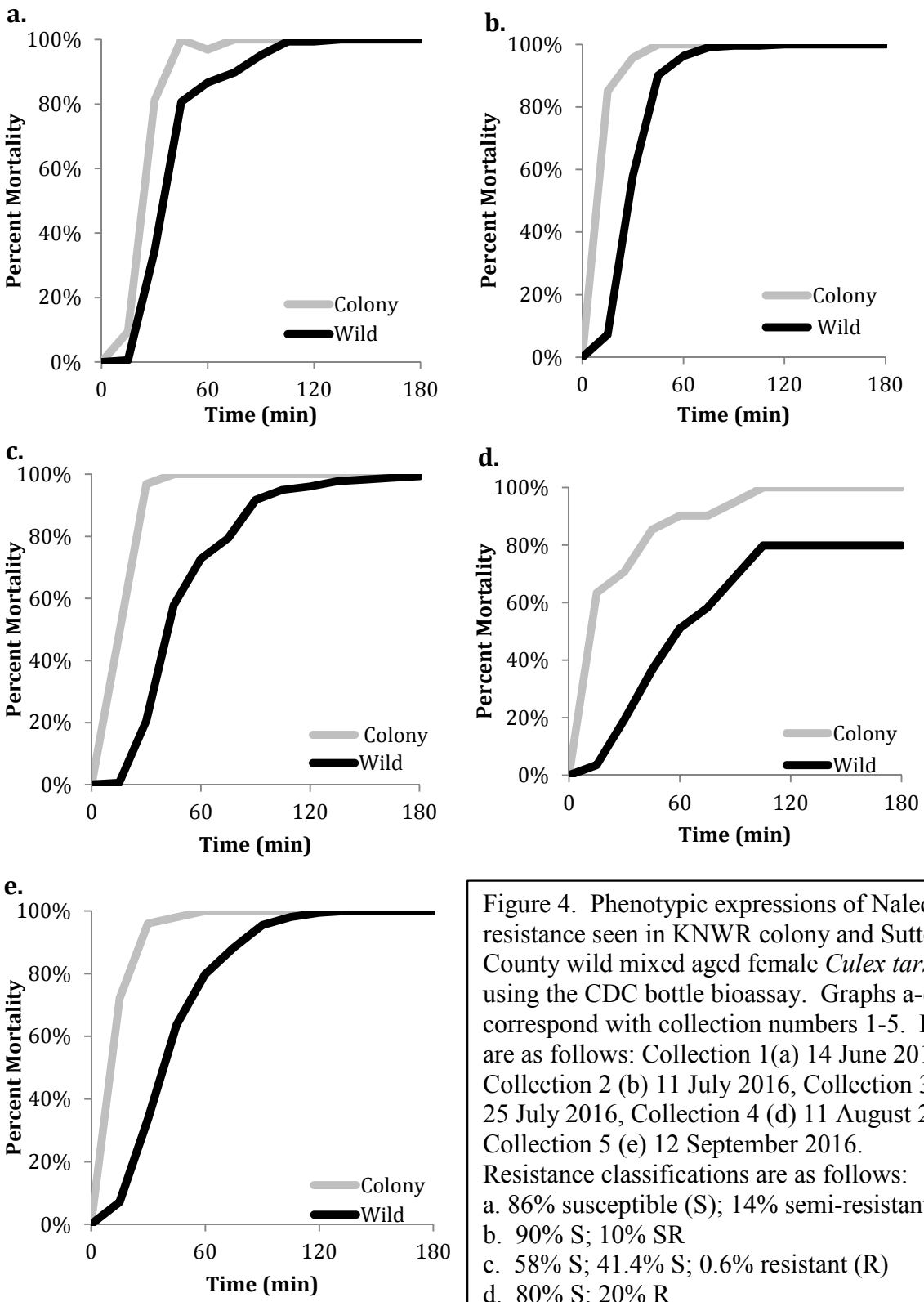


Figure 4. Phenotypic expressions of Naled resistance seen in KNWR colony and Sutter County wild mixed aged female *Culex tarsalis* using the CDC bottle bioassay. Graphs a-e correspond with collection numbers 1-5. Dates are as follows: Collection 1(a) 14 June 2016, Collection 2 (b) 11 July 2016, Collection 3 (c) 25 July 2016, Collection 4 (d) 11 August 2016, Collection 5 (e) 12 September 2016. Resistance classifications are as follows:
a. 86% susceptible (S); 14% semi-resistant(SR)
b. 90% S; 10% SR
c. 58% S; 41.4% S; 0.6% resistant (R)
d. 80% S; 20% R
e. 80% S; 20% SR

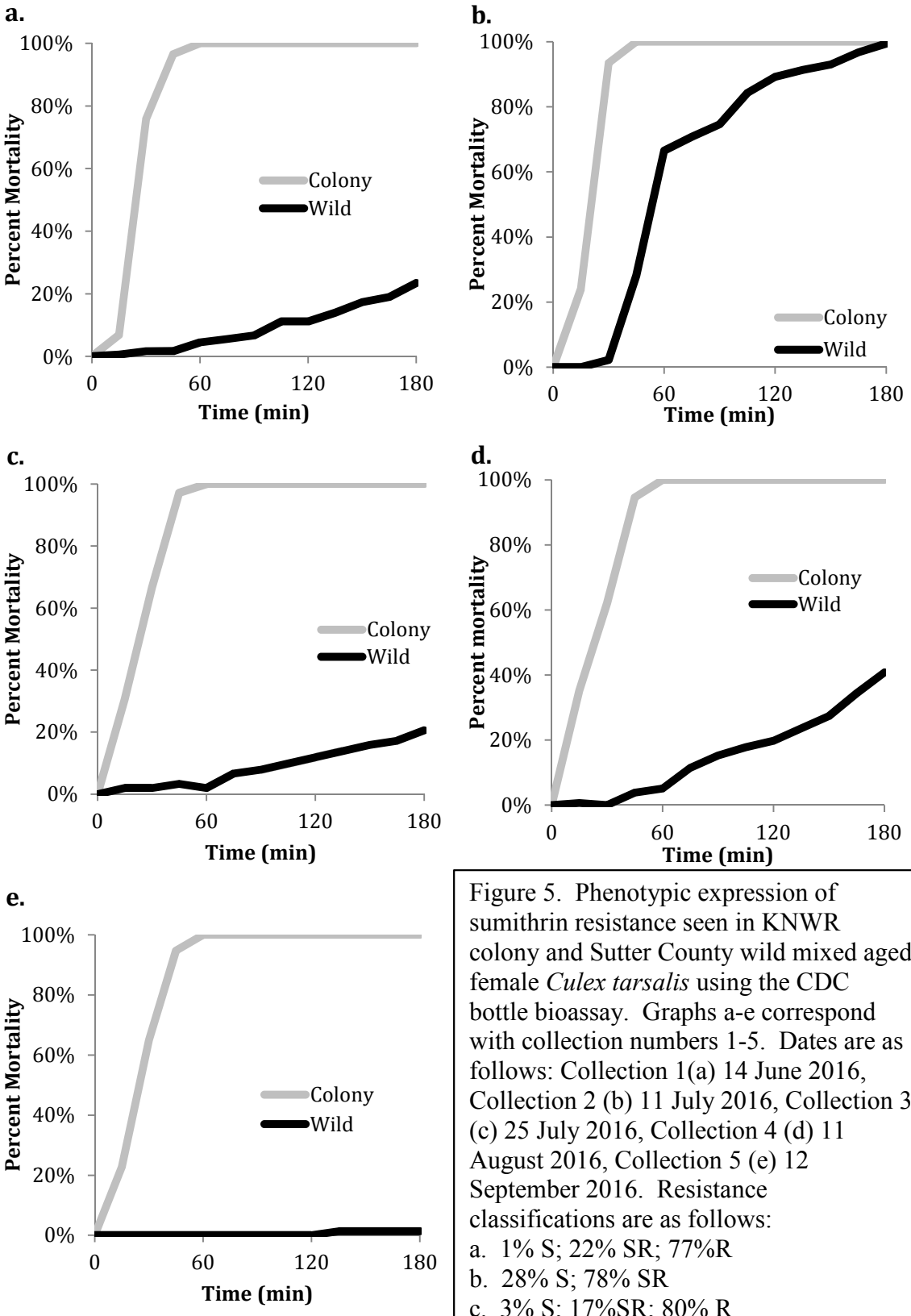
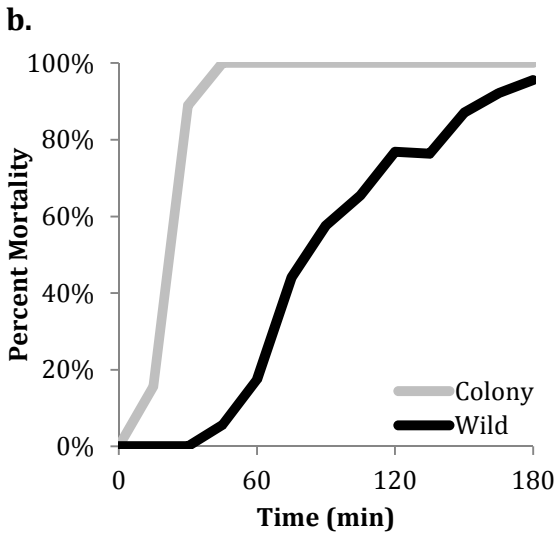
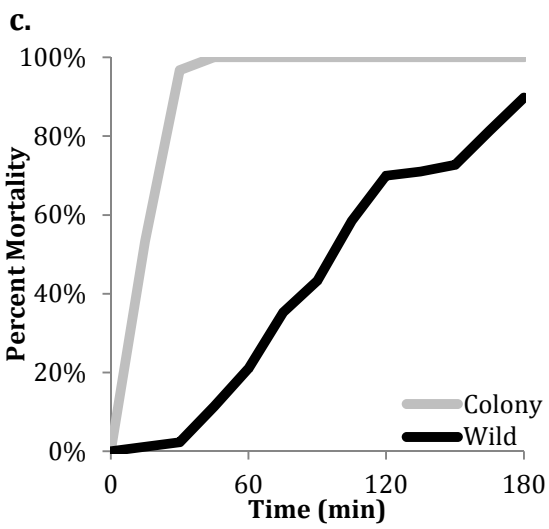


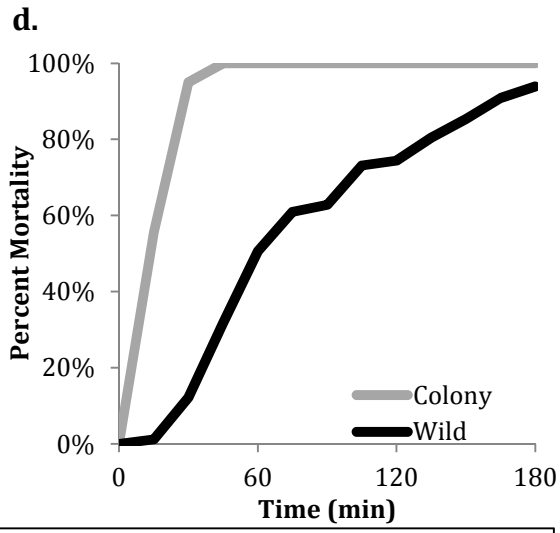
Figure 5. Phenotypic expression of sumithrin resistance seen in KNWR colony and Sutter County wild mixed aged female *Culex tarsalis* using the CDC bottle bioassay. Graphs a-e correspond with collection numbers 1-5. Dates are as follows: Collection 1(a) 14 June 2016, Collection 2 (b) 11 July 2016, Collection 3 (c) 25 July 2016, Collection 4 (d) 11 August 2016, Collection 5 (e) 12 September 2016. Resistance classifications are as follows:

a. 1% S; 22% SR; 77%R
b. 28% S; 78% SR
c. 3% S; 17%SR; 80% R
d. 5% S; 35% SR; 60% R
e. <1% S; >99% R

a.
Culex tarsalis collected at this time point were not treated with Permethrin in CDC bottle bioassay.







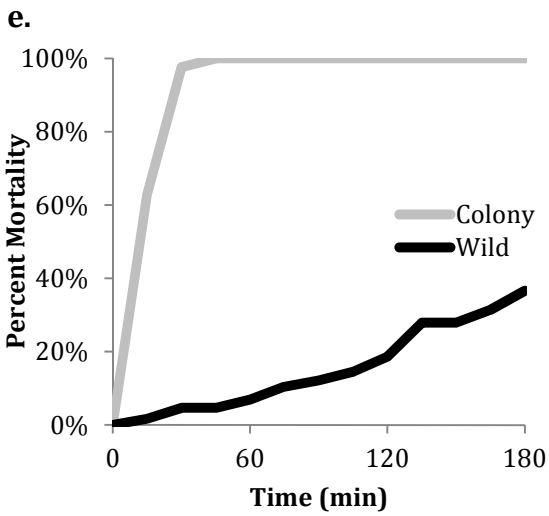


Figure 6. Phenotypic expression of permethrin resistance seen in KNWR colony and Sutter County wild mixed aged female *Culex tarsalis* using the CDC bottle bioassay. Graphs a-e correspond with collection numbers 1-5. Collection 1(a) N/A (b) 11 July 2016, Collection 3 (c) 25 July 2016, Collection 4 (d) 11 August 2016, Collection 5 (e) 12 September 2016. Permethrin was not used in CDC bottle bioassay at the first collection. Resistance classifications are as follows:
b. 6% S; 91% SR; 3% R
c. 11% S; 79% SR; 10% R
d. 32% S; 62% SR; 6% R
e. 5% S; 32% SR; 43%R

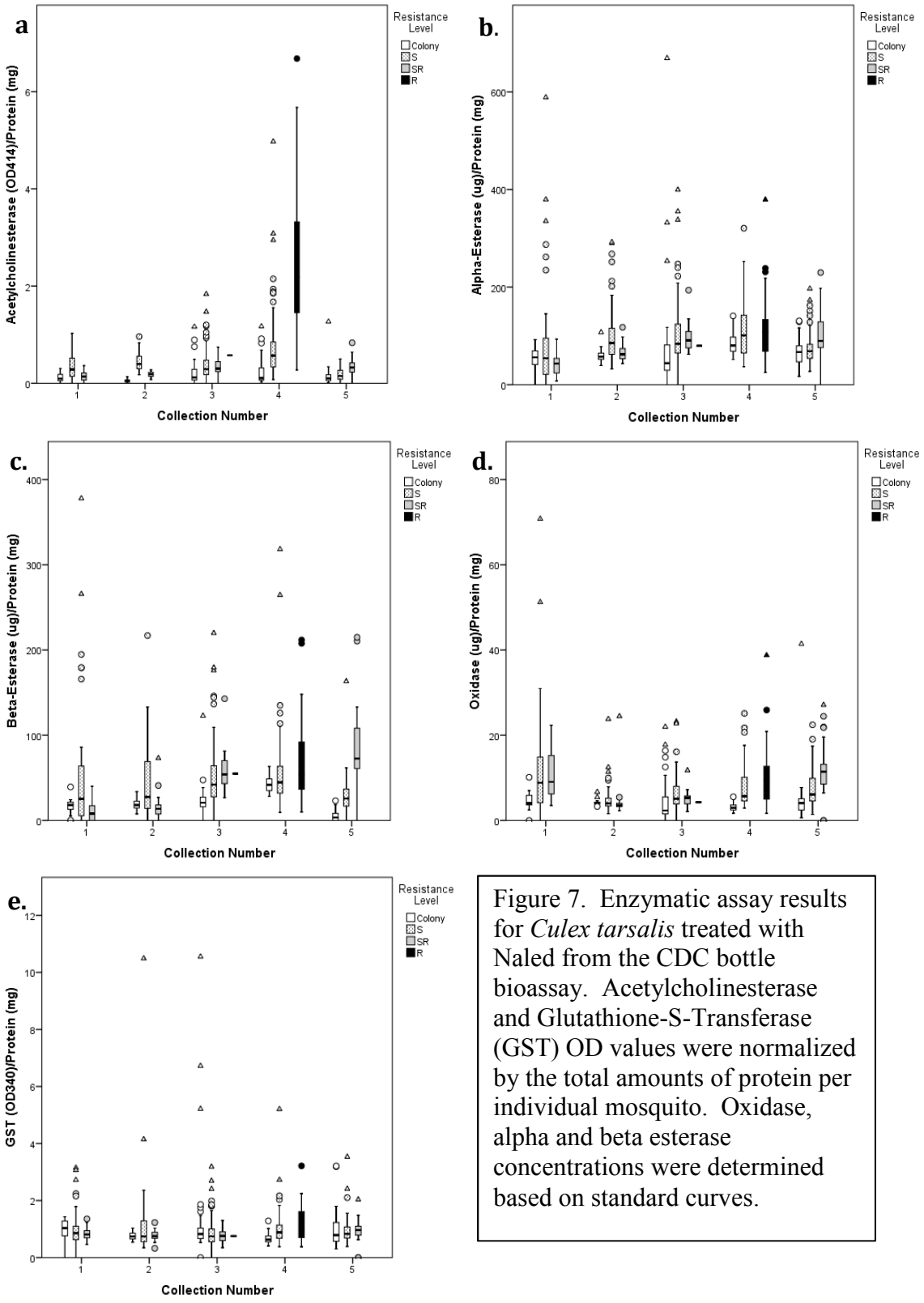


Figure 7. Enzymatic assay results for *Culex tarsalis* treated with Naled from the CDC bottle bioassay. Acetylcholinesterase and Glutathione-S-Transferase (GST) OD values were normalized by the total amounts of protein per individual mosquito. Oxidase, alpha and beta esterase concentrations were determined based on standard curves.

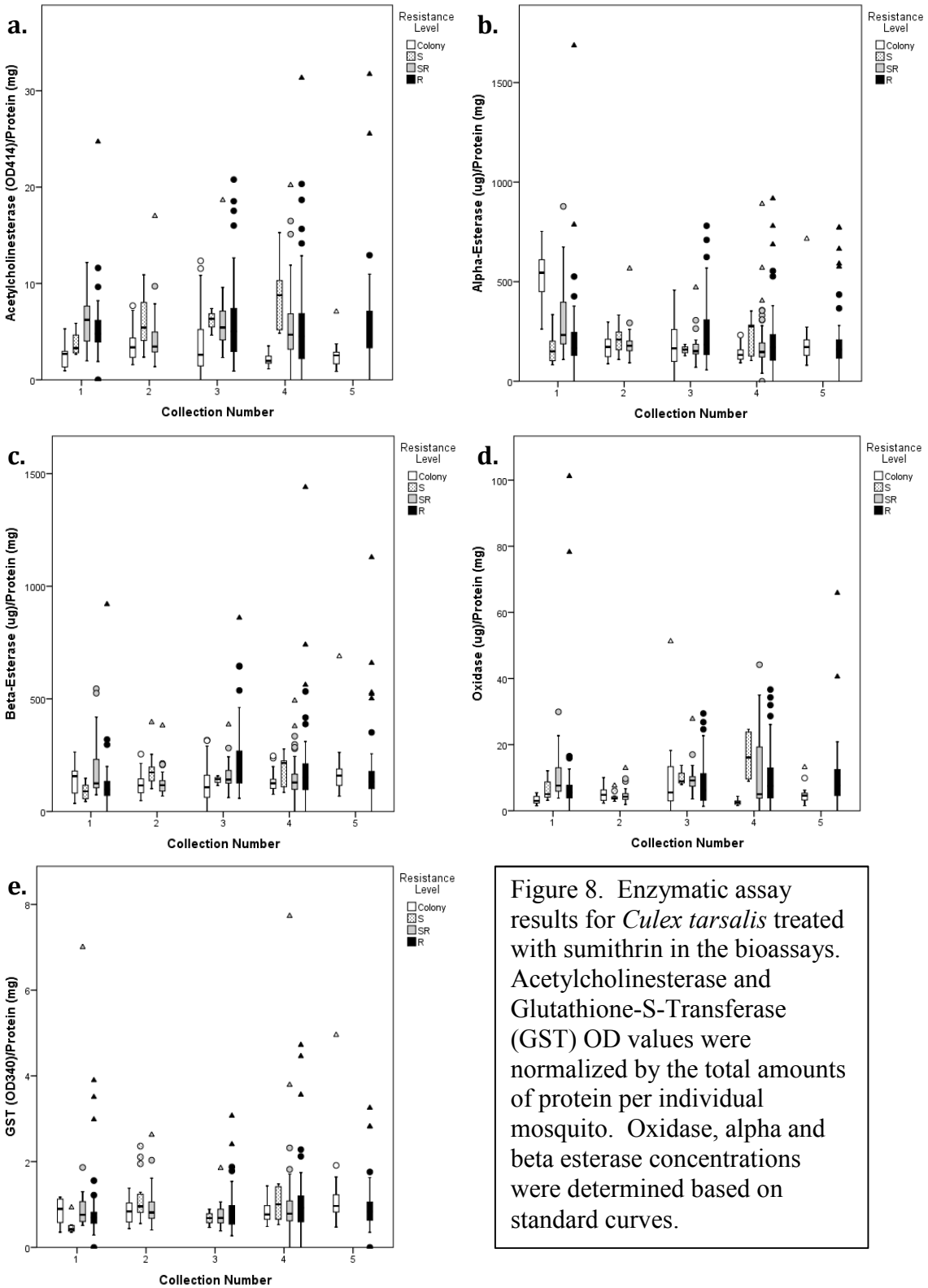


Figure 8. Enzymatic assay results for *Culex tarsalis* treated with sumithrin in the bioassays. Acetylcholinesterase and Glutathione-S-Transferase (GST) OD values were normalized by the total amounts of protein per individual mosquito. Oxidase, alpha and beta esterase concentrations were determined based on standard curves.

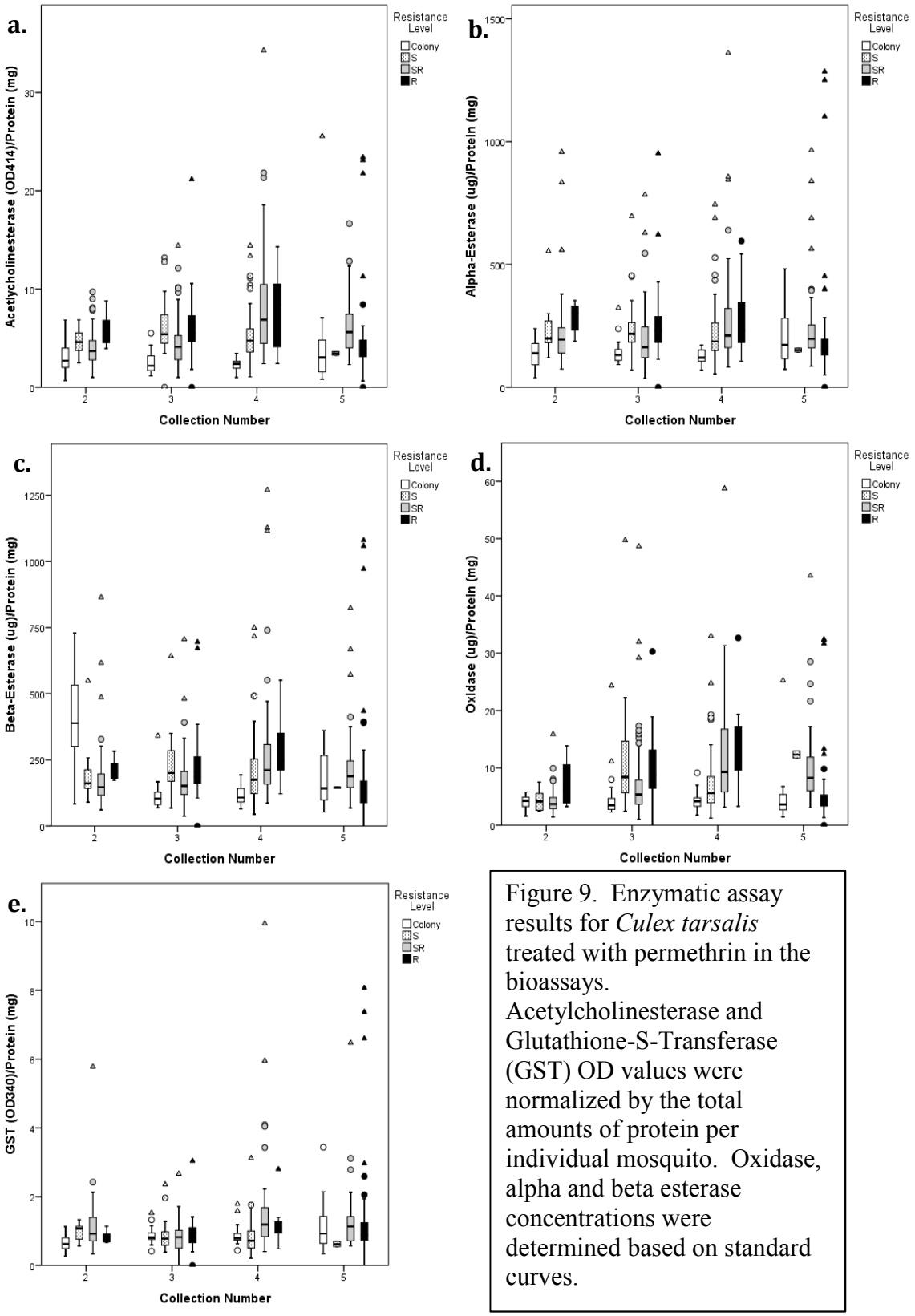


Figure 9. Enzymatic assay results for *Culex tarsalis* treated with permethrin in the bioassays. Acetylcholinesterase and Glutathione-S-Transferase (GST) OD values were normalized by the total amounts of protein per individual mosquito. Oxidase, alpha and beta esterase concentrations were determined based on standard curves.

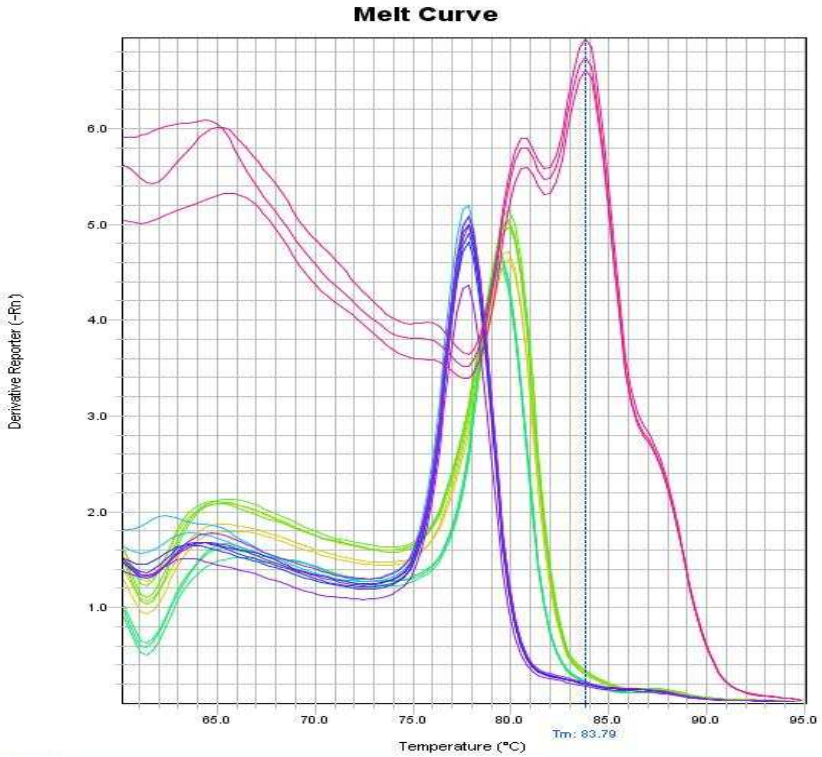


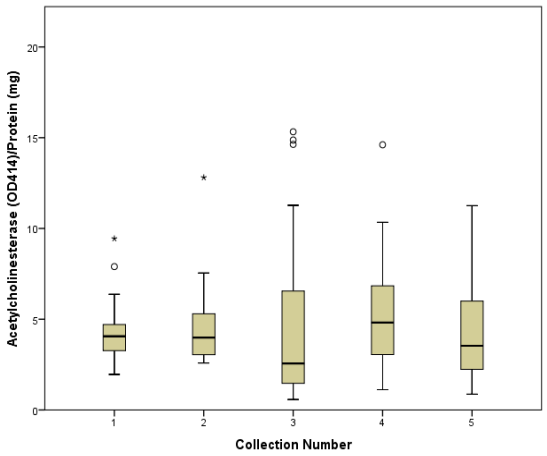
Figure 10. Melt curve graph for all three *kdr* alleles. Range of melt curve temperatures are listed in Table 1.

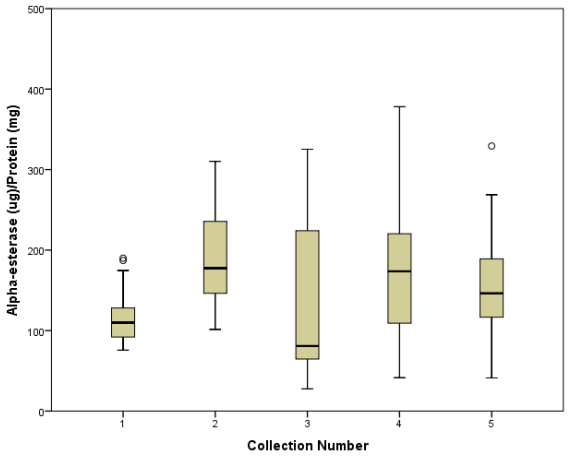
Table 2. *kdr* genotypes in all resistant levels. Mosquitoes treated with pyrethroids. Shown in parenthesis is the percent of the corresponding genotype at each resistance level. The F/L/S indicate which amino acid is being encoded for at the corresponding position 1014 seen in *Anopheles*; whether it be a phenyalanine (F), Leucine(L) or Serine (S).

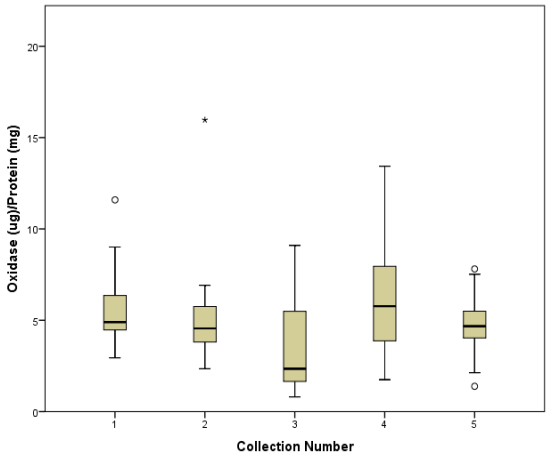
kdr Genotypes in all Resistant Levels							
	F/F (n=387)	**S/S (n=17)**	**F/S (n=95)**	**F/L (n=7)**	**S/L (n=4)**	**L/L (n=4)**	**Totals (n)**
S	55 (61.1%)	4 (4.4%)	24 (26.7%)	3 (3.3%)	3 (3.3%)	1 (1%)	**90 (100%)**
SR	143 (70.1%)	10 (4.9%)	43 (21.1%)	4 (2%)	1 (0.5%)	3 (1.4%)	**204**
R	189 (86%)	3 (1.4%)	28 (12.7%)	0 (0%)	0 (0%)	0 (0%)	**220**

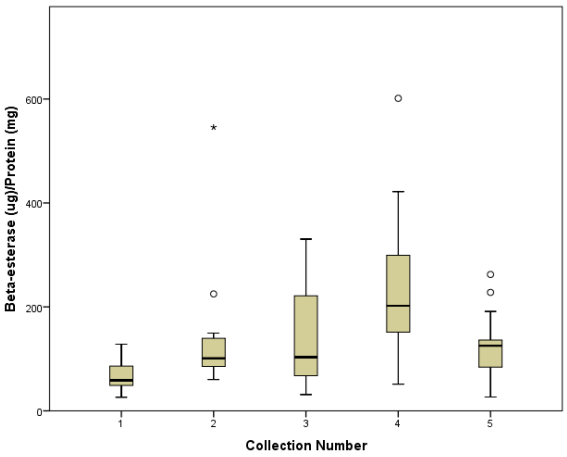
Table 3. Allele frequencies present in every collection. Shown are the kdr alleles present at each collection number. In parenthesis is the percent of that allele present at each collection number. In the total column to the far right, the percent in parenthesis indicate what percent of the total alleles in this collected population.

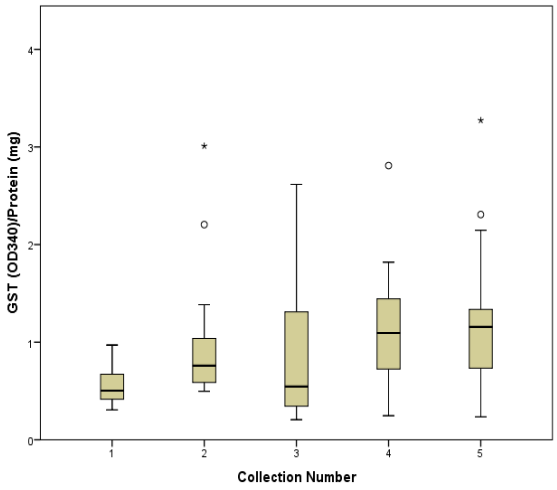
Allele Frequencies Present in Every Collection (%)						
	Collection 1 (n=168)	**Collection 2 (n=182)**	**Collection 3 (n=274)**	**Collection 4 (n=240)**	**Collection 5 (n=164)**	**Overall total alleles n, (%)**
F	137 (81.5%)	157 (86.3%)	233 (85%)	207 (86.3%)	142 (86.6%)	**876 (85.2%)**
S	28 (16.6%)	21 (11.5%)	32 (11.7%)	32 (13.3%)	20 (12.2%)	**133 (12.9%)**
L	3 (1.8%)	4 (2.2%)	9 (3.3%)	1 (0.4%)	2 (1.2%)	**19 (1.8%)**











Supplemental Figure 1. Enzymatic assay results for Culex tarsalis that had not undergone CDC bottle bioassay. GST and Acetylcholinesterase OD values were normalized by the total amounts of protein per individual. Oxidase, alpha and beta esterase concentrations were determined based on standard curves. Circles represent outliers while askterisks represent extreme values. Collection 5 is mosquitoes treated with acetone only during the CDC bioassay.

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