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MOLECULAR AND ENZYMATIC RESISTANCE OF CULEX TARSALIS TO PYRETHROID AND ORGANOPHOSPHATE INSECTICIDES

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MOLECULAR AND ENZYMATIC RESISTANCE OF *CULEX TARSALIS* TO PYRETHROID
AND ORGANOPHOSPHATE INSECTICIDES

By

Billy Michael Mortola

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In Partial Fulfillment of the
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MOLECULAR AND ENZYMATIC RESISTANCE OF *CULEX TARSALIS* TO PYRETHROID AND ORGANOPHOSPHATE INSECTICIDES

Abstract

By Billy Michael Mortola

University of the Pacific
2023

Culex tarsalis, a vector for diseases like West Nile virus, is evolving resistance to pyrethroid and organophosphate insecticides used for agriculture and vector control in Northern California. Common mechanisms of resistance in other *Culex* species include the target-site mutations *kdr* and *ace-1* and increased levels of detoxification enzymes (esterases, GSTs, and oxidases). This study contained two parts: Pyrethroid Individual study and an Organophosphate Preliminary study. For the Pyrethroid Individual study, the goal was to compare the prevalence of *kdr* mutations and detoxification enzymes between pyrethroid susceptible and resistant *Cx. tarsalis* individuals in Northern California. For the Organophosphate Preliminary study, the goal was to survey current organophosphate resistance in Northern California *Cx. tarsalis* populations while identifying possible resistance mechanisms: *ace-1* and variations in detoxification enzymes between populations and individuals. Individual mosquitoes previously categorized by resistance status from CDC bottle bioassays with permethrin or naled were prepared for both molecular and enzymatic testing by separating the legs of a mosquito from the remaining body. Legs were used to test for the presence of *kdr* and *ace-1* mutation by qPCR and PCR respectively and confirmed by DNA sequencing while the bodies were used to test for levels of detoxification enzymes. A subset of individuals in the Organophosphate Preliminary study forwent bottle

bioassays and were tested directly to make comparisons between populations without the stress of going into bottle bioassays. For the Pyrethroid Individual study, the greater number of F alleles present at the *kdr* target-site as well as increased levels of GST significantly increased survival when exposed to permethrin. Individuals with 2 F alleles and an active GST level greater than or equal to 0.052 ug/ml showed a higher survival rate than either mechanism independently demonstrating resistance to pyrethroids in *Cx. tarsalis* is likely the result of multiple resistance mechanisms acting collectively. For the Organophosphate Preliminary study, currently little resistance to naled (3%) was observed in Northern California *Cx. tarsalis* populations. No *ace-1* mutations were found in the 208 individuals tested, however three did possess silent mutations at the target-site, suggesting surveillance is needed in the future. Between the small sample size and little resistance found in this preliminary study, there was no correlation between levels of detoxification enzymes from resistant and susceptible individuals. Due to differences in the biology of *Cx. tarsalis* and other mechanisms of resistance not looked at in this study it is hard to quantify definitive resistance mechanisms in *Cx. tarsalis*. From this study it appears that *kdr* mutation (L1014F) and increased levels of GST likely contribute at some level to resistance to pyrethroid insecticides in *Cx. tarsalis*.

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CHAPTER 1: INTRODUCTION

Background

Mosquitoes as Vectors of Disease

Vectors are any blood-feeding arthropods, such as mosquitoes, ticks, and fleas that are capable of transmitting pathogens to humans. Mosquito-borne diseases alone contribute to over one million human deaths worldwide each year (Mosquito-Borne Diseases, 2021). Mosquitoes are competent vectors of parasites, viruses, and bacteria, which can lead to many diseases such as malaria, dengue, Zika virus, and West Nile virus. The ability of mosquitoes to transmit diseases is referred to as vector competence. Some species are competent vectors of many pathogens, while others can only transmit certain diseases. For example, species belonging to the genus *Anopheles* are the only mosquitoes that can transmit human malaria. In the United States, the most frequent mosquito-borne disease is West Nile virus, for which *Culex* mosquitoes are the most competent vectors (World Health Organization, 2021).

West Nile virus is an encephalitis virus that can cause symptoms ranging from mild flu-like to severe neurological disease and death. While most cases are asymptomatic or mild, the Centers for Disease Control and Prevention reports that 1 in 150 infected people will develop severe, often fatal neuro-invasive disease. Each year cases of West Nile virus peak in the summer and early fall months when mosquito activity is at its highest. There is currently no known cure or treatment for West Nile virus; thus, preventing mosquito-human interactions is the best method to reduce disease transmission (Centers for Disease Control, 2021).

Feeding and Disease Transmission

Mosquitoes have a diet consisting largely of nectar from plants. However, female mosquitoes need protein and iron to properly develop their eggs, thus taking blood meals from various vertebrate species (Zhou, 2007). Different species of mosquitoes prefer to feed on different hosts, seeking out blood meals from mammals, birds, and reptiles (Takken and Verhulst, 2012). Mosquitoes locate suitable hosts using special nerve cells with receptors that detect carbon dioxide and skin odors (Mosquito & Vector Control Association of California, 2021). Mosquitoes can also detect infrared light to help them locate hosts (Raji and DeGennaro, 2017).

Once mosquitoes locate a host, they bite with a specialized mouth part known as a proboscis which is used to pierce a host's skin. They then locate a blood vessel and draw blood up through their mouth. While sucking up blood, the mosquito injects saliva containing an anticoagulant that stops the host's blood from clotting. It is through this saliva that an infected mosquito can transmit a pathogen to the host's bloodstream (Raji and DeGennaro, 2017; Merritt et al., 2019).

Culex tarsalis

Culex tarsalis is an important vector for arboviruses in North America and is characterized by a white band across its proboscis and white bands on its tarsal joints (Reisen, 1993). The species is responsible for the maintenance and transmission of several encephalitis viruses, including West Nile, St. Louis encephalitis, and Western equine encephalitis viruses.

Culex tarsalis is geographically distributed throughout the United States; however, it is much less abundant on the East Coast and has been documented in elevations from sea level to as high as 9,000 feet (Pahk, 2003). Found in both rural and urban habitats, female mosquitoes

are generalist feeders and will seek blood meals primarily from both avian and mammalian hosts. They are most active the first few hours after dusk and are most abundant from late spring to early fall (Pahk, 2003). Some females have been recorded traveling several miles a day in search of hosts (Reisen, 1993).

In northern latitudes, females will overwinter in cool sheltered locations in a state of reproductive diapause awaiting the breeding season starting in spring. In warmer southern latitudes, females can be reproductively active throughout the winter months (Reisen, 1993). *Culex tarsalis* will breed in a variety of wet areas with a wide range of water conditions.

Females will seek a blood meal in order to develop their eggs; however, some are capable of autogeny, a phenomenon where females are able to develop eggs in the absence of a blood meal (Nelms et al., 2013). Females will lay eggs on top of the water's surface in clusters known as rafts, consisting of approximately 100 to 300 eggs. Eggs will typically hatch within 48 hours into larvae, which will feed on organic matter in the water and will molt as they grow, shedding their skin several times. Depending on water temperature and food availability, larvae can fully mature in as little as 5 days. Larvae will then pupate and emerge as adult mosquitoes in 2-3 days (Centers for Disease Control, 2021).

Mosquito Control and Insecticides

Since 1969, California has monitored mosquito abundance and virus activity. The California Department of Public Health alongside the Mosquito and Vector Control Association of California publishes guidelines on surveillance and response to mosquito populations and virus activity (California Mosquito-Borne Virus, 2020). Since many of the pathogens transmitted by mosquitoes have no known cure, controlling mosquito populations is the most effective method to protect people from disease. There are approximately 80 local agencies in

California, consisting of county environmental and health departments, vector control districts, and county agriculture departments that conduct mosquito control practices (California Mosquito-Borne Virus, 2020). When surveillance indicates a need for mosquito control, an approach known as integrated pest management (IPM) is often used. IPM involves habitat modification, biological control, and pesticide application all of which are aimed at controlling pest populations (Vector-Borne Disease Section, 2008). Since adult female mosquitoes are responsible for disease transmission, immediate and direct control of adults is needed when populations reach levels of concern. The application of pesticides is a direct way to effectively target adult mosquito populations (California Mosquito-Borne Virus, 2020).

Pesticides are any substance used to kill or repel organisms deemed as pests. Insecticides are a subcategory of pesticides that are formulated specifically to target a wide variety of insect pests (Vector-Borne Disease Section, 2008). Insecticides contain a combination of “active ingredients” which are the chemicals that specifically target the insect and “inert ingredients” which are substances used to enhance the performance and usability of the product (Environmental Protection Agency, 2021). For adult control, insecticides are often applied as ultra-low volume (ULV) sprays that can be dispersed in the air as fine aerosol droplets that contact flying mosquitoes to kill them. ULV applications contain very small quantities of active ingredients, typically less than 4 ounces per acre to minimize potential toxicity to people and unintended organisms (Environmental Protection Agency, 2021; Vector-Borne Disease Section, 2008). Presently, California authorizes the use of only two classes of insecticides, pyrethroids and organophosphates, for local and government agencies, agriculture, and personal use (Vector-Borne Disease Section, 2008). Both pyrethroids and organophosphates contain active ingredients that target the insect’s nervous system.

Pyrethroids. Pyrethroids are synthetic compounds that mimic naturally occurring pyrethrins, which are insecticidal esters found in the flowers of chrysanthemums. Pyrethroids were first introduced in the 1970s for mosquito control and have been considered some of the most successful insecticides ever created (Davies et al., 2007). Pyrethroids target the insect's central and peripheral nervous system by binding to and opening voltage-gated sodium channels for long periods of time. This creates large influxes of sodium into the cell and causes constant depolarization. This rapidly leads to hyperexcitation of the nervous system, resulting in uncontrolled muscle contractions causing an incapacitating "knockdown" of the insect. (Davies et al., 2007; Field et al., 2017). Type I and Type II pyrethroids differ by their chemical structure. Type I pyrethroids have greater lipophilicity which gives them good knockdown effects as they rapidly bind their target. Type II pyrethroids have an added cyano-group that helps the compound to irreversibly bind to its target, resulting in better kill (Davies et al., 2007). Permethrin, resmethrin, sumithrin, and deltamethrin are the active ingredients used in pyrethroid insecticides in California (Vector-Borne Disease Section, 2008).

Organophosphates. Organophosphates target the enzyme acetylcholinesterase (ACE) by binding to and hindering its normal function, which is to hydrolyze the neurotransmitter acetylcholine at synaptic nerve junctions (Hemingway and Ranson, 2000). The hydrolyzation of acetylcholine terminates a nerve signal, so when organophosphates bind ACE, a build-up of available acetylcholine results, leading to constant depolarization and hyperexcitation of the nervous system (Fukuto, 1990). Malathion and naled are the only two registered organophosphates in California (Vector-Borne Disease Section, 2008). According to the Environmental Protection Agency, naled is widely used in the United States for aerial spraying

and has been applied in metropolitan areas as well as rural and agricultural land since 1959 (2021).

Insecticide Resistance in Culex tarsalis

Shortly after the introduction of Dichlorodiphenyltrichloroethane (DDT) for mosquito control in 1946, it was noticed that mosquito populations started to develop resistance against the insecticide (Hemingway and Ranson, 2000). This was the onset of what is now a continual battle between insecticide use and the development of resistance mechanisms in mosquitoes. As populations of mosquitoes developed resistance to one insecticide, new ones were created and used until their effectiveness diminished. Over time this has led to cross resistance, as some mosquito populations are able to display resistance to multiple insecticides. According to Liu (2015), mosquito resistance to insecticides has been identified in all major vector species and all classes of insecticides in more than 60 countries. Mosquito resistance to insecticides is thought to be pre-adaptive, meaning that individuals with rare mutations or mechanisms that allow them to survive a stressful situation already exist in populations. When exposed to a stressor, those individuals are able to survive and reproduce (Liu, 2015). Since mosquitoes typically have short life cycles, resistance mechanisms can accrue in populations at accelerated rates. A study done by Li and Liu in 2010, looked at inheritance patterns of permethrin resistance in *Culex quinquefasciatus* and found that resistant alleles display incomplete dominance and follow an autosomal inheritance pattern. These results demonstrate that genetic inheritance gives rise to variability in an individual's level of resistance, so populations can consist of individuals with high, moderate, and mild resistance status (Liu, 2015). The two major mechanisms of insecticide resistance in mosquitoes are target-site insensitivities and increased metabolic detoxification of insecticides (Brodgon and McAllister, 1998; Hemingway and Ranson, 2000; Liu, 2015).

Target-site Insensitivities. Target-site insensitivities result from structural modification of target proteins that interact with a given insecticide. This is usually due to mutations to the structural genes of the active site of a target protein, modifying it in a way that prevents interaction with the insecticide (Davies et al., 2007).

For pyrethroid insecticides, mutations to the gene that encodes sodium channel structure can alter the gating properties of the channel, making it insensitive to pyrethroids. This reduces the effectiveness of the insecticide. Any type of resistance to pyrethroids as a result of insensitivity of sodium channels is termed knockdown resistance (*kdr*). A common *kdr* mutation, found in many species of mosquito is L1014F, which is a leucine to phenylalanine amino acid substitution in domain II, segment six of the voltage-gated sodium channel (Williamson et al., 1996). In another mutation, L1014S, the same amino acid leucine is substituted to serine, which also confers resistance to pyrethroids in many mosquito species (Ranson et al., 2000; Rinkevich et al., 2013; Scott et al., 2015). Both mutations at this site have been shown to play some role in pyrethroid resistance in *Cx. tarsalis* (Choi, 2016; Hughes, 2017).

The target site for organophosphate insecticides is acetylcholinesterase (ACE). Two genes, *ace-1* and *ace-2*, encode for ACE in mosquitoes, and are the result of an ancient duplication event. Only *ace-1* has been documented to play a role in the resistance of organophosphates, while the function of *ace-2* remains unknown (Weill et al., 2002; Weill et al., 2004a). An *ace-1* mutation, G119S, is a glycine to serine amino acid substitution that has been reported in several mosquito species including the closely related *Culex pipiens*, *Culex vishnui*, and *Cx. quinquefasciatus* (Liu, 2015). The change to a serine residue at this position causes steric hindrance to the active binding site of the ACE, preventing its interaction with

organophosphates. Conversely, it has been found in some mosquito species that this mutation also incurs a high fitness cost because the serine hinders normal binding of the enzyme to acetylcholine. This G119S mutation has arisen independently 4 times in different species of mosquito, suggesting there are only a few possible mutations that cause ACE insensitivity (Weill et al., 2004a). In 2004, Weill et al.^b examined ACE amino acid sequences from 79 animal species and found that glycine was present at the codon 119 position in all species except some benthic marine invertebrates and trematodes such as Ascidians and Schistosoma, which had a serine at this position. This suggests (1) glycine plays a critical role at this position (2) there is likely a fitness cost associated with having any other amino acid at this position. In *An. gambiae* and *Cx. pipiens* the glycine to serine substitution results from a GGC to AGC base change, which requires only a single nucleotide substitution (Weill et al., 2004b). *Culex tarsalis* is genetically different, the glycine at the *ace-1* mutation site is encoded by a GGA codon (Weill et al., 2004b). So, for glycine (GGA) to change to a serine (AGC) there must be two base pair changes to the codon. Since there is limited literature on this topic, it is unclear to what extent this mutation is contributing to organophosphate resistance in *Cx. tarsalis* populations (Choi, 2016; Hughes, 2017).

Metabolic Detoxification. Many insects have enzymes that can break down xenobiotics such as insecticides into inactive forms that are harmless or less toxic to the insect. These enzymes are found in most insects as a natural defense against plant toxins encountered in their diet. Detoxifying enzymes convert insecticides into non-toxic compounds or forms that can be more rapidly excreted by the insect. The metabolism of insecticides can be broken into phase I and phase II reactions. In phase I reactions, the insecticide is converted into a more polar substance typically through oxidation, reduction, and hydrolysis reactions. In phase II reactions,

phase I products are conjugated to a polar substance that facilitates its excretion from the body. Resistant insects have either (1) forms of enzymes with higher catalytic activity or (2) have higher quantities of enzymes due to increased transcription or gene amplification (Hemingway et al., 2004; Panini et al., 2016). Mosquitoes have three enzymatic families responsible for metabolic detoxification: oxidases, esterases, and glutathione-S-transferases (Brodgon and McAllister, 1998; Hemingway et al., 2004; Panini et al., 2016).

Oxidases are a large, complex family of enzymes found in insects. Cytochrome P450 monooxygenases are the most common type of oxidase found in insects that are associated with the detoxification of many insecticides, plant toxins, and other chemicals. Upregulation or overexpression of oxidase genes play an important role in the detoxification of insecticides and mosquito resistance. Some oxidase genes can be induced by certain compounds, an adaptation that allows insects to rapidly respond to change in their environments. In general, oxidases perform phase I metabolism, mediating resistance by binding oxygen molecules to the insecticide (David et al., 2013; Liu et al., 2011). Overexpression of oxidases is strongly correlated to pyrethroid resistance in many mosquitoes including *Culex* species (Hardstone et al., 2007; Komagata et al., 2010).

Esterases are another large, diverse family of enzymes that can metabolize a wide range of insecticides. Esterases are phase I enzymes that hydrolyze ester bonds on insecticides through the addition of water to generate an alcohol and acid. Most esterases belong to the α/β -hydrolase fold protein superfamily (Brodgon and McAllister 1998; Montella et al., 2012). In as early as 1987, Mouches et al. showed that there were high levels of α/β -esterases in organophosphate resistant *Cx. tarsalis* strains compared to susceptible strains found in California. In *Cx. tarsalis* larvae, it has been demonstrated that two esterase-based resistance mechanisms exist: elevated

levels of esterases and non-elevated, metabolically enhanced esterases that are able to metabolize malathion (Ziegler et al., 1987).

Glutathione-S-transferases (GSTs) are phase II proteins that metabolize many endogenous and exogenous compounds. These enzymes catalyze the conjugation of reduced glutathione to electrophilic substances like insecticides. This transforms the insecticide into a water-soluble metabolite that is more readily excreted by the insect. GSTs have been shown to protect against pyrethroid toxicity in many insects (Kostaropouloset et al., 2001). These enzymes can also fluctuate widely throughout an insect's lifespan (Hemingway et al., 2004). Increased transcription and gene amplification cause elevated GST activity, which has been shown to play a role in resistance to multiple insecticide classes, especially DDT (Enayati et al., 2005; Hemingway et al., 2004).

CHAPTER 2. PYRETHROID INDIVIDUAL STUDY

Introduction

In the United States, West Nile virus (WNV) is the most common mosquito-borne disease (Centers for Disease Control, 2022). *Culex tarsalis*, known as the Western Encephalitis Mosquito, is a primary vector for WNV in California and many other areas of the Western United States (Reeves and Hammon, 1962). Since there are no human-approved vaccines or antivirals for WNV, reducing human-mosquito interaction and therefore transmission remains the best way to avoid disease (Centers for Disease Control, 2022). Local and state authorities rely on insecticides to control the abundance of vector populations (Mosquito & Vector Control Association of California, 2021). Since *Cx. tarsalis* inhabits both rural and urban areas, the species faces insecticide pressure from agriculture, vector control, and home-use. Over time, selective pressures placed by insecticides have led to the evolution of resistance across many mosquito species to these chemicals (Richards et al., 2020).

Pyrethroids are one of two classes of insecticides approved for use in California (Vector-Borne Disease Section, 2008). Pyrethroids function by binding to and opening voltage-gated sodium channels of the mosquito's neurons. This disruption of the gating properties allows for abnormally large amounts of sodium ions to flow into the cell channels causing excessive nerve signaling. Pyrethroids cause the mosquito to have a hyper excited nervous system leading to uncontrolled muscle contractions, physical exhaustion, knockdown, and death (Davies et al., 2007).

Mosquitoes have evolved several mechanisms of resistance as a result of insecticide pressure. The two common ones are target-site insensitivities and enzymatic detoxification (Brodgon and McAllister, 1998).

Target-site insensitivities to pyrethroid insecticides are any genetic mutations to the voltage-gated sodium channel gene that reduces the binding ability of the insecticide. Knockdown resistance (*kdr*) mutations resulting from amino acid substitutions from leucine to phenylalanine (L1014F) or a leucine to serine (L1014S), originally described in *Anopheles gambiae* by Martinez-Torres et al. in 1998 and Ranson et al. in 2000, have been documented in many mosquito species. The 1014 position in *An. gambiae* is in domain II, segment six of the voltage-gated sodium channel. Mutations to this region alter the gating properties of the channel, allowing prolonged entrance of sodium causing an overactive nervous system (McAbee et al., 2003; Xu et al., 2006).

Enzymes such as P450 oxidases, esterases, and glutathione-S-transferases are capable of detoxifying insecticides by either converting them into non-toxic forms or rapidly excreting them from the body. Elevated levels of these enzymes can create resistance to pyrethroid insecticides (Hemingway and Ranson, 2000; Hemingway et al., 2004).

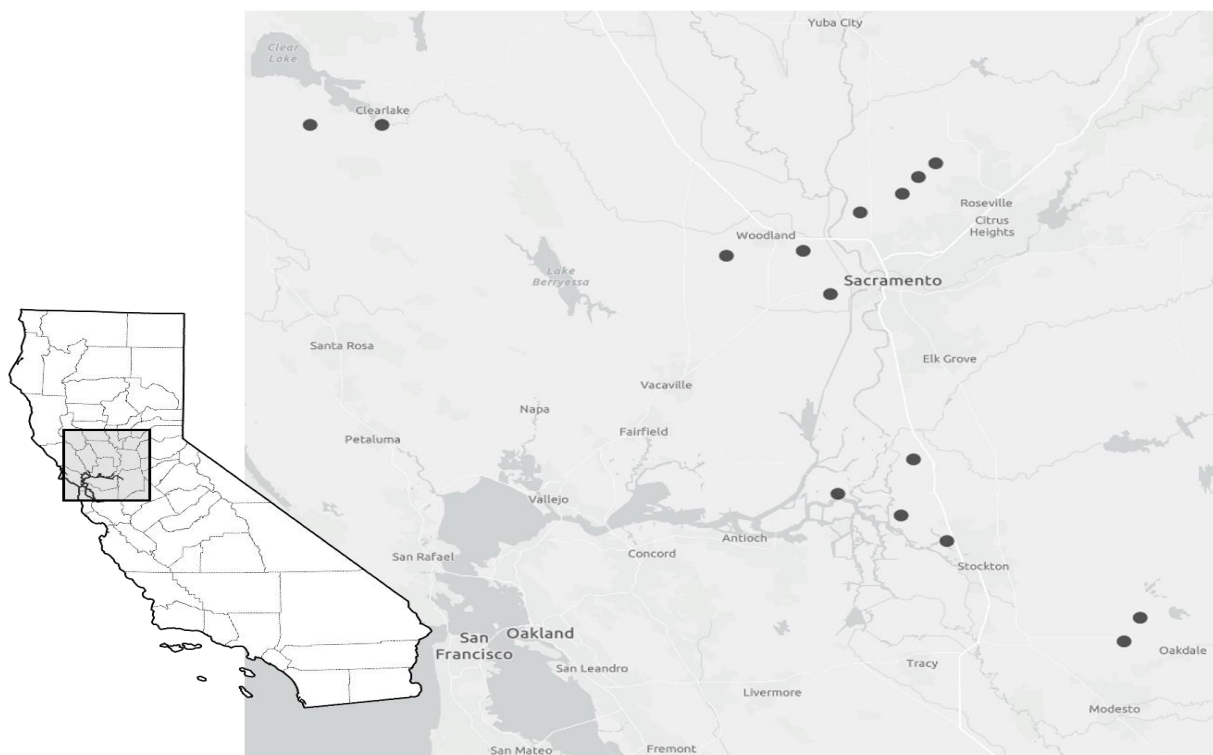
In other vector species, target-site insensitivities and enzymatic detoxification have been studied extensively. Due to differences in the biology of individual species as well as environmental influences, it is important to understand how these mechanisms of resistance are specific to *Culex tarsalis*. Work by Choi (2016) and Hughes (2017) showed that pyrethroid resistant *Cx. tarsalis* populations displayed both *kdr* mutations and elevated detoxification enzymes. While previous work looked at pyrethroid resistance mechanisms in *Cx. tarsalis* populations, it is still unclear as to the extent to which these mechanisms are contributing to

resistance at the individual level (De La Vega and Ryan, 2022). The objective of this study is to compare the prevalence of target-site mutations and enzyme levels between individual *Cx. tarsalis* that are resistant to pyrethroids and individuals that are susceptible, in an effort to elucidate the role of these potential resistance mechanisms in functional insecticide resistance.

Methods

Collections and Sample Preparations

The following collaborating vector control districts, Sacramento-Yolo, Lake County, Placer County, and San Joaquin County, collected mixed-age female mosquitoes from 15 Northern California study sites using CO₂ baited traps during the summer of 2018. Resistance status was established using modified CDC bottle bioassays (McAllister, 2014) with mosquitoes being exposed to permethrin (43 µg/bottle) for 2 hours. Individuals were categorized by their survival status from the bottle bioassays; labeled as alive or dead at the 2-hour mark, placed in 2ml Eppendorf tubes, and stored at -80°C for subsequent testing. From these samples, 30 individuals from each population were randomly selected while maintaining an equal as possible ratio of dead and alive mosquitoes to give a total of 450 individuals.

Figure 1*Map of 15 Northern California Study Sites*

To prepare the mosquitoes for both molecular and enzymatic testing, the legs of each individual mosquito were separated from the remainder of the body using a clean microscope slide as a cutting surface and clean razor blade for each individual. An empty pipette tip box was filled with dry ice and used as a stand for the microscope slide to cut the mosquitoes on while keeping them as cold as possible. Legs, used for *kdr* detection, and bodies, used for enzyme assays, were put into their respective 2ml safe lock Eppendorf tubes (Hamburg, Germany) and stored on dry ice until used or returned to a -80°C freezer.

Molecular Detection: KDR

A GeneJET Genomic DNA purification kit (Vilnius, Lithuania) with modified manufacturer's protocols was used to extract DNA from the legs of the mosquitoes. Modifications included: (1) Two 3.5mm UFO stainless steel beads by Next Advance (Troy, New York) and a solution of 20 μ l Proteinase K and 180 μ l Digestion Solution were used to homogenize the legs of mosquitoes in a bead homogenizer (2) to concentrate the DNA, DNA was eluted in a spin column with only 75 μ l of elution buffer, incubated for 5 minutes, and centrifuged at 6,000g for 1.5 minutes. The flowthrough was collected and returned to the same spin column for repeated incubation and centrifugation. A quantitative polymerase chain reaction (qPCR) assay with melt curve analyses described by Hughes (2017) was used for allele detection. Allele-specific primers shown in Table 1 correspond to either the wildtype or one of the *kdr* alleles and were labeled with GC rich tails to separate the amplified products based on their melting temperature. The PCR reaction mixture consisted of 10 μ l SYBR Master Mix by Applied Biosystems (Vilnius, Lithuania), 0.2 μ l CxTkdrMC_R5 (10 μ M), 0.2 μ l CxTkdrMC_LeuF3 (10 μ M) leucine forward primer, 0.2 μ l CxTkdrMC_Ser4 (10 μ M) serine forward primer, 0.25 μ l CxTkdrMC_PheF2 (10 μ M) phenylalanine forward primer, 4.15 μ l nuclease free H₂O, and 5 μ l of template DNA. An Applied Biosystems StepOnePlus Real-Time PCR thermocycler was used. The thermocycler conditions were as follows: (1) 50°C for 2 minutes, (2) 95°C initial denaturation for 2 minutes, (3) 95°C denaturation for 15 seconds, (4) 61°C annealing and elongation for 1 minute. Steps 3-4 were repeated 40 times. The melt curve parameters were as follows: 95°C for 15 seconds, (6) 60°C for one minute, then temperature increased 0.2°C every 10 seconds until complete denaturation of the amplicons. Due to similarities between the leucine primer and serine template, this assay cannot accurately

distinguish between leucine/serine heterozygotes and serine homozygotes. Individuals with both a leucine and serine peak, as well as samples that had atypical melting temperatures, were sequenced to determine the genotype. Additionally, 4% of samples identified by the melt curve assay were sequenced for confirmation.

For sequencing confirmation, standard PCR was used to amplify the relevant region of the voltage-gated sodium channel gene. The reaction mixture consisted of 2.5 μ l 10X Buffer (Thermo Fisher Scientific), 2.5 μ l $MgCl_2$ (25 μ M), 0.5 μ l dNTP's (10 μ M), 0.25 μ l 8VGSCF1 forward primer, 0.25 μ l CxtVGSCR1 reverse primer (Choi, 2016; Zhou et al., 2009), 0.2 μ l BSA, 0.2 μ l Amplitaq Gold, 13.6 μ l of nuclease free H_2O , and 5 μ l template DNA. An Applied Biosystems ProFlex PCR thermocycler was used with the following conditions: (1) 94°C initial denaturation for 10 minutes, (2) 94°C denaturation for 45 seconds, (3) 55°C annealing for 45 seconds, (4) 72°C extension for 1 minute, (5) repeated steps 2-4 40 times, (6) 72°C final elongation for 7 minutes. Samples were sequenced by Quintara Biosciences (Berkeley, CA).

Enzyme Assays

The bodies of the mosquito samples, all parts except legs, were used to determine levels of detoxification enzymes. A modified protocol from Sac-Yolo Mosquito and Vector Control District was followed to determine the levels of α -esterase and β -esterase, P450 oxidases, glutathione-S-transferases, and acetylcholinesterase. Individual mosquito protein levels were measured to normalize for differences in mosquito size. Individual mosquito bodies were homogenized with two 3.5 mm UFO stainless steel beads by Next Advance (Troy, New York) in 2 ml of potassium phosphate buffer (8.3 g/L KPO_4) adjusted to a pH of 7.2. Homogenate (100 μ l) was loaded into 96 well microtiter plates in triplicate for each test, except protein in which 10 μ l was used.

For α -esterase, 100 μ l of α -naphthyl acetate solution (28 mg α -naphthyl acetate, 10 ml acetone, 40 ml KPO_4 buffer) was added to each well and incubated for 15 minutes. Then 100 μ l of 0-dianisidine solution (50 mg 0-dianisidine tetrazotized, 50 ml deionized water) was added and incubated for 2 minutes. An absorbance reading was taken at 540 nm using a BioTek Synergy microplate reader with Gen5 2.0 software. The same procedure was followed for β -esterase differing only in β -naphthyl acetate solution (28 mg β -naphthyl acetate, 10 ml acetone, 40 ml KPO_4 buffer) was used. Standard curves for both α/β esterase were constructed for concentration ranges of 0-100 μ g/ml of product.

For oxidase, 200 μ l of TMBZ (25 mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride, 12.5 ml methanol, 37.5 ml 0.25 M Sodium acetate buffer pH 5.0) was added to 100 μ l of mosquito homogenate and 25 μ l of 3% hydrogen peroxide and incubated for 5 minutes. An absorbance reading was taken at 620 nm. The standard curve ranges for oxidase were 0-2 μ g/ml of product.

For glutathione-S-transferase, 100 μ l of CDNB (10 mg 1-chloro-2,4-dinitrobenzene, 5 ml acetone, 45 ml KPO_4 buffer) and 100 μ l of reduced glutathione (30.5 mg reduced glutathione, 50 ml KPO_4 buffer) were added to 100 μ l of mosquito homogenate and incubated for 5 minutes. Absorbance was measured at 340 nm. The standard curve ranges for GST were 0-8 μ g/ml of enzyme.

Two assays were done for acetylcholinesterase. The first was used to measure the amount of acetylcholinesterase present, where 100 μ l of ATCH (37.5 mg Acetylthiocholine iodine, 5 ml acetone, 45 ml KPO_4 buffer) and 100 μ l DTNB (6.5 mg Dithio-bis-2-nitrobenzoic acid, 50 ml KPO_4 buffer) are added to 100 μ l of mosquito homogenate and incubated for 20 minutes. An absorbance reading was taken at 414 nm. The standard curve ranges were 0-0.25

$\mu\text{g/ml}$ of enzyme. The second assay was used to detect the presence of insensitive acetylcholinesterase, which is an altered form of the enzyme with a decreased sensitivity to organophosphate inhibition (Devonshire and Moores, 1984). This assay followed a similar procedure with the exception that 10.5 mg propoxur (Baygon) was added to ATCH and absorbance readings were taken immediately following the addition of DTNB (T_0) and after a 24-hour incubation at 4°C (T_{24}). The (T_0) was subtracted from (T_{24}) reading and after the protein concentration was applied the insensitive acetylcholinesterase test was used as a presence/absence test and therefore no standard curve was applied.

For quantification of total protein concentration, 200 μl of Protein dye (10 ml Bio-Rad Protein Assay Dye Reagent diluted with 40 ml deionized water) was added to 10 μl of mosquito homogenate and incubated for 5 minutes. An absorbance reading was taken at 595 nm. Standard curve ranges were 0-0.5 $\mu\text{g/ml}$ of bovine serum albumin (BSA).

Statistics

For molecular detection, the genotypes of all individuals were categorized, and a comparison of proportions was used to compare the wildtype allele frequencies between alive and dead individuals. The quantity of the most common *kdr* allele, phenylalanine, was compared to survivorship using logistic regression. Lastly, a comparison of survival rates between different genotypes was performed using chi-squared tests. The significant groups which consisted of the homozygous genotypes were further analyzed with additional chi-squared tests.

For enzyme analysis, box and whisker plots were created for Alpha (α -esterase), Beta (β -esterase), GST, Oxidase, and Ace respectively to compare alive and dead individuals. To determine if there was a significant difference between enzymatic value means and medians of alive and dead individuals, Two-sampled t-tests and Wilcoxon-tests were used.

To relate *kdr* status and enzyme levels as a predictor of survival, a Classification and Regression Tree (CART) was created. Backward stepwise selection on an Akaike Information Criterion model was first used to select the variables that showed the most significance from all enzyme/*kdr* variables to create an optimal logistic model. Since the number of phenylalanine alleles and amount of GST were independently important for survivorship from logistic regression analysis and Two-sampled t-test (see results section), these two parameters were used in the CART model.

Results

Molecular Detection: KDR

Of the 450 individuals tested, 342 were homozygous for phenylalanine (F) at the *kdr* locus. The second most observed genotype was phenylalanine/serine heterozygous (FS) with 75 individuals. The remaining genotypes were as follows: 15 leucine homozygotes (LL), 12 serine homozygotes (SS), 4 phenylalanine/leucine heterozygotes (FL), and 2 leucine/serine heterozygotes (LS) (Table 2). When comparing the allele frequencies between alive and dead individuals, the phenylalanine allele comprised 94% of the total alleles found in the alive individuals compared to 75% in the total alleles for dead individuals. The proportion of S alleles was 6% of the total alleles in alive individuals compared to 17% of the total alleles in dead individuals. When comparing the proportions of alive and dead individuals, the susceptible allele L, varied significantly between groups with a normal distribution z-score 5.98, p-value 0.001 (Figure 2). The number of F alleles an individual possessed was compared to observed survival using logistic regression analysis. From the individuals tested, those without an F allele had a survival rate of just 7%, while those with one F allele had a 34% survival rate, and those with two F alleles had a 61% survival rate. When compared to individuals without an F allele,

those with one and two F alleles displayed a significantly better chance of survival F(n=1) p-value 0.01 and F(n=2) p-value 0.001 (Figure 3). A chi-squared test was used to compare survival of homozygous genotypes LL, SS, and FF. When comparing FF individuals to LL individuals, survival was significantly greater for phenylalanine homozygotes (LL survival rate 7% and FF survival rate 61%, $\chi^2 = 22.8$, p-value 0.0005). When comparing FF individuals to SS individuals, survival was again significantly greater for phenylalanine homozygotes (SS survival rate 8% and FF survival rate 61%, $\chi^2 = 26.8$, p-value 0.0015) (Figure 4).

Enzyme Assays

When comparing detoxification enzyme levels between alive and dead individuals, both the Two-sampled t-test and the Wilcoxon-test for GST showed a significant difference in mean and median values, p-values: 0.029 and 0.018 respectively, between alive and dead individuals. While not statistically significant, β -esterase displayed a greater difference between means and medians of alive vs. dead individuals, with p-values of 0.066 and 0.075, compared to the remaining enzymes tested (Figure 5). There was no significant difference for the other enzymes tested. In all enzymes tested, alive individuals on average seemed to have more high outliers compared to their dead counterparts for each respective enzyme. This was not the case with oxidase however, as both dead and alive individuals were similar (Figure 6).

The CART model was used as a tool to relate both molecular and enzymatic resistance variables together to predict survivorship. Backward stepwise selection on a Akaike Information Criterion model was used to select the variables that showed the most significance. The number of F alleles and the value of GST enzyme were the two most significant variables. The greater number of F alleles an individual possessed, and a high GST level corresponded to increased survival, as 76% of the individuals with two F alleles and a GST level above 0.052 $\mu\text{g/ml}$

survived. Comparatively, 57% of the individuals with two F alleles and GST below 0.052 $\mu\text{g/ml}$ survived. Survival decreased in individuals that had only one or zero F alleles with only 36% and 7% survival respectively (Figure 7.)

Discussion

Molecular Detection: KDR

In this study, there were individuals that comprised every genotypic combination for the *kdr* target-site, however the vast majority were homozygous for the phenylalanine (F) allele. This was consistent with previous studies on *Cx. tarsalis* where the most common *kdr* allele associated with pyrethroid resistance was phenylalanine (Choi, 2016; Hughes, 2017). For this study, having a phenylalanine allele did not automatically confer resistance as 152 individuals that contained one or two F alleles died in the bottle bioassays. Rather, it was the number of phenylalanine alleles an individual possessed that factored more in determining resistance status (Figure 3). These results make sense in comparison to another study, where lab-reared *An. gambiae* were introduced with the L1014F mutation with CRISPR/CAS9 (Grigoraki et al., 2021). This study found that individuals containing two F alleles showed resistance to pyrethroids, while individuals with only one F allele, displaying a F/L genotype, did not show resistance to WHO discriminating doses of pyrethroids. Interestingly, this study also found that carrying both F alleles resulted in a fitness disadvantage with individuals having increased mortality during larval stages, reduced fecundity, and decreased adult lifespan (Grigoraki et al., 2021). These disadvantages may be a selective factor for why other resistance alleles are seen at position 1014 or why other resistance mechanisms exist.

Having the susceptible L allele was also important for survival status. All heterozygous individuals containing an L allele died during bottle bioassays, and all but one homozygous

susceptible individual containing two L alleles died as well. There could be many possible reasons for the survival of the one homozygous susceptible individual. This could be attributed to an elevated GST level (0.046 μg GST/ μg protein) that this individual had, other resistance mechanisms not tested in this study, or even by chance. Hunt et al. in 2005 suggested that factors such as age, time of blood meals, and mating can all influence the survival of female *Anopheles funestus* exposed to pyrethroids. Grigoraki et al. saw a similar pattern in their study where individuals that possessed a L allele in the genotype were susceptible to pyrethroids. Previous work on *Cx. tarsalis* by Hughes in 2017 also did not document any resistant individuals containing an L allele.

Due to the low sample size of individuals containing an S allele, it is hard to draw conclusions on its importance in pyrethroid resistance in *Cx. tarsalis* from this study. From the individuals tested in this study, it seemed the S allele played a lesser role in contributing to resistance. This is consistent with Hughes in 2017, where *Cx. tarsalis* with the L1014S mutation showed less resistance to pyrethroids compared to individuals with the L1014F mutation. Both this study and Hughes in 2017 also found the L1014S mutation to be less common in wild populations of *Cx. tarsalis* in Northern California with a frequency of just 11% and 13% respectively. The low frequency of L1014S mutants in Northern California *Cx. tarsalis* populations may be indicative of its lesser role or the mutation may confer resistance in the field but not to CDC discriminating doses found in bottle bioassays. This has similarly been suggested by Reimer et al. in 2008: while *kdr* mutations alone (L1014S and L1014F) in *An. gambiae* may not resist doses of pyrethroids in WHO bottle bioassays, they may offer resistance to lesser doses mosquitoes encounter in their environment.

Enzyme Assays

In this study GST was the only detoxification enzyme that was statistically different between alive and dead groups. This class of enzyme has been known to metabolize a wide range of insecticide classes, including pyrethroids (Hemingway et al., 2004). Previous work on *Cx. tarsalis* has shown elevated levels of GST to be associated with resistance to pyrethroids (Hughes, 2017; Strong et al., 2008). Importantly, this study found a relationship between elevated levels of GST and the *kdr* allele F. Individuals that contained both F alleles and a GST level above 0.052 $\mu\text{g GST}/\mu\text{g protein}$ showed increased survival. Similarly, *An. gambiae* with L1014F in conjunction with overexpression of glutathione-S-transferase, *Gste2*, increased resistance to permethrin even further than either mechanism alone (Grigoraki et al., 2021). While GSTs facilitate excretion of insecticides through enhanced solubility, further research is looking at their potential role in mediating oxidative stress response in insects as a mechanism of resistance (Enayati et al., 2005). Interestingly, GSTs vary throughout the life stages of mosquitoes. In *Aedes aegypti*, for example, GST levels decline as adult mosquitoes age (Hazelton and Laing, 1983). Since this study is composed of mixed-age mosquitoes, further study could be useful in determining the influence of age on GST activity and resistance in *Cx. tarsalis*.

β -esterase levels were higher in individuals that survived the bottle bioassay, though it was not significant. The p-values were very close to the 95% confidence intervals (Two-sampled T-test p-value=0.066 and Wilcoxon-test p-value=0.075). It is not completely clear whether β -esterase is impacting survival. Previous work on *Cx. tarsalis* has shown no correlation of esterase-mediated resistance (Hughes, 2017). However, in 1987, Ziegler et al. showed *Cx. tarsalis* larvae displayed non-elevated metabolically enhanced esterases that were

capable of metabolizing malathion. While this resistance mechanism exists for organophosphate insecticides, the same could be true for pyrethroids as both contain ester bonds. However, the enzymatic testing done in this study detected quantitative changes in nonspecific esterases and therefore would not detect any esterases that may have higher metabolic activities.

It was interesting that all enzymes except oxidase had the general trend of alive groups having a few individuals with very high levels of enzymes compared to the dead groups. This was surprising because oxidase is generally the enzyme class most associated with pyrethroid resistance in other mosquito species (Scott et al., 2014). In this study, the role of oxidase remains inconclusive as no correlation was found between the level of oxidases and resistance status from our data. Also, we currently don't have a good reference of baseline oxidase levels in wild *Cx. tarsalis* from this area. Others have documented increased mortality in bottle bioassays with the addition of Piperonyl butoxide (PBO), a synergist added to insecticides to inhibit oxidases (De La Vega and Ryan, 2022). Further studies on *Cx. tarsalis* have linked oxidases to pyrethroid resistance (Hughes, 2017; Strong et al., 2008). There may be other variables affecting oxidase-mediated pyrethroid resistance in *Cx. tarsalis* which provide possible explanations for the discrepancies seen between this study and aforementioned ones. Since oxidases are a large family of enzymes responsible for catabolizing many substances by introducing oxygen atoms to create polar products ready for excretion from the body, they are mainly found in the digestive system (Panini et al., 2016). Oxidase levels could vary between individuals due to differences in the midgut. In 2019, Machani et al. showed that younger-aged females and females that were blood fed had significantly increased oxidase levels that contributed to pyrethroid resistance in *An. gambiae*. In 2021, Muturi et al. showed permethrin resistant *Aedes aegypti* to have higher gut microbial richness compared to susceptible individuals

and in 2008, Spilling et al. demonstrated that blood fed *An. funestus* displayed pyrethroid resistance compared to their unfed counterparts. Since the samples used in this study were wild, mixed-aged females stored at -80°C, blood meal consumption, age, and the effect of freezing on microbial communities in the midgut are all variables that may contribute to the different oxidase levels seen in this study.

It is clear that one mechanism alone is likely not enough to confer resistance to pyrethroids. There are many enzymes responsible for the detoxification of insecticides coupled with many molecular mutations at different loci. While it is difficult to collectively relate these independent variables to an individual's ability to resist pyrethroid insecticides, it is likely a combination of mechanisms that contribute to resistance seen in *Cx. tarsalis* in Northern California.

CHAPTER 3: ORGANOPHOSPHATE PRELIMINARY STUDY

Introduction

While pyrethroid insecticides are widely used in Northern California for adult mosquito population control, in recent years there has been documentation of *Cx. tarsalis* populations from this geographic region displaying resistance to this class of insecticide (Choi, 2016; Hughes, 2017). Continual reliance on pyrethroid insecticides places selective pressure on this species. Organophosphates are the only other class of insecticide apart from pyrethroids allowed for use in California (Vector-Borne Disease Section, 2008).

Organophosphates act on insect nervous systems by binding to and inhibiting acetylcholinesterase (ACE), the enzyme responsible for the breakdown of the neurotransmitter, acetylcholine, across nerve synapses which terminate nerve signals. The inhibition of ACE results in mosquitoes with uncontrolled nerve signaling that causes knockdown, physical exhaustion, and death (Hemingway and Ranson, 2000). Studies in other mosquito species demonstrated that a mutation to the gene encoding ACE, known as the *ace-1*, confers resistance to organophosphates (Weill et al., 2003). In *An. gambiae* the *ace-1* mutation is encoded by a single nucleotide polymorphism at position 119 where the codon GGC (glycine) changes to AGC (serine). *Culex tarsalis* is genetically different at this locus where GGA (glycine) is the wildtype codon. In order for *Cx. tarsalis* to obtain the *ace-1* mutation two single nucleotide polymorphisms must occur changing either the first and third position or the first and second position of the codon to create the amino acid serine (Weill et al., 2003). The *ace-1* target-site mutation coupled with enzymatic detoxification from α/β esterases, oxidases, and GSTs could be

playing a role in any possible resistance in *Cx. tarsalis* (Mouches et al., 1987; Panini et al., 2016; Hemingway et al., 1991).

According to the Environmental Protection Agency and California Department of Public Health, naled, a highly effective organophosphate, is occasionally used to quickly control adult populations throughout the state (2022). Since this class of insecticide is currently being used in Northern California, this study provides preliminary data to get an idea of current organophosphate resistance in *Cx. tarsalis* populations and possible underlying mechanisms that may be contributing to resistance.

Methods

Study Methodology

The purpose of this study was to get a baseline of organophosphate resistance in Northern California populations for *Cx. tarsalis*. This study was designed to have two parts: a population level study and an individual level study.

The population level study was designed to look broadly at 6 populations of *Cx. tarsalis* from Northern California to detect any measurable levels of resistance to organophosphates. Individuals from these populations were simply collected and tested for the target-site mutation: *ace-1* and tested for levels of detoxification enzymes. All individuals in the population study were frozen individually directly from the field and did not go into CDC bottle bioassays, therefore having no prior exposure to insecticides in the laboratory or the stress of the bottle bioassay itself. We hoped this would replicate detoxification enzyme levels more similar to what *Cx. tarsalis* experiences in the natural environment.

The individual study was designed to identify possible underlying mechanisms of resistance in the event that organophosphate resistance was seen among any individuals. This

study consisted of individuals from each population that underwent CDC bottle bioassays and showed phenotypic resistance to organophosphates. Any individuals from a population that showed resistance by surviving the 2-hour timepoint in the bottle bioassay were collected and compared to an equal number of susceptible individuals from the same population to identify any differences between levels of detoxification enzymes or *ace-1* mutation status.

Collections and Sample Preparations

Collaborating vector control districts: Sacramento-Yolo, Placer County, and San Joaquin County collected mixed-age female mosquitoes from 6 Northern California study sites using CO₂ baited traps during the summer of 2020. Study sites were selected by the districts as areas of concern due to possible exposure to organophosphates. A portion of the collected individuals at each study site underwent CDC bottle bioassays with exposure to 22 µg (Sacramento-Yolo) or 25 µg (San Joaquin and Placer County) for 2 hours. These individuals were categorized by their survival status labeled as dead or alive at the two-hour mark, placed individually in 2 ml Eppendorf tubes (Hamburg, Germany), and stored at -80°C for subsequent testing. All survivors from each study site as well as an equal number of dead individuals from each site were used to give a total of 28 individuals, which will be referred to as the ACE individual study. The remaining collected individuals were left untreated, and 30 individuals were selected at random from each of the six study sites and placed in 2 ml Eppendorf tubes and stored at -80°C for subsequent testing. These untreated individuals will be referred to as the ACE population study.

The preparation of mosquitoes followed the same protocol as Chapter 2. Pyrethroid Individual Study: legs of the mosquitoes were separated and used for molecular detection of *ace-1* and the bodies were used for enzyme assays.

Bottle Bioassays

CDC bottle bioassays were performed by personnel at the individual vector control districts where mosquito collections were made. Approximately 20 mosquitoes were put into 3 separate 250 ml glass bottles evenly coated with 25 μg naled dissolved in acetone, except for Natomas and Brannan Island, which used 22 μg naled. The bottles are set to dry completely before the addition of any mosquitoes. To serve as a control, an equal number of mosquitoes are placed into 250 ml glass bottles coated with acetone only and allowed to dry/dissipate. Mortality was recorded at 15 minutes intervals until 120 minutes, at which mosquitoes are separated based on survival status. All populations are compared to susceptible lab-reared populations that underwent the same bottle bioassay procedure: Bakersfield laboratory colony (BFS) for Empire and Wright populations or Kern National Wildlife Refuge laboratory colony (KNWR) for the remaining populations.

Molecular Detection: ace-1

Extraction of DNA followed the same methods outlined in Chapter 2. Pyrethroid Individual Study: *Molecular Detection: KDR* apart from the elution step which used 100 μl of elution buffer instead of 75 μl . PCR was used to amplify the approximate 300 base pair region of the *ace-1* gene. The PCR reaction mixture followed Choi, 2016 and consisted of 2.5 μl 10X Buffer (Thermo Fisher Scientific), 2.5 μl MgCl_2 (25 μM), 0.5 μl dNTP's, 0.25 μl ACE300 forward primer (20 μM), 0.25 μl ACE300 reverse primer (20 μM)(Choi, 2016; Weill et al., 2004b), 0.1 μl Amplitaq, 5 μl of template DNA, and 13.9 μl of nuclease free H_2O . An Applied Biosystems ProFlex PCR thermocycler was used with the following conditions: (1) 94 $^\circ\text{C}$ initial denaturation for 5 minutes, (2) 94 $^\circ\text{C}$ for 30 seconds, (3) 59 $^\circ\text{C}$ for 15 seconds, (4) 72 $^\circ\text{C}$ for 20

seconds, repeat steps 2-4 30 times, (5) 72°C for 5 minutes. All samples were sequenced by Quintara Biosciences (Berkeley, CA).

Enzyme Assays

The bodies of the mosquitoes were used to determine levels of detoxifying enzymes and followed the same methods as previously described in Chapter 2. Pyrethroid Individual Study:

Enzyme Assays.

Statistics

For the ACE individual study, a Kruskal-Wallis test was used to determine if there were significant differences among the median levels of enzymes when comparing alive and dead individuals. For the ACE population study, Kruskal-Wallis in conjunction with post hoc Dunn's test was used to determine if there were significant differences between median enzyme levels among each of the 6 populations. The results were represented with compact letter displays (CLD's) that denoted which populations were significant from one another.

Results

Bottle Bioassays

The results of the CDC bottle Bioassays are displayed in (Figure 8). For Sacramento-Yolo County, the susceptible colony KNWR reached 100% mortality at 60 minutes. Interestingly, the Brannan Island population reached 99% mortality at 120 minutes. For Placer County, the susceptible colony KNWR reached 100% mortality at 75 minutes. Both the Locust and Phillip populations reached 100% mortality at 75 minutes and 120 minutes respectively. For San Joaquin County, the BFS susceptible colony only reached 98% mortality at 120 minutes. There may have been an issue with this bottle bioassay as this population should have reached 100% mortality. The Empire population reached 96% mortality at 120 minutes, while Wright

reached 85% mortality at 120 minutes with only 23% mortality at 90 minutes. From the individuals in the 6 populations tested from Northern California, 97% were susceptible to naled while only 3% showed resistance (Figure 9).

Molecular Detection: ace-1

From both the ACE individual and population study, no *ace-1* mutations were found in the 208 individuals tested. However, three individuals, two from the population study individuals from Natomas (N3 and N28) and one from the individual study Wright (NW14) did have a single nucleotide polymorphism (SNP) at the 119 codon (Table 4). All three SNPs occurred at the third position in the codon which did not change the amino acid from glycine GGA to serine AGC.

Enzyme Assays

For ACE individual study, there was no significant difference in levels of detoxifying enzymes between alive and dead individuals found (Figure 10). Due to the low sample size of individuals tested it is hard to determine significance, but the range of acetylcholinesterase levels in the alive group was greater than the dead group.

For the ACE population study, levels of α -esterase, oxidase, GST, and ACE varied significantly among the populations tested while β -esterase showed only minor differences in median enzyme values among the populations (Figure 11). Some general trends observed were that Brannan Island and Empire populations typically had the lowest median values and ranges for all enzymes tested, while the Locust and Phillip populations had higher median values and ranges for all enzymes tested. Phillip in particular had individuals with both the highest recorded values as well as outliers in all enzymes tested, except GST, even though Phillip still had the greatest range for this enzyme.

Discussion

Bottle Bioassays

From the 6 Northern California populations of *Cx. tarsalis* tested in this study, there is currently little resistance (3%) to the organophosphate naled (Figure 8). This was consistent with previous work where *Cx. tarsalis* from Sutter county showed little resistance to naled (Hughes, 2017). From a vector control standpoint, little change in naled resistance in Northern California *Cx. tarsalis* populations from 2016 to 2020 is encouraging. With new grant funding for the Thiemann Lab, this will be assessed again around 2025.

Molecular Detection: ace-1

Even though there were no *ace-1* mutations observed in the 208 individuals tested in this study, there were some individuals with SNPs resulting in silent mutations at the *ace-1* locus. In the Natomas individual (N3), the SNP resulted in a genotype that was homozygous for GGC and would therefore only need one SNP at the first codon position with adenine to have the G119S mutation. It is possible that others like this exist in the population and could be a risk for future resistance. The *ace-1* locus is a highly conserved region among all insects, in mosquito species such as *Cx. pipiens* and *An. gambiae*; individuals with the *ace-1* mutation experience severe fitness disadvantages, which is likely a reason for the low frequency seen in nature (Weill et al., 2004a). Interestingly, it has been reported that gene duplication of *ace-1*, which combines a susceptible and resistant copy of the *ace-1* gene on the same chromosome, has occurred independently in both *Cx. pipiens* and *An. gambiae*, and offers individual's resistance to organophosphates without the fitness disadvantage (Labbé et al., 2007; Djogbénou et al., 2008). While this has not been documented thus far in *Cx. tarsalis*, it may provide a potential mechanism of resistance if organophosphate selection pressure increases in the future.

Enzyme Assays

Due to the low sample size in the ACE individual study, it is hard to draw conclusions based on the results of this study. The low sample size was a result of low survival among the populations which means there is little organophosphate resistance in the Northern California populations tested. The enzyme acetylcholinesterase, though not significant, seemed to have a wider range of enzyme levels among the alive group. Since organophosphates target the enzyme acetylcholinesterase and increased level of activity in the alive individuals could indicate a potential role in their survivorship and should be watched in future studies moving forward. Quantitative increases in acetylcholinesterase have been documented in organophosphate resistant *Drosophila melanogaster* (Fournier et al., 1992). Previously, naled resistant *Cx. tarsalis* had increased levels of acetylcholinesterase compared to semi-resistant and susceptible individuals (Hughes, 2017).

The ACE population study showed variation among the populations for each enzyme. Wright was clearly the population with the most potential naled resistance from the bottle bioassays. However, the enzyme assay results for this population do not suggest the detoxification enzymes we tested are playing a role in resistance for these individuals. Rather, other mechanisms not accounted for in this study may be contributing to the resistance seen in this population. Interestingly, both Phillip and Locust were the populations with comparatively higher levels of each enzyme even though no resistance was seen in either population. This could be attributed to geographical differences as these populations are both from Placer County and could just be a result of higher enzyme levels for this particular location. In mosquitoes, α/β -esterases, oxidases, and GSTs are responsible for many physiological processes other than detoxification of xenobiotics.

The goal of this study was to get a better understanding of current organophosphate resistance in Northern California *Cx. tarsalis*. Since we only found a small amount of isolated resistance in San Joaquin County, it is hard to outline potential mechanisms for organophosphate resistance in *Cx. tarsalis*. Both nucleotide substitutions around the *ace-1* target-site and levels of acetylcholinesterase could be two potential mechanisms to look into moving forward. For future work on *Cx. tarsalis*, looking at the potential mechanisms presented in this study or others may give insight to organophosphate resistance in this species.

Table 1

List of Primers and Sequences for kdr qPCR and PCR.

Sequence Name	Target site	Sequence	Melt Temp
8VGSCF1	VGSC	5' TGGA ACTTC ACCGACTTC 3'	
CxtVGSCR1	VGSC	5'CTGGAGATTGCTATAGCGATG 3'	
CxTkdrMCLeuF3	KDR	5' GGGGCGGGGC ACCGTAGTGATAGGA AACTTA 3'	78.5 - 81.3 °C
CxTkdrMCPheF2	KDR	5'GGCACCGTAGTGATAGGAACTTT 3'	76.7 -78.0 °C
CxTkdrMCSerF4	KDR	5' GGGCGAGGCGGGCGGGGGGGCGC GGGCGAGGGC ACCGTAGTGATAGGAA ACTC 3'	83.1 - 84.2 °C
CxTkdrMCR5	KDR	5' TACAGACTCCTACCTCCGGA 3'	

Table 2

kdr Genotypes of Alive and Dead Individuals Exposed to Pyrethroids.

kdr Genotypes of Alive and Dead Individuals							
	FF (n=342)	FS (n=75)	FL (n=4)	LL (n=15)	LS (n=2)	SS (n=12)	Totals (n=450)
Alive	208 (46.2%)	27 (6%)	0 (0%)	1 (0.2%)	0 (0%)	1 (0.2%)	237 (52.7%)
Dead	134 (29.8%)	48 (10.7%)	4 (.9%)	14 (3.1%)	2 (0.4%)	11 (2.4%)	213 (47.3%)

Shown is the number of individuals of each genotype and in parenthesis is the percentage of the total individuals tested.

Table 3*List of Primers and Sequences for ace PCR*

Sequence Name	Target site	Sequence
Ace300F1	Ace-1	5' ARATGGACSGGWGTGCTGAACG3'
Ace300R1	Ace-1	5' CGRTACTGCAGYGAAACYACG 3'

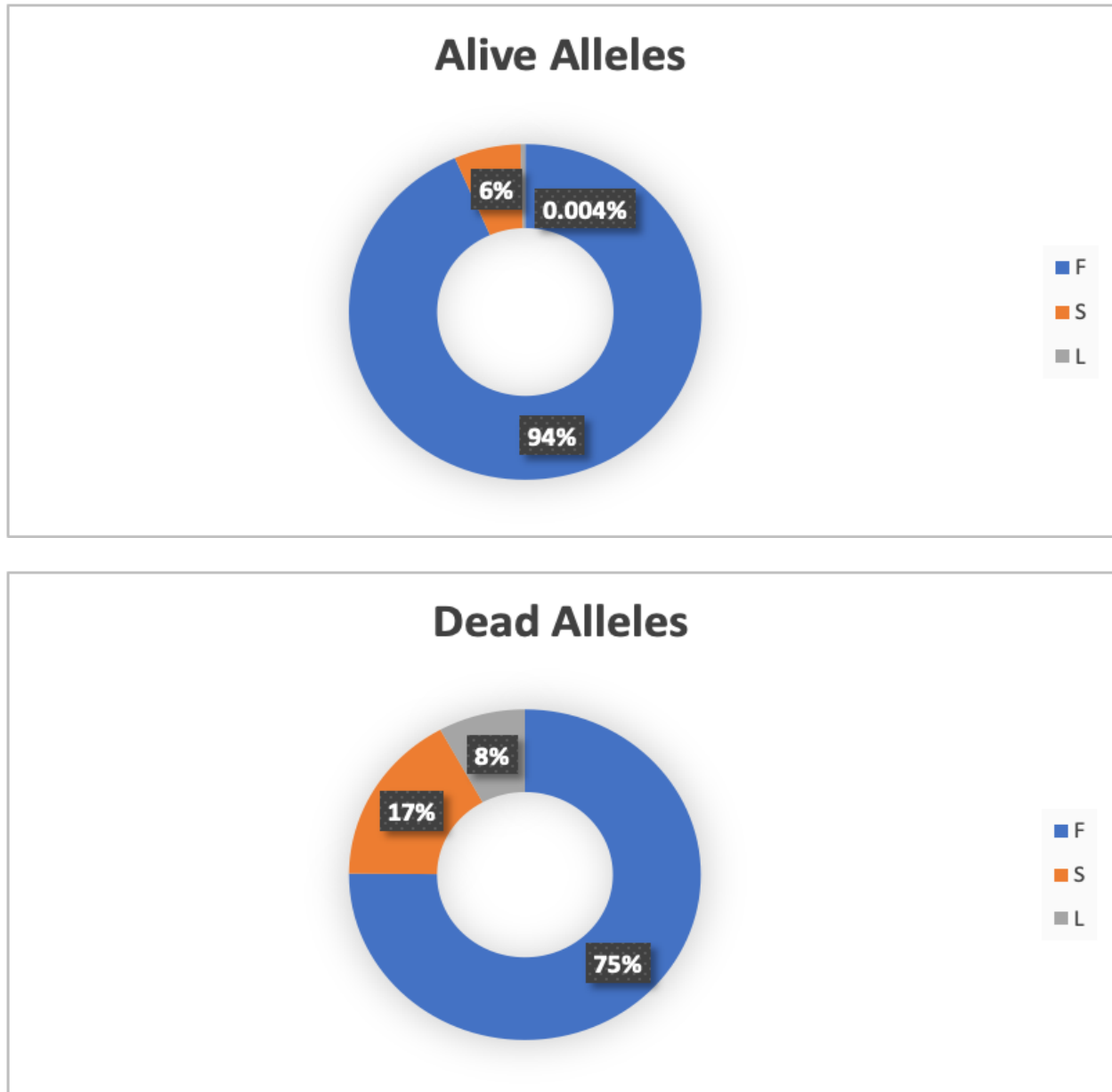
Table 4*Sequencing Results for ace-1 Mutation*

>N30_WT	GCCGCCGTCATGCTGTGGATCTTCGGGGGCGGATTCTACTCCGGAA
>N3	GCCGCCGTCATGCTGTGGATCTTTGGGGGTGGCTTCTACTCCGGGA
>NW14	GCCGCCGTCATGCTGTGGATCTTCGGGGGTGGA/CTTCTACTCCGGAA
>N28	GCCGCCGTCATGCTTTGGATCTTCGGGGGTGGA/CTTCTACTCCGGAA

Shown are segments of the *ace-1* locus from sampled individuals. N30-WT denotes the wildtype sequence. N3, NW14, N28 are individuals in the study that possessed single nucleotide polymorphisms (SNPs) at the *ace-1* locus (highlighted in green). Yellow highlights denote SNPs at nearby base pairs. All SNPs resulted in silent mutations.

Figure 2

Comparison of Allele Frequencies between Alive and Dead Individuals



A comparison of the proportions of L alleles between alive and dead groups using a standard normal distribution gave a Z score value of 5.98. P-value 0.001.

Number of alive alleles: F=443, S=29, L=2

Number of dead alleles: F=320, S=72, L=32

Figure 3

Comparison of the Number of F Alleles between Alive and Dead Individuals



When compared to F(n=0); F(n=1) and F(n=2) significantly differed in logistic regression.

F(n=1) p-value 0.01. F(n=2) p-value 0.001.

Figure 4

Comparison of Survival Among Homozygotes and Heterozygotes of Different Allele

Combinations

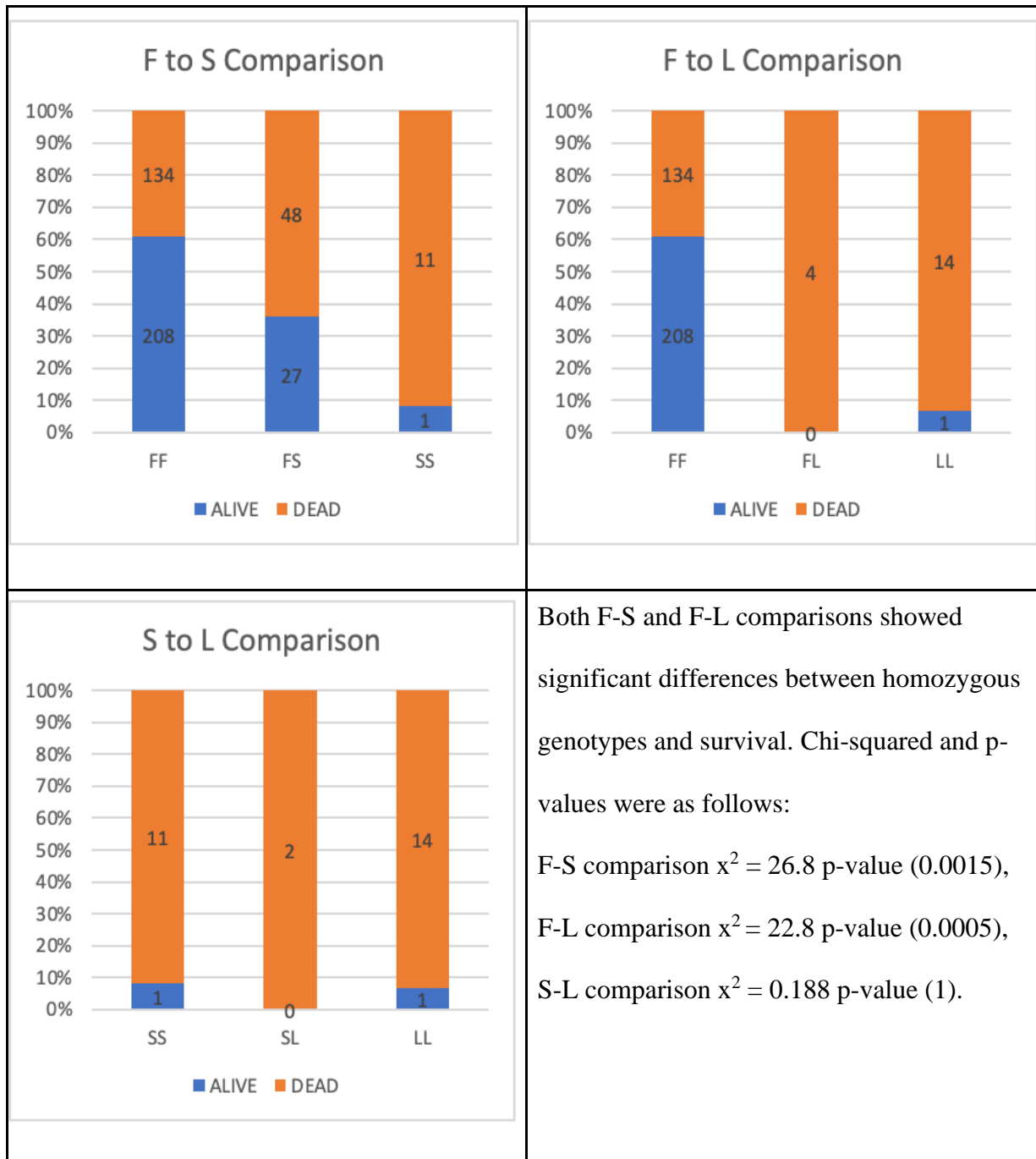
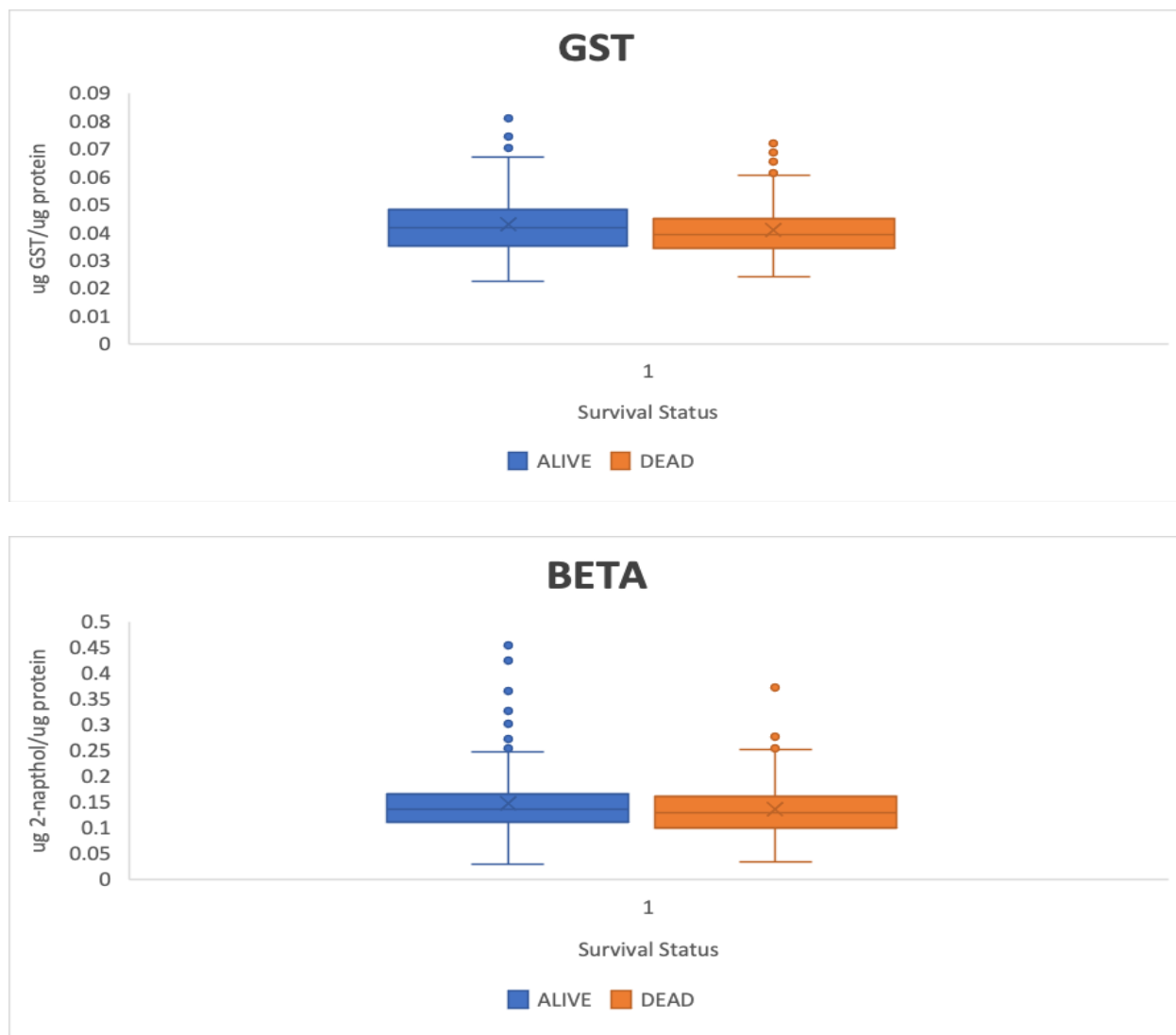


Figure 5

Box and Whisker Plots for Permethrin Exposed Individuals: GST and BETA



Box and Whisker plots showing differences in detoxification enzymes between alive and dead individuals. Two-sample t-tests p values: GST 0.029 and Beta 0.066. Wilcoxon-test p-values: GST 0.018 and Beta 0.075. GST showed significant difference in both mean and median values between alive and dead individuals. Though not significant, Beta showed greater difference between mean and median values as compared to other enzymes tested.

Number of individuals tested: GST: A=234 D=212 Beta: A=227 D=211

Figure 6

Box and Whisker Plots for Permethrin Exposed Individuals: ALPHA, OXIDASE, ACE

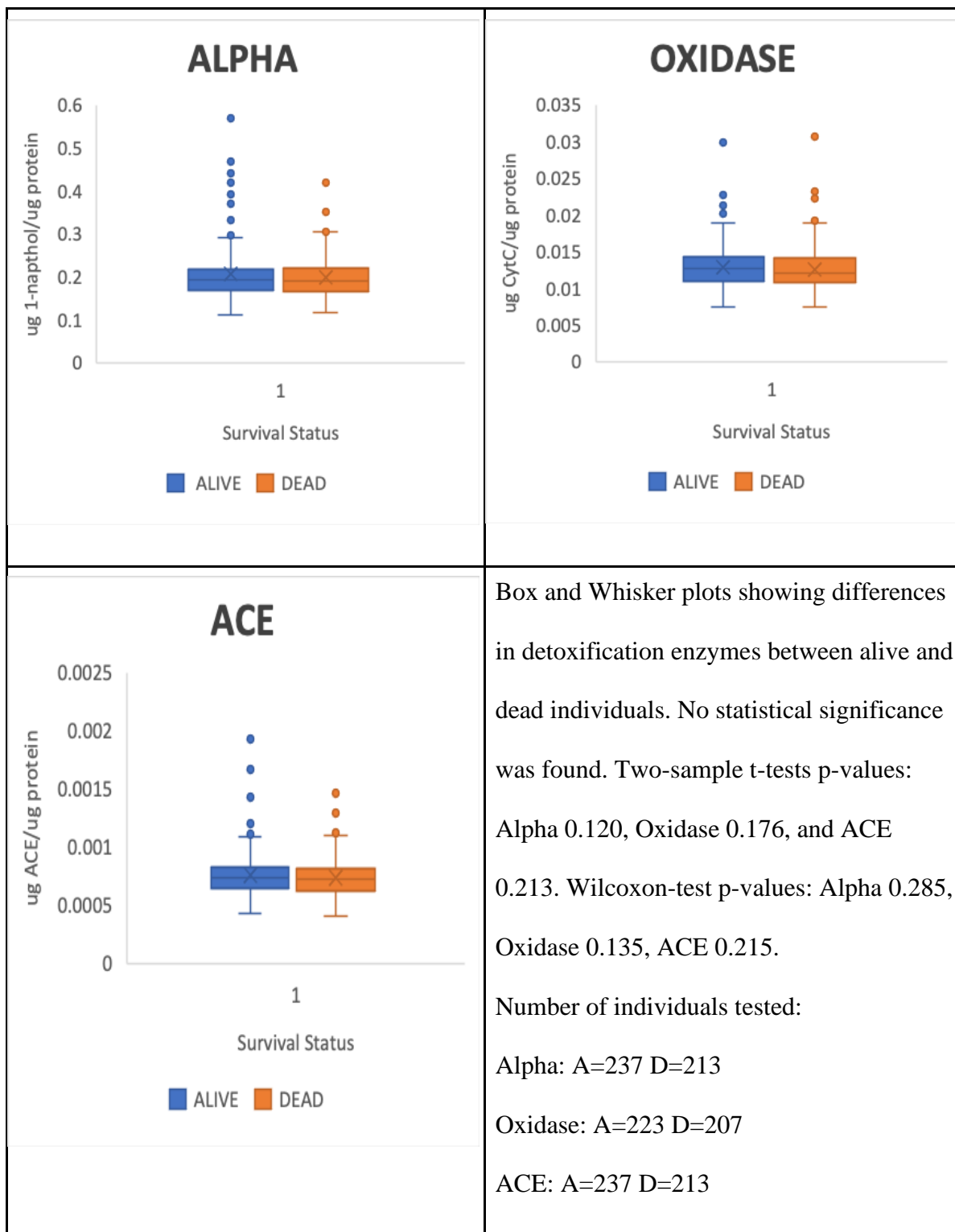
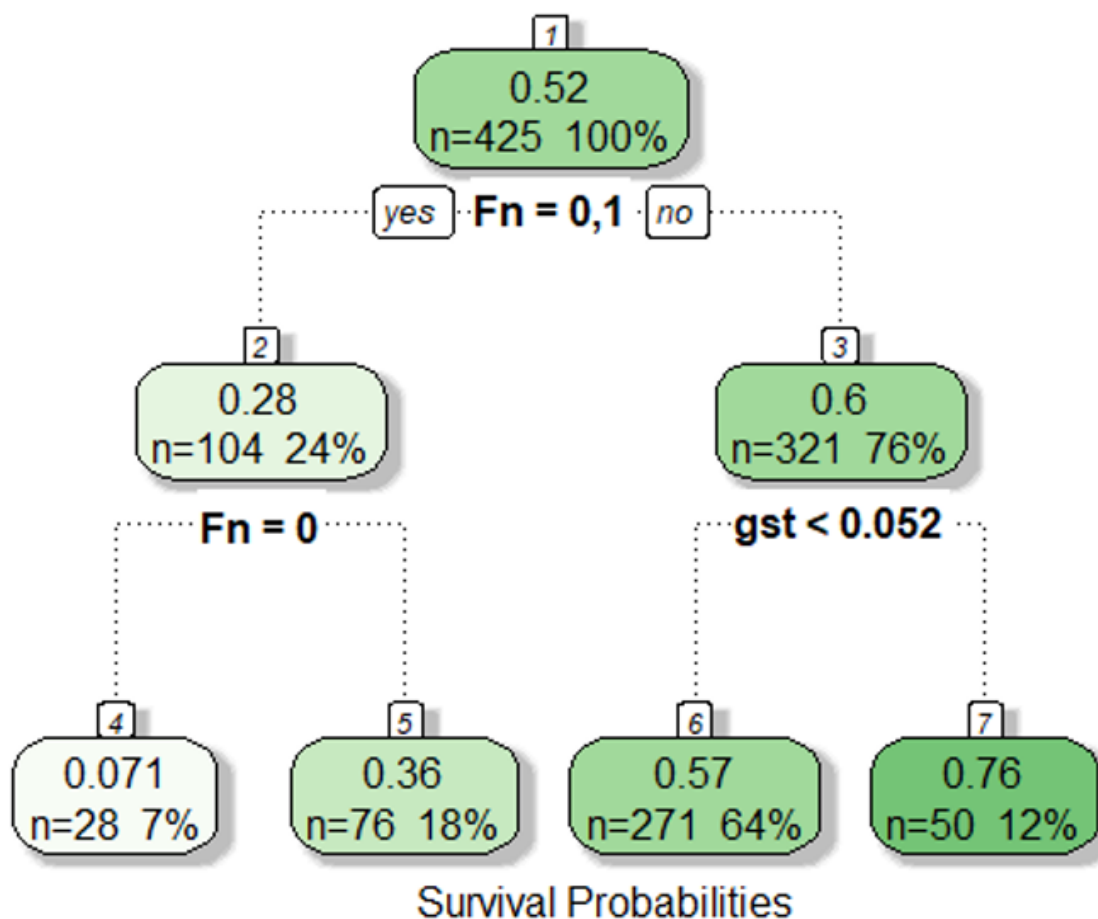
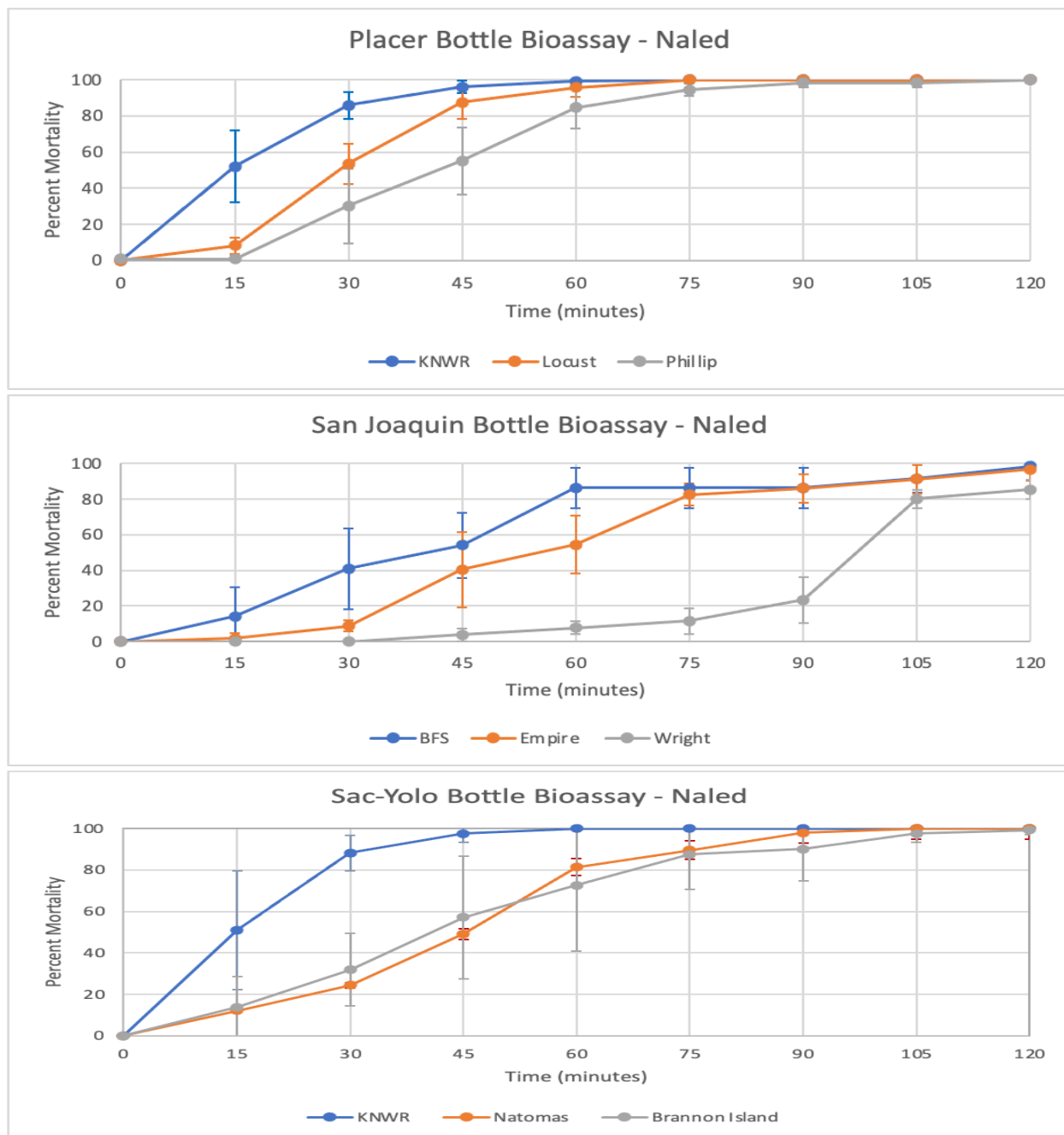


Figure 7

CART Model of Survival Probabilities for Permethrin Exposed Individuals



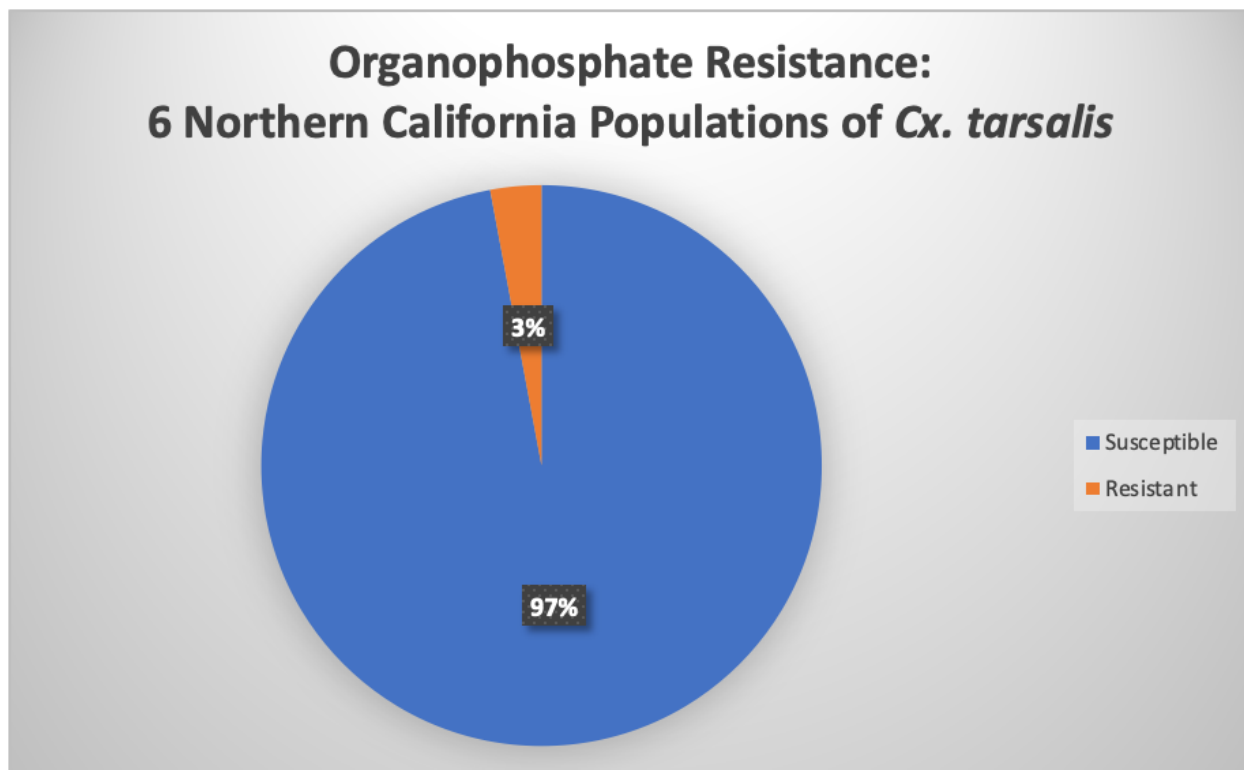
CART model showing survival probabilities in relation to number of F alleles and amount of GST present for Permethrin exposed individuals. Backwards stepwise selection on Akaike Information Criterion (AIC) model was used to select the two variables with most significance. Each node displays: survival rate for the group, number of individuals, percentage of total individuals.

Figure 8*Naled Bottle Bioassay Results*

Bottle Bioassays for the 6 Northern California populations of *Cx. tarsalis* during the summer of 2020. 25 ug Naled was used for Placer and San Joaquin Counties and 22 ug Naled was used for Sac-Yolo County.

Figure 9

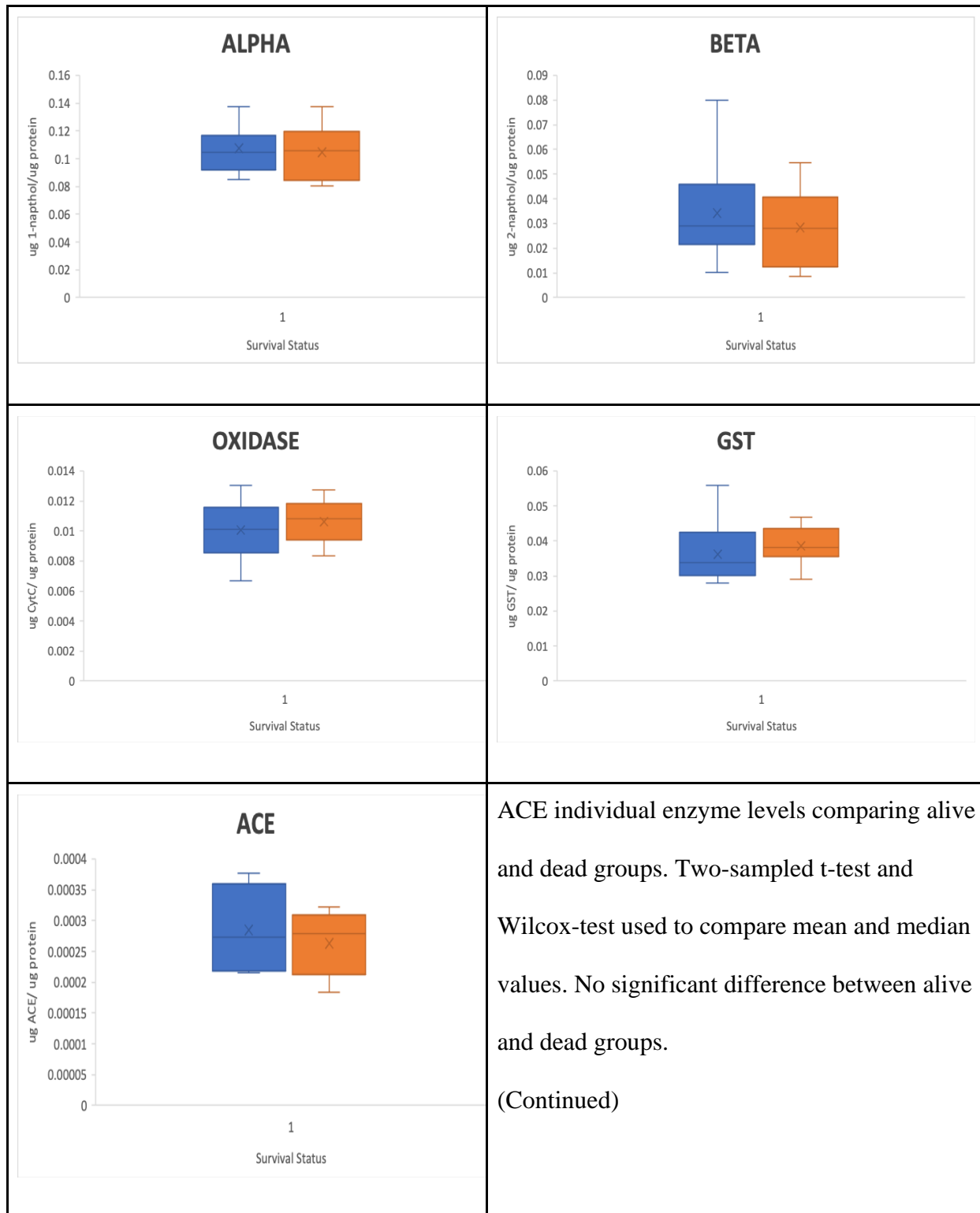
Organophosphate Resistance in 6 Northern California Populations of Cx. tarsalis



Number of individuals = 476

Figure 10

Box and Whisker Plots for ACE Individual Study



Two sampled t-test p-values: Alpha 0.737, Beta 0.408, Oxidase 0.328, GST 0.253, ACE 0.345.

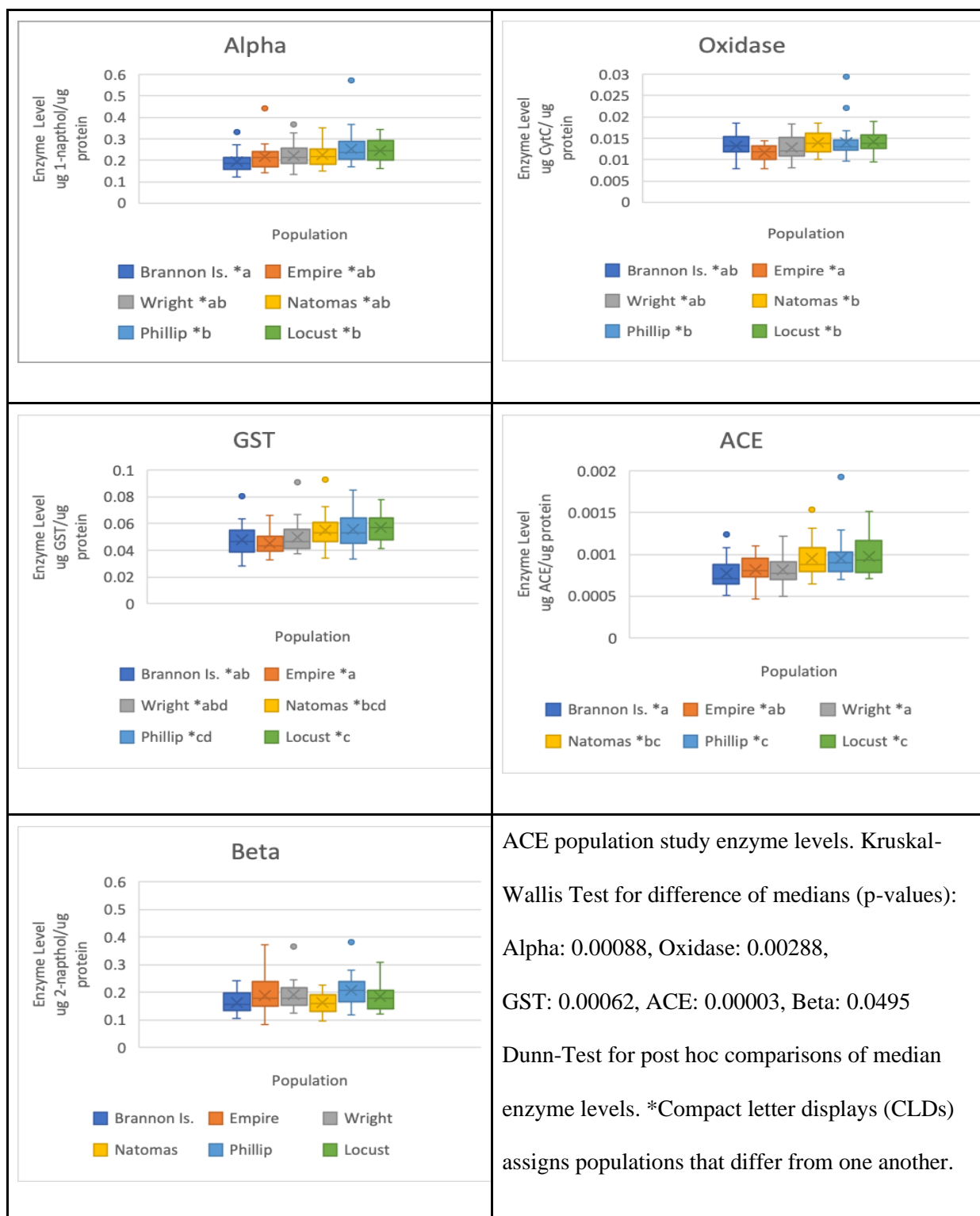
Wilcox-test p-values: Alpha 0.750, Beta 0.527, Oxidase 0.427, GST 0.164, ACE 0.454

Number of individuals tested:

Alpha A:12 D:6, Beta A:14 D:12, Oxidase A:14 D:14, GST A:14 D:14, ACE A:14 D:14

Figure 11

Box and Whisker Plots for ACE Population Study



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