THESIS

INVESTIGATING THE RESISTANCE STATUS TO PERMETHRIN AND TEMEPHOS IN *AEDES AEGYPTI* (THE YELLOW FEVER MOSQUITO)

Submitted by

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ABSTRACT

INVESTIGATING THE RESISTANCE STATUS TO PERMETHRIN AND TEMEPHOS IN *AEDES AEGYPTI* (THE YELLOW FEVER MOSQUITO)

Aedes aegypti (*Ae. aegypti*) is the principle urban vector of several viruses of high medical significance which carry a disease burden on a global scale. *Ae. aegypti* is anthropophilic and lives in close association with humans. This places nearly half of the global population at risk of becoming infected with an arboviral pathogen every year. Therefore, emphasis must be placed on investigating methods for controlling this vector to combat and reduce the spread of human disease. This is especially true in areas where socioeconomic factors promote sustained transmission cycles.

While vector control programs use a variety of strategies, the primary method of reducing vector populations is through insecticide use. Widespread use of insecticides has placed intense selection pressures on *Ae. aegypti* populations and resistance mechanisms have developed. Target site modifications and the expression of detoxifying enzymes are the most significant resistance mechanisms to date. Several single nucleotide polymorphisms resulting in amino acid changes within the voltage-gated sodium channel (VGSC) have been shown to reduce binding site sensitivity and confer resistance to pyrethroids. Specifically, mutations at the knockdown-resistant (kdr) 410, 1,016, and 1,534 sites have been associated with a reduction in pyrethroid sensitivity.

I investigated the resistance status to permethrin and temephos at five locations in Hidalgo County, Texas. I determined the presence of permethrin resistance using a well-characterized susceptible colony as a reference for insecticide sensitivity. The resistant allele $C_{1,534}$ reached fixation at all sites and L_{410} and $I_{1,016}$ were found at high frequencies. The permethrin resistance was over 40-fold when compared to the reference colony. The sites were less resistant to temephos at approximately 6-fold to 12-fold, which I attributed to cessation of this insecticide in the continental United States since 2016.

In the absence of selection pressures mosquito populations trend towards susceptibility, which suggests that there are potential fitness costs associated with insecticide resistance. Studying these associations is important to public health as they may support different strategies to reduce vector populations. I used two collections from Tapachula, Mexico, that were free of pyrethroid exposure since 2013, to determine the presence of two previously described fitness cost metrics: wing length and egg production. I found that the average wing length of V410L and V1,016I homozygous resistant individuals were significantly smaller compared to homozygous susceptible individuals. The interaction between wing length and genotype had no effect on egg production. Wing length had no significant effect on egg production. Most notably, L_{410} and $I_{1,016}$ resistant alleles had no effect on egg production.

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ARTHROPODS AS VECTORS

Arthropods are extremely diverse and can establish and thrive in nearly every environment. Some arthropods have become human-habitat specialists as a result of human migration and destruction of their natural habitats (Keller, 2007; Schofield et al., 1999). Arthropod-borne viruses, or arboviruses (ABVs), describes a diverse group of viruses that are transmitted by hematophagous arthropods. More than 100 ABVs are known to infect humans (Miller, 2008). These viruses develop complex interspecific relationships with the arthropod vector (e.g. ticks, mosquitoes, and sand flies) and the vertebrate host. Some of these viruses persist and amplify in the sylvatic cycle within non-human vertebrate hosts and are then transmitted incidentally to human hosts (urban cycle) (Monath & Vasconcelos, 2015). These viruses are propagative, multiplying within the vector prior to transmission.

Vector competence is described as the ability of the vector to become infected by a virus leading to replication, dissemination, and transmission to a host (Kramer & Ciota, 2015). The time between vector infection and viral transmission is described as the extrinsic incubation period (EIP). This period is impacted by factors internal to the vector, such as the innate immune response and tissue barriers, as well as environmental factors, such as temperature and relative humidity (Chamberlain & Sudia, 1955; Franz et al., 2015). Vectorial capacity describes the potential of a vector to transmit a virus based on the EIP combined with extrinsic factors, such as vector population density, vertebrate host density, vector longevity, and feeding patterns. (Hardy et al., 1983). Extrinsic factors have a varying degree of influence on the vectorial

capacity. For instance, in the MacDonald classical model of vectorial capacity, the probability of surviving through one day (p) is represented as 1/(-ln(p)) (Brady et al., 2016). This means that vector longevity has an exponential effect on vectorial capacity.

A typical ABV transmission cycle begins with the vector acquiring the virus from a blood meal that the vector imbibes from an infected host. Arthropods have developed diverse modified mouth parts and feeding mechanisms which facilitate blood acquisition. Convergent evolution of biting-chewing mouthparts led the hematophagous arthropods to develop a variety of piercing-sucking mouthparts. Hematophagous piercing-sucking arthropods have a separate canal for food and for the salivary duct (Krenn & Aspöck, 2012). In addition to modified mouthparts, the blood meal acquisition is aided by salivary secretions which are composed of a variety of proteins such as anticoagulants, prostaglandins and numbing agents (Ribeiro, 1987). In mosquitoes, the secretion of saliva takes place during the intradermal probing phase and contains antiplatelet molecules, vasodilators and aides in probing site lubrication (Martin-Martin et al., 2022; Orr et al., 1961; Ribeiro et al., 1984). More specifically, in *Aedes aegypti* (*Ae. aegypti*), the peptide sialokinin serves as a vasodilator, thereby increasing the feeding success by reducing time spent at the bite site (Martin-Martin et al., 2022). Salivary secretions suppress the adaptive immune response of the host, promoting viral transmission and infection (Schneider & Higgs, 2008). Once within the vector, the virus propagates and must overcome midgut and salivary gland barriers to be transmitted to the next host by bite (horizontal transmission). If the virus can infect the ovaries, it may also be transmitted to the offspring of the vector (vertical transmission).

The first barrier that must be overcome is the midgut infection barrier (MIB). The peritrophic matrix is a semipermeable membrane which is usually secreted by midgut columnar cells and envelopes the imbibed blood meal acting as a physical barrier to infection of the midgut epithelium. A primary role of the peritrophic matrix is to protect midgut epithelium by detoxifying heme within the blood bolus (Pascoa et al., 2002). Furthermore, the type of receptor on midgut epithelial cells can determine viral binding, facilitating viral entry into the midgut (Hardy et al., 1983). The next barrier is the midgut escape barrier (MEB). This barrier prevents the virus from leaving the midgut epithelial cells into the hemolymph by passing through the basal lamina. Once overcoming the MEB, the virus enters the hemolymph, circulates, and infects subsequent tissues and secondary organs. The last barriers are the salivary gland infection barrier (SIB) and salivary gland escape barrier (SEB). Similarly to the midgut barriers, the virus must both invade the salivary gland from the hemolymph, and then exit the cells of the salivary gland into the saliva. Once overcoming the salivary gland barriers, the virus travels to the lumen and is transmitted through the saliva when the vector takes a blood meal (Carpenter & Clem, 2023). After the EIP, the next blood meal exposes the host to the virus and the potential for transmission arises. Of all the arthropods, mosquitoes are the most medically significant ABV vector.

MOSQUITO-BORNE DISEASES

Mosquitoes are found in nearly every terrestrial habitat, even utilizing snow melt pools to inhabit Arctic areas (Reiter, 2001). Females from most species require a blood meal to acquire enough protein to produce offspring. A small proportion of the 3,500 documented mosquito species are associated with mosquito-borne diseases (MBD)

(Reiter, 2001). It is estimated that over one million people die each year from MBD globally, with infection cases likely reaching 700 million (Caraballo & King, 2014). Furthermore, from a global perspective, children and adolescents have the highest amount of risk associated with MBD (Tolle, 2009). Malaria, West Nile encephalitis (WNE) and West Nile meningitis (WNM) are three examples of MBD, not associated with *Ae. aegypti*, that carry a heavy disease burden. However, these examples do not encompass the magnitude of burden all MBD place on our planet. There is a vast amount of literature available that discusses specific vectors and the pathogens that they transmit.

Malaria, caused by the protozoan parasite genus *Plasmodium* (*P.*) and vectored by *Anopheles* (*An.*) mosquitoes, is responsible for the most deaths annually. While there are four *P.* spp. known to cause malaria, *P. falciparum* and *P. vivax* are responsible for 95 % of the cases reported globally (Qureshi, 2018). Symptoms of malaria can be mild and flu-like, and more serious cases can lead to anemia, jaundice, kidney failure, coma, and eventual death. While antimalaria drugs are still widely used, resistance has been observed (Sinha et al., 2014). African regions suffer the most from malaria, representing 95 % of reported cases and 96 % of deaths. Unfortunately, children under the age of 5 account for 80 % of these cases (World Health Organization [WHO], 2021b). The United States reports approximately 1,500 cases of malaria per year, and while these are typically associated with travelers returning to the United States from endemic areas, autochthonous malaria has been reported in Florida and Texas as recently as May 2023 (Blackburn et al., 2023; Mace et al., 2021).

WNE and WNM are neuro-invasive diseases caused by the West Nile virus (WNV), a mosquito-borne *Flavivirus* (Family: *Flaviviridae*) (Campbell et al., 2002). WNV is vectored by ornithophilic *Culex* (*Cx.*) mosquitoes and is typically maintained within enzootic cycles by avian species (amplification hosts) (Work et al., 1955). Symptoms of WNV infection can range from fever and headaches to more severe symptoms like cerebral dysfunction which can lead to a coma (Campbell et al., 2002). Unlike malaria infections, older patients are more likely to suffer from encephalitis and death (Nash et al., 2001). Currently, there are no treatments or preventative medicines for WNV infections (Centers for Disease Control and Prevention [CDC], 2023b). WNV was discovered in the West Nile region of Northern Uganda in 1937 and first appeared in New York City in 1999 (Campbell et al., 2002; CDC, 1999). The United States reported 2,406 cases in 2023, and since appearing in New York City, there have been 2,776 related deaths (CDC, 2024b, 2024a).

ARBOVIRUSES TRANSMITTED BY *AEDES AEGYPTI*

Ae. aegypti is a globally significant arbovirus vector, being principally responsible for the transmission of several viruses of high medical significance. For example, yellow fever virus (YFV), dengue viruses (DENV), chikungunya virus (CHKV), and Zika virus (ZIKV), are all transmitted by *Ae. aegypti* and carry a heavy disease burden globally (Liu-Helmersson et al., 2019). As of 2013, an estimated 3.9 billion people are at risk of dengue infection worldwide. It is estimated that that nearly 400 million people are infected with DENV each year, and 96 million of these cases present symptoms with varying degrees of severity (Bhatt et al., 2013). Studying ways to control this vector

species would simultaneously combat and reduce human disease by several arboviral pathogens.

Yellow Fever

YFV is a positive-sense, single-stranded RNA virus that belongs to the genus *Flavivirus* and is endemic to Africa and South America. Yellow fever (YF) is maintained within three transmission cycles: (1) a sylvatic cycle between mosquitoes from the genera *Haemagogus* or *Aedes* and non-human primates, (2) an urban cycle between humans and *Ae. aegypti,* and (3) an intermediate cycle between peri-domestic *Aedes* spp. and humans/primates (Monath & Vasconcelos, 2015).

North America was plagued by YF epidemics between 1668 and 1905, and the cause of these epidemics leading up to the end of the 19th century was unknown (Reiter, 2001). However, in 1881, Carlos Finlay proposed *Ae. aegypti* as the principal vector that transmitted YFV. This hypothesis was left unconfirmed for nearly 20 years until 1900, when Walter Reed accompanied by the United States Army Yellow Fever Commission, conducted human trials in Cuba (Cutter, 2016; Kelly, 2018). YFV was not isolated until 1927, and nearly a decade later, the YFV vaccine (17D) was made available by The Rockefeller Foundation sponsorship (Theiler & Smith, 1937). To date, endemics are still present in South America and Africa. Symptoms include anemia, fever, and jaundice. YF jaundice has a case fatality rate of 20 – 50 % (Monath, 2005). Despite mass vaccination efforts, it is estimated that 30,000 deaths still occur each year, and the majority of these cases stem from Africa (90 %) (CDC, 2018).

Dengue

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are caused by four distinct serotypes (DENV1-4). DENV, like YFV, are members of the genus *Flavivirus*, and originated from sylvatic transmission cycles in Africa, involving non-human primates and forest dwelling mosquitoes approximately 500 years ago (Halstead, 2007). Unlike YFV, DENV have undergone genetic changes that have allowed for persistence within urban cycles without input from sylvatic cycles (Brady & Hay, 2020). Because of this, half of the world's population is at risk of being infected with DENV (Bhatt et al., 2013).

While serological tests for DENV were not available until the 1950s, descriptions of dengue-like illnesses dated back to 992 in China (Gubler et al., 2014). In 1947, the Pan American Health Organization (PAHO) launched an eradication plan, which was initiated when *Ae. aegypti* was associated with DF and YF. While this eradication plan was deemed successful between the 1960s and 1970s, countries became non-compliant (Slosek, 1986). From the 1970s to 1999, there was a resurgence of DF and the re-establishment of *Ae. aegypti* populations (Gubler & Clark, 1996). In 2024 alone, over a million cases have already been reported in the Americas, of which, 207 have resulted in death (PAHO, 2023b). Low levels of confirmed cases compared to estimates can be attributed to asymptomatic or less-severe infections which go unreported. Additionally, a study conducted within the last decade showed that asymptomatic and presymptomatic individuals, with certain viral loads, are more infectious to mosquitoes and can contribute significantly to the transmission of DENV. This can be attributed to

the unaltered daily routine of asymptomatic and presymptomatic individuals who maintain exposure to biting mosquitoes (Duong et al., 2015).

It is crucial to note that infection of DENV manifests in two syndromes: DF and DHF/DSS. Most commonly, a fever is accompanied by elevated liver enzymes, an increased cytokine response and increased vascular permeability. An increase in vascular permeability leads to fluid imbalances which results in shock if the balance is not restored (Halstead, 2007). Recovery from one serotype leads to life-long immunity from that serotype, however reinfection of a different serotype increases disease severity (Kularatne & Dalugama, 2022). In 2022, a tetravalent live-attenuated vaccine was made available for children from 9 – 16 years of age who have previously been infected and live in endemic regions (CDC, 2021).

Chikungunya

CHKV belongs to the genus *Alphavirus* (Family: *Togaviridae*) and is vectored by *Ae. aegypti* and *Ae. albopictus* (Nunes et al., 2023). CHKV originated in Africa and was maintained in enzootic transmission cycles with occasional spillover. It was first identified in 1952 in Tanzania, and *Ae. albopictus* became a competent vector of CHKV in 2015 due to a single mutation in the E1 envelope protein. Global distribution of CHKV has been attributed to climate change, vector-host dynamics, anthropogenic activity, and mutations which improve viral fitness (Montalvo Zurbia-Flores et al., 2023). Symptoms of CHKV infection include fever, rash, headaches, polyarthralgia (a condition that leads to pain in several joints in the body), with deaths occurring mostly in patients with comorbidities (Gasque et al., 2015). There are limited therapeutics, and no approved vaccines are available. In the last decade, nearly 3.7 million cases (suspected

or confirmed), were reported to PAHO. In 2023, nearly 200,000 cases were confirmed in the Americas with 700 associated deaths (PAHO, 2023a).

Zika

ZIKV is a single-stranded RNA *Flavivirus*, vectored by *Ae. aegypti* and *Ae. albopictus*. Like YFV, DENV, and CHKV, ZIKV was first isolated in Africa. ZIKV isolation occurred in 1947 from a non-human primate (Dick et al., 1952). It primarily remained endemic to African regions until 2014 when it rapidly expanded through the Polynesian Islands. Symptoms of ZIKV infection are non-specific and include fever, headache, and congenital microcephaly with neurological complications (Musso & Gubler, 2016). Congenital Zika syndrome symptoms include seizures, cognitive impairment, and neurological deficits (CDC, 2019). There are no approved vaccines against ZIKV. Currently, accurate prevalence and incidence reports can be difficult to obtain as cases are confused with other ABV infections due to non-specific clinical presentations. Additionally, under-resourced communities lack available therapeutic supplies and are unable to perform diagnostic tests (Musso & Gubler, 2016). Between 2015 and 2016, the Americas had over 500,000 suspected cases of ZIKV infections (PAHO & WHO, 2016). In 2016, locally acquired cases within U.S. territories exceeded 36,000, and in the same year, autochthonous Zika cases from Texas and Florida exceeded 200 (CDC, 2023c). Over 2,000 congenital syndrome cases associated with ZIKV infections were reported in the Americas in the 2015 - 2016 outbreak (PAHO & WHO, 2016).

DISTRIBUTION OF *AEDES AEGYPTI*

A comprehensive approach to describe the global distribution of *Ae. aegypti* combines genetic analyses with historical and epidemiological records. Evidence

suggests that the extant subspecies *Aedes aegypti formosus* (*Ae. formosus*) is the ancestral form of *Aedes aegypti aegypti* (*Ae. aegypti*). *Ae. formosus* dwells in Sub-Saharan Africa, lays eggs in tree-holes and prefers a non-human blood source (Failloux et al., 2002). However, *Ae. aegypti* is a cosmotropical species that thrives in close association with humans. This anthropophilic mosquito preferentially feeds on humans and oviposits in artificial containers (Harrington et al., 2001; Ponnusamy et al., 2008). The following section details a comprehensive hypothesis, presented by Powell et al. (2018), as to how *Ae. aegypti* dispersed throughout the tropics.

Several hypotheses on the origin of domesticity in *Ae. aegypti* have been proposed. Dr. John Petersen was the first to propose this may have happened thousands of years before being distributed to the New World (Petersen, 1977). However, epidemiological records and advancements in genetic analyses support the more likely scenario that these adaptations happened leading up to the Transatlantic slave trade (Powell et al., 2018).

The land that is now the Saharan Desert was once home to tropical plant taxa and conditions suitable for the establishment of *Ae. formosus* populations (Kröpelin et al., 2008). By 2000 B.C., the drying of these suitable lands could have geographically segregated populations of *Ae. formosus* in Northern Africa. Tabachnick (1991), in agreement with Dr. Petersen, proposed that these mosquitoes may have developed into human specialists at this time, preferentially feeding on human blood and ovipositing in man-made containers that were readily available. Historical and epidemiological records do not support this hypothesis. Travel and trade by the Egyptians, Phoenicians, and Carthaginians had long been established within the Mediterranean region. This region

would have had favorable conditions for the establishment of transported *Ae. aegypti* (Holstein, 1967; Soren et al., 1990). However, there were no reports in the Mediterranean of DF or YF until the 18th and 19th centuries. Genetic analyses also do not support this hypothesis. These analyses suggest the domestic form of *Ae. aegypti* is monophyletic and likely split apart from the Old World approximately 500 years ago (Crawford et al., 2017; Gloria-Soria et al., 2016; Kotsakiozi et al., 2018; Powell et al., 2018).

Powell et al. (2018) hypothesized that the origin of the domestic *Ae. aegypti* was initiated by a combination of annual droughts and human migration into previously uninhabited areas of West Africa. Man-made water containers would have been stored as a reserve during droughts, and as female mosquitoes fled drought-stricken forest ecotones in search of oviposition sites, they would have entered these disrupted habitats to oviposit their eggs. As previously described, the ancestral form would likely have sought out humans as the most readily available blood source, thereby strengthening their domestic bond. *Ae. aegypti* mosquitoes likely lost the need for acquiring sugar from plants during this domestication. The ability to synthesize and build reserves of triglycerides from only human blood allowed for sedentary lifestyles (Harrington et al., 2001; Van Handel, 1965).

The 16th century brought about the Transatlantic slave trade. During this time, Europeans gathered slaves from West Africa and brought them to the New World. With only access to water and human blood during the 2-to-4-month voyage, domestic traits would have been optimal for survival (Powell & Tabachnick, 2013). Based on EIP, vector longevity, and length of voyage, multiple YFV transmission cycles likely occurred

(Powell et al., 2018). Yellow fever was present in Sub-Saharan Africa prior to the 1400s, and the first credible YF epidemics in the New World were reported in Yucatan and Havana in 1648 (McNeill, 1998). This suggests *Ae. aegypti* populations were widespread in the New World and stable enough to sustain multiple transmission cycles.

It is likely that the same scenario that brought *Ae. aegypti* from the Old World to the New World, was responsible for the establishment of populations within the Mediterranean. The Transatlantic slave trade has been described as triangular; ships first traveled from Portugal and Spain to West Africa, then crossed the Atlantic, and returned to Portugal and Spain. The first major reports in the Mediterranean of diseases associated with *Ae. aegypti* appeared in the early 1800s (Sawchuk & Burke, 1998). Throughout the 19th and 20th centuries, the Mediterranean was plagued by DF and YF (Schaffner & Mathis, 2014). In 1869, the Suez Canal was opened and soon after, the first reports of CHKV and DENV infections surfaced in Asia. Trade routes between Asia and Australia were well established during the late 1800s, and the first case of dengue in Australia appeared in 1897. It is likely soldiers during World War II were responsible for the transportation of *Ae. aegypti* to the Pacific Islands (Calvez et al., 2016). Cosmotropical distribution of *Ae. aegypti* within the last 500 years makes this mosquito a globally significant arbovirus vector. Therefore, population reduction is vital to stopping the spread of diseases which carry a heavy burden (Liu-Helmersson et al., 2019).

CURRENT VECTOR CONTROL STRATEGIES

This section is not intended to be an exhaustive list but rather to serve as a broad summary of vector control techniques that are currently in use.

Source Reduction

Source reduction involves making habitats unsuitable for larvae and ovipositing adult females by physically removing potential and existing breeding sites. Source reduction dates back to the early 20th century and is performed on a variety of different scales, ranging from rain receptacles in private back yards to impoundment management practices (Howard, 1911). Knowing species-specific larval requirements and oviposition preferences makes source reduction a cost-effective means to reduce vector populations by targeting the most productive breeding sites (Baldacchino et al., 2015). For instance, Unlu et al. (2014) observed that *Ae*. *albopictus* favored ovipositing eggs in corrugated extension spouts over catch basins. In urban areas, *Ae. aegypti* primarily oviposit in man-made containers, which makes community engagement critical (Floore, 2006). The primary drawback of using source reduction to reduce *Ae*. aegypti populations is that larvae inhabit cryptic areas and small artificial containers, such as bottle caps (Baldacchino et al., 2015; Norris, 2004).

Biological Control

Bacillus thuringiensis israelensis (*Bti*), a naturally occurring soil bacteria, is a commonly used non-chemical pesticide. Toxins found in the parasporal inclusions of *Bti* are ingested during feeding. This disrupts the midgut lining of larval mosquitoes causing osmotic imbalances and eventually leads to cell lysis (Knowles & Ellar, 1987; Lacey, 2007). It is not known to be toxic to humans or other non-target invertebrates such as

honeybees (Lagadic & Caquet, 2014; McClintock et al., 1995). The use of *Bti* and *Bt* ssp. are a popular abatement method because of their high level of specificity. Since *Bt* spp. target immatures as a larvicide, they should be used in conjunction with an adulticide to reduce the density of biting females. This is the most effective method to reduce transmission rates during a disease outbreak (Burattini et al., 2008).

Other notable biological control strategies include the introduction of predators to the immature mosquito's environment. Examples include *Toxorhynchites* (*Tx.*) spp. mosquitoes and the Mosquitofish (*Gambusia affinis*). Since *Tx.* spp. are not hematophagous, they do not pose the risk of being arbovirus vectors. Some limitations for using *Tx.* spp. as a biological control agent includes temperature restrictions, predator/prey interactions, and water body preferences (Focks, 2007). Scalability is also a limitation due to *Tx.* spp. not producing enough eggs in nature to be effective, and laboratory rearing in large numbers is challenging (Donald et al., 2020).

With regards to Mosquitofish, caution should be taken because their introduction may impact ecosystem dynamics and eutrophication (Hurlbert et al., 1972). The use of Mosquitofish as a biological control strategy to combat *Ae. aegypti* is limited because larval habitats may be small, cryptic, and numerous, thereby making them unsuitable for the introduction of this larvivorous organism (Benelli et al., 2016).

Mosquito Modifications

The use of genetically modified mosquitoes (GMM) is a relatively new concept in the field of mosquito control. GMM can target either the reproductive success (population control) or the vector competence (population alteration) and requires a localizing or non-localizing approach (WHO, 2021a). The localizing approach is spatially

restricted while the non-localizing approach is meant to disperse between interbreeding populations. An example of a GMM is the OX513A *Ae. aegypti* strain which uses a RIDL (Release of Insects carrying a Dominant Lethal) system (Harris et al., 2012). This system works by modulating the expression of a toxic tetracycline-repressible transcriptional activator (Phuc et al., 2007). OX513A *Ae. aegypti* larvae are exposed to tetracycline in a laboratory and reared to adulthood. When OX513A males are released, they copulate with wild females, and the resulting larvae die due to lack of tetracycline exposure.

Another mosquito modification (MM) that has been utilized is the introduction of *Wolbachia* spp. into wild *Ae. aegypti* populations. *Wolbachia* is an endosymbiotic bacterium that is present in the reproductive tissues of nearly all arthropods and can interfere with reproductive success by cytoplasmic incompatibility. Some *Wolbachia* strains, like wMelPop-CLA and wMel, have not only been shown to reduce vector longevity, but also disrupt infection by viruses known to cause human disease (Walker et al., 2011).

A major consideration when implementing these strategies to reduce vector populations is whether the modification is self-limiting or self-sustaining, which describes how persistent the modification will be in natural populations over time. Examples of self-limiting modifications includes sterile male and female-killing systems, while an example of self-sustaining modification is the use of *Wolbachia* spp. (Burt, 2014). In urban environments, MM follow the natural behaviors of wild-type mosquitoes making otherwise unreachable cryptic habitats exposed to control efforts (WHO, 2021a). One of the advantages to MM strategies is that they do not require the

community to alter their normal behavior (e.g. actively reducing breeding sites by emptying receptacles). Caution needs to be taken when implementing MM strategies. This is especially true for non-localizing approaches, where mosquito populations are not restricted by national borders, making ethical considerations of paramount importance. Additionally, fitness plays a major role in the ability to insert desired genes into the population; laboratory strains may experience a reduction in fitness, subjecting them to increased selection pressures in the wild (T. Scott et al., 2002).

Oils and Surface Films

Since the 1950s larvicidal oils and surface films have been used as an effective means to target immature mosquitoes. Oils and surface films work by altering normal air-water interface interactions, which floods the respiratory system and exposes immature mosquitoes to toxic substances (Corbet et al., 2000). Respiratory function in immatures is largely dependent on the properties of the air-water interface. Larvae utilize a respiratory siphon which contains spiracular lobes that spread open at the surface of the water, exposing the spiracle to air. When the larvae leave the surface, the lobes fold in and close off, preventing spiracle flooding (S. Lee et al., 2017). Pupae utilize respiratory trumpets that project through the surface of the water (Snodgrass, 1959). When an insoluble monolayer is spread across the air-water interface, it changes the contact angle of the trumpet, causing the inner trumpet to flood and the pupae eventually drowns (McMullen et al., 1977).

One major advantage to this strategy is that there is a low environmental impact. Another major advantage is the ability to reach cryptic environments as the oils and surface films spread rapidly on contact (Nayar & Ali, 2003). A limitation is that oils and

surface films target immatures and not adults. Furthermore, while no evidence suggests that pupae can maintain respiration while submerged, studies suggest *Ae. aegypti* larvae can live for over fifty days and can reach the 4th instar while submerged (Clements, 1992).

Chemical Control

Insect growth regulators (IGRs) act by interfering with the natural process of molting and metamorphosis. Fluctuating juvenile hormone (JH) levels in the hemolymph of larvae play an important role in larval molting and metamorphosis into pupae (Fain & Riddiford, 1975). Small lipophilic analogs such as methoprene mimic JH, prohibiting metamorphosis to the adult stage (Riddiford, 1994). Only a small amount of JH is required for this process to take place. IGRs are favored due to low mammalian toxicity. However, they are not species-specific and are relatively unstable in the environment (Minakuchi & Riddiford, 2006). Globally insecticides are the most utilized type of chemical control. They are crucial to vector control because they are efficacious, are offered in a variety of classes, and can be applied using different delivery methods (Van Den Berg et al., 2021).

INSECTICIDES AND VECTOR CONTROL

The most used insecticide classes for mosquito control are carbamates, neonicotinoids, organochlorines, organophosphates, and pyrethroids. These classes can be broken into three groups dependent on their modes of action; the groups are voltage-gated sodium channel (VGSC) modulators, acetylcholinesterase inhibitors, and nicotinic acetylcholine receptor competitive modulators (Van Den Berg et al., 2021). The VGSC, discussed in greater detail in the following section, is the target site for

pyrethroids and organochlorines like dichloro-diphenyl-trichloroethane (DDT) (Davies et al., 2007). DDT was mass produced during the 1940s as a synthetic insecticide. DDT was produced and distributed worldwide due to its extremely low production cost. However, DDT is an organic pollutant, and has been phased out of use in developed countries due to its persistence within the environment and associated adverse health effects (Turusov et al., 2002).

Carbamates and organophosphates act as acetylcholinesterase (AChE) inhibitors. The neurotransmitter acetylcholine (ACh) is released by transporting vesicles to the presynaptic cleft. AChE is responsible for breaking down ACh via hydrolysis into acetic acid and choline. When AChE becomes blocked by either class of insecticides, ACh breakdown is inhibited (Fukuto, 1990). The buildup of ACh increases nerve and muscle impulses via muscarinic and nicotinic receptor site activation (Goel & Aggarwal, 2007). While both classes degrade rapidly, carbamates and organophosphates are highly toxic to vertebrates (Jayaraj et al., 2016). The two classes of insecticides are distinguishable by binding reversibility. Carbamate binding in AChE is reversable, and hydrolysis typically occurs within 48 hours (Goel & Aggarwal, 2007). However, organophosphates are eventually irreversible AChE inhibitors (Jayaraj et al., 2016).

As the name suggests, Neonicotinoids act as nicotinic acetylcholine receptor competitive modulators. Neonicotinoids are receptor agonists which have a high binding affinity to insect nicotinic receptor sites compared to mammalian receptors (Tomizawa & Casida, 2003). For this reason, they are favored for being efficacious while remaining relatively non-toxic to mammals. While the use of neonicotinoids gained popularity for malaria control in Africa in 2019, its global use remains relatively low compared to other

classes (Van Den Berg et al., 2021). A variety of insecticide classes are used on a global scale to reduce vector populations, however organophosphates and pyrethroids are the only two classes of insecticides available for use to reduce adult mosquito populations in the continental United States (CONUS) (CDC, 2022).

Organophosphates

Organophosphates, derived from phosphoric acid, are a broad class of organic compounds containing phosphorous. There was a rapid development of organophosphates during World War II to be used as insecticides and chemical warfare agents (Jaga & Dharmani, 2003). Since my study includes the use of temephos, it is beneficial to discuss the historical use and development of resistance.

Temephos (O,O,O',O'-tetramethyl O,O'-thiodi-p-phenylene bis(phosphorothioate)) or temefos, is an organophosphate that has been used since the 1960s as a cost-effective means to target immature mosquitoes. Temephos, first registered in the United States in 1965 by the American Cyanamid Company, was transferred to Clarke Mosquito Control Products Incorporated in 1997 and was marketed as Abate® (Environmental Protection Agency [EPA], 2016). Community acceptance of temephos led to its widespread use. It was popular because it was available in multiple forms (e.g. granules and emulsifiable concentrates) and was originally accepted for use in potable water due to its low hazardous rating (WHO, 2009). However, documentation of temephos resistance is now widespread in Central and South America (Da Silva Soares et al., 2003; Grisales et al., 2013; Rodríguez et al., 2007). Unfortunately, the lack of comprehensive data may have contributed to the development of high resistance levels (Ranson et al., 2010).

Pyrethroids

Pyrethroids are a large class of plant-based synthetic analogs derived from natural pyrethrins found in the pyrethrum daisy, *Chrysanthemum cinerariaefoium* (Khambay & Jewess, 2005). Efforts in the 1950s, 1960s and 1970s developed pyrethroids enhanced with increased insecticidal efficacy and photostability while maintaining relatively low levels of mammalian toxicity (**Figure 1**) (Elliott, 1989). Tetramethrin (**Figure 1A**), among the first pyrethroids to reach the market, did not have the desired photostability (Soderlund, 2020). Phenothrin (**Figure 1B**) was discovered by keeping the chrysanthemic acid moiety. Permethrin (**Figure 1C**), the first photostable pyrethroid, was produced by replacing methyl groups with chlorines. Cypermethrin (**Figure 1D**) was created with the addition of an alpha-cyano group, which proved to be extremely potent and had new effects (Soderlund, 2020). There are two types of pyrethroids (type I and type II), and the presence of the alpha cyano-3-phenoxybenzyl alcohol moiety classifies a type II pyrethroid (Nasuti et al., 2003).

Exposure to a type I pyrethroid elicits neurological hyperactivity, movement incoordination, rapid paralysis (knockdown), and metabolic disruption (Khambay & Jewess, 2005). Type I pyrethroids preferentially bind to closed VGSCs producing repetitive discharges along the axon, while type II pyrethroids preferentially bind to open VGSCs, blocking synaptic conduction (Hu et al., 2011). Exposure to a type II pyrethroid elicits a delayed neurological response followed by convulsions and subsequent paralysis. Knocked down mosquitoes are left vulnerable to predation and desiccation. Type I and type II pyrethroids are effective by altering the normal function of the VGSC.

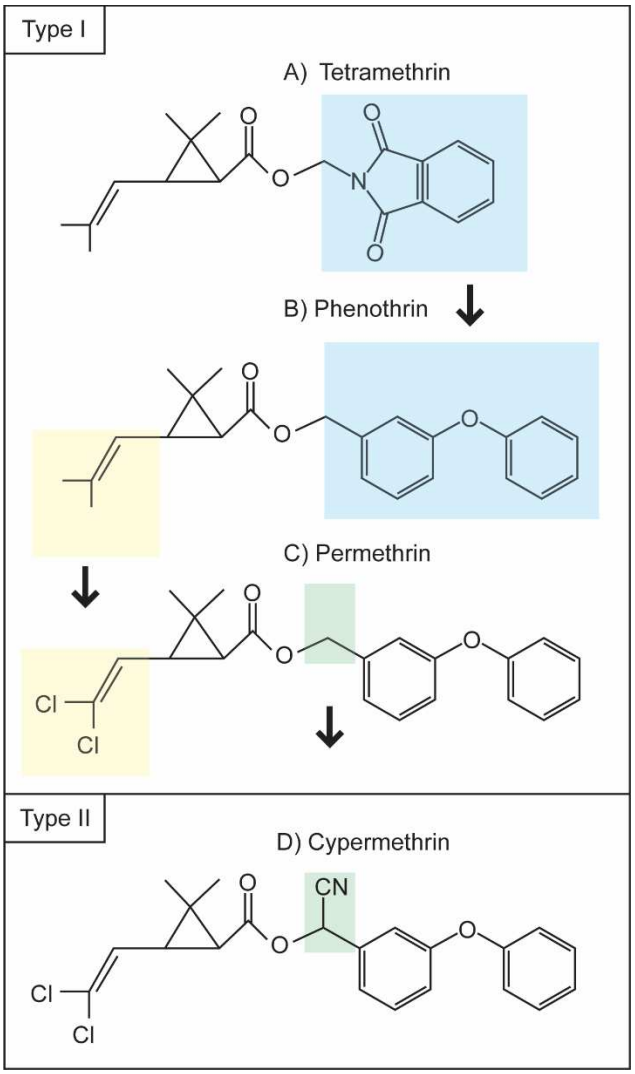

Figure 1. Development of pyrethroids. The blue squares represent the development in the alcohol moiety of the molecule which produced compounds like phenothrin (B) from tetramethrin (A). The yellow squares represent the substitution of methyl groups with chlorine which produced permethrin (C). The green squares represent the addition of the alpha-cyano substituent which produced cypermethrin (D). The presence of the alpha-cyanogroup classifies a type II pyrethroid. Chemical structures obtained from (Soderlund, 2020).

FORM AND FUNCTION OF THE VOLTAGE-GATED SODIUM CHANNEL

The VGSC is a transmembrane protein which is largely responsible for the initiation and propagation of an action potential within the nervous system of insects (Du et al., 2013). The VGSC is made up of 4 homologous domains (I - IV), each containing

6 α-helical segments (S1 - S6), as depicted in **Figure 2**. Segments 1 - 4 are known as the voltage-sensing module, while segments 5 and 6, with the reentrant loop, are known as the outer pore (Catterall, 2000). To date, two pyrethroid binding sites have been identified and modeled: PYR-1 and PYR-2. The PYR-1 binding site is located between the linker helix connecting IIS4 and IIS5 (IIL45), and domains IIS5, IIS6, and IIIS6. The PYR-2 binding site is between IL45, and domains IS5, IS6, and IIS6 (Du et al., 2013; O'Reilly et al., 2006; Saavedra-Rodriguez et al., 2018).

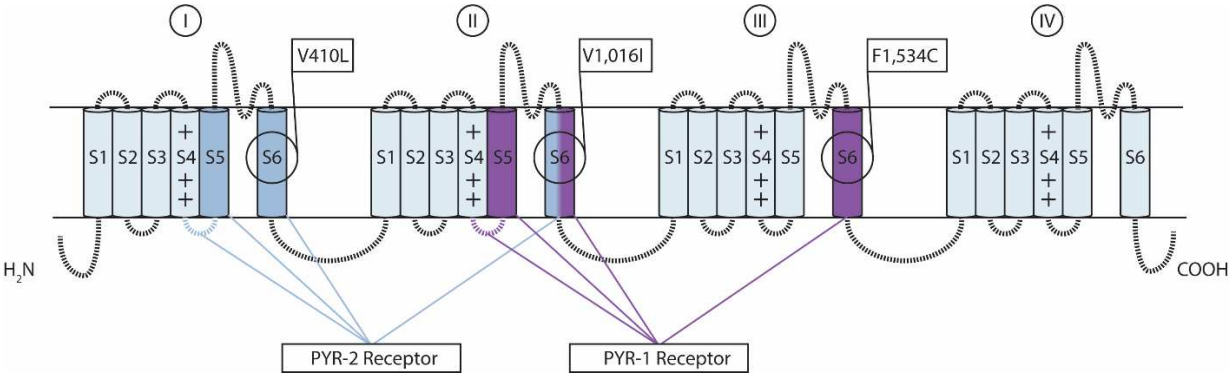

Figure 2. Depiction of the three mutations within the voltage-gated sodium channel (VGSC) of *Aedes aegypti* (*Ae. aegypti*). PYR-1 binding site is depicted in purple and PYR-2 binding site is depicted in dark blue. The V410L kdr site is in domain I, subunit 6 (IS6). The V1,016I kdr site is in domain II, subunit 6 (IIS6). The F1,534C kdr site is in domain III, subunit 6 (IIIS6). This figure was modified from Saavedra-Rodriguez et al. (2018)

The *Xenopus* oocyte expression system was instrumental in locating binding sites and determining the effects of pyrethroids on the VGSC of *Ae. aegypti* (Haddi et al., 2017; Loughney et al., 1989). The two binding sites were located by studying the impact of mutations in the VGSC on pyrethroid sensitivity. First, the insect VGSC gene, para or $DmNa_v$, was cloned in *Drosophila melanogaster* in 1989. The system was used to confirm that the $DmNa_v$ gene encoded for functional VGSCs. This system also determined that $DmNa_v$ had elevated pyrethroid sensitivity compared to mammalian sodium channels (Warmke et al., 1997). Then, it was used to functionally express the

$DmNa_v$ ortholog for *Ae. aegypti* ($AaNa_v$) and identify the second pyrethroid binding site (Du et al., 2013).

INSECTICIDE RESISTANCE MECHANISMS

Widespread use of insecticides has placed intense selection pressures on mosquito populations and resistance mechanisms have developed. The development of insecticide resistance in mosquitoes is described as pre-adaptive; the resistant allele or alleles that confer resistance to an insecticide are already present in a small proportion of the individuals prior to a selection pressure (Crow, 1957). As the number of surviving individuals increases, so does the proportion of individuals that carry a resistant allele or alleles. The two main insecticide resistance mechanisms are target site modifications and metabolic detoxification (Liu, 2015).

Target Site Modifications

Target site modifications are natural replacement mutations that encode specific regions within AChE and the VGSC which lower the binding site sensitivity to organophosphates and pyrethroids, respectively. Mutations within the AChE1 and AChE2 genes (Ace1 and Ace2, respectively) have been correlated with resistance to organophosphates in *An. gambiae*, *Cx. pipiens* and *Cx. tritaeniorhynchus*. However, this has yet to be determined in *Ae. aegypti* as a resistance mechanism (Alout et al., 2007; Nabeshima et al., 2004; Weill et al., 2004). Knockdown resistance (kdr) is produced by the presence of replacement mutations within the VGSC. Identification of these mutations have occurred across a variety of agricultural pests, disease vectors, and nuisance arthropods (Rinkevich et al., 2013). Evidence also suggests that point mutations within the VGSC can influence gate kinetics, which impacts preferential

binding. Eleven mutations have been identified in the *Ae. aegypti* VGSC that can confer resistance to pyrethroids (**Figure 2**).

V1,016I kdr Site

Saavedra-Rodriguez et al. (2007) discovered a mutation at the 1,016 codon, domain II, subunit 6 (IIS6). This mutation is a guanine (G) to adenine (A) transition in the first position of the 1,016 codon. This single nucleotide substitution results in an amino acid replacement from valine (V) to isoleucine (I). Evidence suggests that V1,016I does not, on its own, reduce sensitivity to pyrethroids. It has been suggested to have co-evolved with F1,534C, and the co-occurrence of these mutations may further impact binding site sensitivity (Dong et al., 2014; Du et al., 2013).

F1,534C kdr Site

Harris et al. (2010) sequenced *Ae. aegypti* from the Grand Cayman Islands and Yanola et al. (2011) discovered a mutation at the 1,534 codon, domain III, subunit 6 (IIIS6). This mutation is a transversion of thymine (T) to G at the second position of the 1,534 codon which results in an amino acid replacement from phenylalanine (F) to cysteine (C). The presence of the F1,534C mutation has been shown to provide protection against type I but not type II pyrethroids (Hu et al., 2011).

V410L kdr Site

Haddi et al. (2017) identified a new mutation in an *Ae. aegypti* laboratory colony from Brazil. This mutation was discovered at the 410 codon, domain I, subunit 6 (IS6). However, the authors did not observe this mutation in field strains (Haddi et al., 2017). This mutation is a G to T transversion in the first position of the 410 codon which encodes an amino acid replacement from V to leucine (L). V410L, either alone or

accompanied by F1,534C, confers resistance to both type I (permethrin) and type II (deltamethrin) pyrethroids (Haddi et al., 2017). In 2002, a genome-wide association study found the first appearance of heterozygote alleles at the 410 kdr site. By 2016, this frequency increased to 0.64 in Tapachula, Mexico (Saavedra-Rodriguez et al., 2018). This corresponds to an increase in resistant allele frequencies of V1,016I and F1,534C from 2000 to 2016. Additionally, a strong correlation was observed between the genotype and phenotype of V410L when exposed to pyrethroids (Saavedra-Rodriguez et al., 2018).

Metabolic Detoxification

Differences in esterase activity between susceptible and resistant *Ae. aegypti* have been recognized as causes of insecticide detoxification (Mazzarri & Georghiou, 1995). Specifically, the overexpression of cytochrome P450 monooxygenases (CYP), carboxyl/cholinesterases (CCE), and glutathione S-transferases (GST) have been observed in multiple resistant populations (Goindin et al., 2017; Grisales et al., 2013; Saavedra-Rodriguez et al., 2014). It is likely that multiple groups of enzymes make up a metabolic system capable of detoxifying insecticides. For example, CYP have been demonstrated to process secondary pyrethroid metabolites first hydrolyzed by CCE (Chandor-Proust et al., 2013). Additionally, metabolic detoxification can provide cross-resistance to multiple classes of insecticides regardless of the target site (J. Scott, 1999).

INSECTICIDE RESISTANCE AND FITNESS COSTS

Understanding the effect that resistance mechanisms have on mosquito populations is vital to mosquito control because they can impact vectorial capacity,

disease transmission, and the reversion to susceptibility (Brito et al., 2013). Fitness costs associated with insecticide resistance have long been a topic of conversation.

Crow (1957) hypothesized that resistant alleles would be disadvantageous in the absence of insecticide selection pressures, which would, in time, cause a resistant population to return to susceptibility (Cochran, 1993). In the absence of selection pressures, a reduction in resistant allele frequencies is evidence of fitness costs being associated with insecticide resistance.

This type of comprehensive multi-generational fitness cost study has been performed in *Ae. aegypti* (Brito et al., 2013; Vera-Maloof et al., 2020). However, targeting a specific component of fitness requires the observation of specific parameters between susceptible and resistant individuals. Examples of fitness costs include adult longevity, host-seeking characteristics, and reproductive characteristics (e.g. egg production, egg viability, and sex ratio).

Caution must be taken when making comparisons between susceptible and resistant individuals for a particular mechanism because other mechanisms responsible for resistance may differ between the two groups. Additionally, mosquito collections from separate geographical locations may have different life history traits (Rivero et al., 2011). Lastly, making comparisons between an insecticide-resistant field strain and a susceptible lab colony may yield skewed results as field strains are not well adapted to optimal laboratory conditions (Bourguet et al., 2004).

Recall that the two major types of resistance mechanisms come in the form of metabolic detoxification enzymes and target site modifications. An example of a fitness cost associated with detoxifying enzymes was shown in *Cx. pipiens,* which exhibited a

reduction in longevity due to resource-based trade-offs (RBTO) and oxidative stress. As described in the Arthropods as vectors section within this chapter, vector longevity can have an impact on the vectorial capacity. Evidence suggests RBTO can also influence immunocompetence when resource allocation is directed towards detoxifying enzymes rather than eliciting an immune response against a pathogen (Rivero et al., 2010).

Foundational examples of fitness costs associated with kdr mutations in non-vector insects have been described (Foster et al., 2003). It has been proposed that the VGSC plays a role in olfactory signal transduction pathways. These kdr mutations may impact host seeking or mating behaviors, however this has yet to be described (Rivero et al., 2010; Zwiebel & Takken, 2004). Brito et al. (2013) suggested that the presence of V1,016I and F1,534C resistant alleles were associated with increased female locomotive activity, reduction in number of eggs laid, and longer larval developmental times.

Further investigation is needed as the understanding of fitness costs associated with kdr mutations are less extensive compared to RBTO. Additionally, multi-generational studies can help determine how resistant alleles impact fitness in the presence or absence of insecticides. Studying fitness costs associated with insecticide resistance offers two primary benefits. The first being that it can help describe how resistant populations return to a susceptible status in the absence of selection pressures. The second benefit is understanding how fitness costs may impact vectorial capacity and pathogen transmission.

CONCLUSIONS

Ae. aegypti is principally responsible for the transmission of several viruses which carry a heavy disease burden globally (Liu-Helmersson et al., 2019). Every year, nearly half of the world's population is at risk of being infected with one of these viruses (Bhatt et al., 2013). Mosquito control entities primarily rely on insecticides more than any other method to decrease population densities and prevent the spread of diseases. In the last decade, pyrethroids and organophosphates were used the most in the Latin American and Caribbean regions to reduce the spread of dengue (Van Den Berg et al., 2021). Consistent and widespread use of the same classes of insecticides exposes populations to intense selection pressures, which leads to the development of resistance mechanisms (Liu, 2015). Many areas, conducive to *Ae. aegypti* habitation, are underreported or lack resistance status data entirely. Furthermore, methodology is not standardized across resistance status studies (Moyes et al., 2017). Areas in Southern Texas, along the Mexican border, are conducive for mosquito habitation and virus transmission (Brunkard et al., 2007; Rivera, 2014). Texas has reported dengue virus activity in Hidalgo County as recently as 2019 (Texas Department of State Health Services [TDSHS], 2022). Therefore, I investigated the resistance status to permethrin and temephos using standardized methodologies and a well-characterized susceptible colony. I performed this investigation to supplement an underreported area with insecticide resistance data which can be used to develop vector control and arbovirus mitigation strategies. I then determined if there were associations between two fitness costs (wing length and egg production) and resistant alleles known to confer resistance to pyrethroids.

CHAPTER 2: RESISTANCE TO PERMETHRIN AND TEMEPHOS IN A*EDES AEGYPTI* WITHIN SOUTHERN TEXAS

SUMMARY

Aedes aegypti (*Ae. aegypti*) being the principal urban vector of a variety of arboviruses that carry severe disease consequences, makes the species a primary target for vector control in the Americas. However, prolonged insecticide use has exposed mosquito populations to strong selection pressures, and as a result, a host of resistance mechanisms have developed. The two most significant types of resistance in mosquitoes are the expression of detoxification enzymes that aid in metabolism, and target site modifications that alter the binding affinity of insecticides. In *Ae. aegypti*, single nucleotide substitutions at the 410, 1,016, and 1,534 loci within the voltage-gated sodium channel (VGSC) have been described to confer resistance to pyrethroids. Studying the status of resistance in underreported or resource-limited areas is crucial for public health. I investigated the status of resistance to permethrin and temephos in *Ae. aegypti* collected from Southern Texas using reputable criteria for resistance monitoring, and a well-characterized susceptible colony for reference. Permethrin is widely used in the continental United States (CONUS) as an insecticide, while the use of temephos has been phased out since 2016. An initial susceptibility test revealed the presence of permethrin resistance in all collection sites. All the sites that I performed permethrin intensity testing on exhibited high levels of resistance. I observed high resistant-allele frequencies for all collections at the 410, 1,016, and 1,534 loci. Most notably, the resistant allele C_{1534} reached fixation at every site. Lower levels of temephos resistance at all the collection sites was attributed to the cessation of

temephos production in the CONUS since 2016. In summary, this study provides resistance data for an underreported area, emphasizing the use of a well-characterized susceptible colony as a reference for insecticide sensitivity.

INTRODUCTION

Aedes aegypti (*Ae. aegypti*) is the principal vector of a variety of viruses including, but not limited to chikungunya virus (CHKV), dengue viruses (DENV), yellow fever virus (YFV), and Zika virus (ZIKV), which carry a heavy disease burden globally (Liu-Helmersson et al., 2019). *Ae. aegypti* are anthropophilic and have established populations within the southern regions of the continental United States (CONUS) (Christophers, 1960; Hahn et al., 2016; Howard, 1903; Kelly, 2018; Morlan & Tinker, 1965). While autochthonous disease outbreaks are not frequent in south-western states, bordering regions report higher disease prevalence (Centers for Disease Control and Prevention [CDC], 2023c, 2023a; Ehrenkranz et al., 1971). Pyrethroids and organophosphates are two classes of insecticides that are globally used to reduce *Ae. aegypti* populations (Zhang, 2018). Exposure to these insecticides create selection pressures, wherein resistance mechanisms develop (Dong et al., 2014; Du et al., 2013; Mazzarri & Georghiou, 1995). Southern Texas is an area that promotes mosquito habitation, and when coupled with a viral presence, poses a public health risk (Brunkard et al., 2007; Rivera, 2014). As such, it is critical for public health entities to implement control strategies to reduce vector populations. When mosquito control entities are decentralized and funding is limited, it would be beneficial to supply resistance data to an area that is underreported.

The establishment of *Ae. aegypti* populations in the southern regions of the CONUS has been well documented since first being described by Wiedemann in 1828 (Christophers, 1960). The importance of conducting distribution surveys in the CONUS was highlighted in 1900, when the U.S. Army Yellow Fever Commission confirmed *Ae. aegypti* as the causative agent of yellow fever (Kelly, 2018). Reports shortly thereafter documented established populations in most southern states, in addition to more northern states, such as Maryland and Illinois (Howard, 1903). Extensive surveys were conducted by the CDC, state, and local public health entities leading up to the Pan American Sanitary Organization *Ae. aegypti* eradication program in 1964 (Morlan & Tinker, 1965). These surveys indicated similar results to the reports conducted in the beginning of the 20th century, finding that *Ae. aegypti* infestations were prevalent in Texas, Florida, Alabama, and Georgia. Present day distribution studies (1995 to 2016) have observed that the majority of *Ae. aegypti* populations are in Florida, Texas, Arizona, and California (Hahn et al., 2016).

A mosaic of persistent and intermittent populations of *Ae. aegypti* have been associated with disease throughout the history of the United States. There are accounts of yellow fever plaguing northern states like Pennsylvania and New York as early as the mid-17th century (Reiter, 2001). Major dengue outbreaks have occurred in the southeast since the beginning of the 20th century (Texas; 1922, Miami/South Georgia; 1934, and Texas; 1941) (Ehrenkranz et al., 1971). The next dengue outbreak did not occur until 2004 in Texas, and then again in Florida in 2010. Autochthonous chikungunya was reported in Texas and Florida in 2014 and 2015, whereas in 2016, autochthonous Zika cases in the United States exceeded 200 (CDC, 2023c). In the last twelve years, there

have been over 300 confirmed cases of locally acquired dengue transmission within the CONUS (CDC, 2023a). While travel-associated cases of dengue, Zika, and chikungunya far exceed these statistics, controlling local *Ae. aegypti* populations is critical to limit autochthonous disease spread.

Mosquito abatement entities utilize insecticide applications as a primary means to reduce *Ae. aegypti* populations and other mosquito vectors known to transmit viruses that cause human diseases (Kondapaneni et al., 2021). This is especially true in communities where lack of engagement makes source reduction practices unfeasible or impractical (e.g. large areas of control or accessibility restrictions). In a broad sense, *Ae. aegypti* populations are exposed to insecticides in one of two ways: (I) a target event, and (II) a non-target event. A target event occurs when the primary goal is to specifically control *Ae. aegypti* populations via insecticide applications. For instance, the application of organophosphates and pyrethroids to treat *Ae. aegypti* within Miami-Dade County in response to Zika outbreaks in 2016 (Stoddard, 2018). In contrast, a non-target event occurs when the primary goal is to control other mosquito species in an area that is coinhabited with *Ae. aegypti* populations. In this case, *Ae. aegypti* populations are exposed to the same selection pressures. For example, the application of pyrethroids to treat *Culex* spp. within Dallas County, an area known to have existing *Ae. aegypti* populations, to reduce West Nile virus (WNV) infections in 2012 (Chung et al., 2013). Additionally, routine applications to reduce nuisance species is also an example of a non-target event (Kondapaneni et al., 2021).

Widespread pyrethroid exposure, through target or non-target applications, has created intense selection pressures on *Ae. aegypti* knockdown-resistant (kdr) genes,

making future generations less susceptible to conventional control strategies. The three voltage-gated sodium channel (VGSC) mutations discussed in the previous chapter, which confer resistance to pyrethroids, have been described in many southeastern states in a variety of combinations. No V1,016I resistant alleles were found in a 1998 collection of *Ae. aegypti* from Houston, TX (García et al., 2009). However, collections in 2018 from central and southern California exhibited high frequencies of V410L, V1,016I, and F1,534C. F1,534C reached fixation in nearly all the collections, and V410L and V1,016I were almost always linked (Mack et al., 2021). Collections in 2016 and 2017 from a variety of locations within Florida also exhibited F1,534C frequencies nearing fixation. The frequency of resistant alleles at the V1,016I position did fluctuate throughout the state, and while the linkage between V410L and V1,016I was discussed, the study did not perform V410L genotyping (Estep et al., 2018). Resistant alleles (L_{410}, $I_{1,016}$, and $C_{1,534}$) were also found in high frequency in multiple locations in California (Merced, San Diego, Tulare, and Los Angeles), as well as in New Orleans, LA (Fan et al., 2020). However, unlike the study performed by Estep et al. (2018), Fan et al. (2020) did not identify the presence of these resistant alleles in Florida (St. Augustine). A study published in 2023, reported the presence of V410L for the first time in Texas, from *Ae. aegypti* collected in Harris County between 2017 and 2019 (Hernandez et al., 2023). The presence and frequency of these mutations can be variable; therefore, it is important to perform location specific genotyping when attempting to draw conclusions on what factors may be impacting insecticide resistance for a given locality.

Temephos (O,O,O',O'-tetramethyl O,O'-thiodi-p-phenylene bis(phosphorothioate)) or temfos, is a globally used organophosphate larvicide

preferred for its low vertebrate toxicity and low production cost (Martínez-Mercado et al., 2022; World Health Organization [WHO], 2009). Temephos (trade name Abate®), was largely used by mosquito abatement entities from 1965 until 1995, when reports of resistance began to surface. By 2013, resistance to temephos had been documented around the world (Da Silva Soares et al., 2003; Grisales et al., 2013; Mazzarri & Georghiou, 1995; Tikar et al., 2009). In 2014, a study from southern Mexico associated the expression of detoxifying enzymes, such as carboxyl/cholinesterase esterase (CCE), with high levels of resistance to temephos (Saavedra-Rodriguez et al., 2014). Its continued use can unfortunately be attributed to a lack of resistance data. Studies have shown that the selection pressures induced by temephos exposure can significantly increase resistance levels within a few generations (Ranson et al., 2010; Saavedra-Rodriguez et al., 2014). This may be the case within the CONUS, where published data on temephos resistance is lacking. However, the Environmental Protection Agency (EPA) ceased the production and distribution of temephos by the end of 2015 (McGregor & Connelly, 2020). Therefore, it would be beneficial to investigate the presence and magnitude of temephos resistance in the absence of selection pressures, in addition to supplying resistance data to an area that is underreported.

The Lower Rio Grande Valley (LRGV) is composed of the three southernmost counties at the Texas – Mexico border and is home to an estimated 1,400 impoverished communities called Colonias (Rivera, 2014; Texas Department of Housing and Community Affairs [TDHCA], 2023). These predominantly low-income Hispanic communities commonly lack public works infrastructure, which limits the development of adequate sewage systems and can leave residents without potable water (Hargrove et

al., 2015; Rivera, 2014). Additionally, short distances between dwelling units, lack of air-conditioners, and poor water drainage systems can create ideal conditions for mosquito habitation. These factors have been described as predictors of past dengue infections (Brunkard et al., 2007). Hidalgo County, positioned in the middle of the LRGV, has nearly 1,000 Colonias which house over 150,000 people (TDHCA, 2023). While this region has the largest concentration of mosquito control entities per county in the state, they are decentralized, with extremely low funding (Bolling, 2019; Juarez et al., 2022). Routine insecticide applications have taken place in Weslaco and Mercedes (southern Hidalgo County), and resistance to pyrethroids was documented in the surrounding towns as recent as 2019 (Bolling, 2019; City of Weslaco, 2022).

For my study, an insecticide resistance status is defined as a summary of data depicting resistance levels and mechanisms to one or more classes of insecticides within a population. The benefits of investigating an insecticide resistance status are two-fold: (I) it informs mosquito abatement entities with the most effective way to allocate resources towards routine applications, and (II) by obtaining this information prior to an endemic outbreak, swifter and more effective response efforts can occur. Currently, an insecticide resistance status for the towns of Mercedes and Weslaco has not been described, but this would prove beneficial given the present factors which promote the persistence of *Ae. aegypti* populations and disease endemicity.

GOAL

The goal of my study was to gather preliminary data on permethrin and temephos resistance in *Ae. aegypti* populations collected in Mercedes and Weslaco, TX.

First, I utilized the CDC bottle bioassay to perform an initial susceptibility test. This method involved using a CDC recommended diagnostic dose (DD) to obtain the diagnostic time (DT), which is the time required to knockdown 100 % of the known susceptible colony (CDC, 2012). The knockdown percentage of the suspected resistant field collections at the DT could then be compared to the WHO standards which indicates resistance status (CDC, 2012). I hypothesized that the results of the initial susceptibility test would indicate resistance to permethrin (< 80 % knockdown at the DT) (**H_1**). This was hypothesized due to the history of pyrethroid use in the region, as well as documented pyrethroid resistance in the surrounding towns in 2018 and 2019 (Bolling, 2019).

Second, the results from the initial susceptibility test were confirmed by calculating resistance ratios (RR_{50}), which determines the magnitude of resistance to a particular insecticide, in this case permethrin (Cui et al., 2006). Allele-specific polymerase chain reaction (AS-PCR) assays were utilized to identify the presence of kdr alleles, which are known to confer resistance to pyrethroids. I hypothesized that if the initial susceptibility test produced a knockdown percentage less than 80 in the collection sites at the DT, then permethrin RR_{50} values would be larger than 1 (**H_{2a}**) and resistant alleles in the VGSC would be present in those populations (**H_{2b}**). Due to selection pressures in the region, I hypothesized that the collections would exhibit deviations from Hardy-Weinberg (HW) equilibrium (**H_{2c}**).

Third, larval bioassays were conducted to calculate temephos RR_{50} values, which would indicate the magnitude of organophosphate resistance at these collection sites. I hypothesized that the larval bioassays would indicate resistance to temephos at

the collection sites (RR_{50} > 1) (**H_3**) due to selection pressures from past and present organophosphate use (CDC, 2017; Rodríguez et al., 2002).

METHODOLOGY

Mosquito Collections

Ae. aegypti mosquitoes used in this study were collected by the Dr. Gabriel Hamer Laboratory (Texas A&M University) in the Spring of 2018 via oviposition cups. The five collection sites in Hidalgo County, TX were as follows: Mercedes Cemetery (MC, 26°09′20″N, 97°55’31″W ± 0.5 km), Mercedes La Mesa (MLM, 26°13′51″N, 97°57’29″W ± 0.5 km), Valley Nature Center (VNC, 26°09’44’’N, 97°59’48”W ± 0.5 km), Weslaco Christian Court (WCT-03, 26°08′31″N, 97°58’44″W ± 0.5 km), and Weslaco Mile 5 (WM5, 26°07′37″N, 97°58’08″W ± 0.5 km). Five oviposition cups (500 mL black plastic cups lined with seed germination paper and filled with a water and hay mixture) were placed 100 m apart at each collection site. Collections were conducted weekly between March and July. The F_1 eggs were dried in an open Ziploc® bag, exposed to ambient air temperature and humidity, and then shipped to Colorado State University (CSU). The New Orleans (NO) laboratory colony was used as the reference for susceptibility. The NO colony, originally collected in New Orleans, LA, by the CDC, was donated to the William C. Black IV laboratory (CSU) in 2005 by Dr. William Brogdon. Since 2005, the colony has been free of insecticides, exhibits no presence of kdr alleles, and is routinely confirmed as insecticide-susceptible via bottle and larval bioassays. The laboratory reports a lethal concentration 50 % (LC_{50}) range of 0.4 to 0.6 µg/bottle for permethrin, and 0.005 µg ai/mL for temephos (Saavedra-Rodriguez et al., 2014; Vera-

Maloof et al., 2020). **Figure 3** depicts the geographical location of each collection site.

Table 1 depicts the collection site information and characteristics.

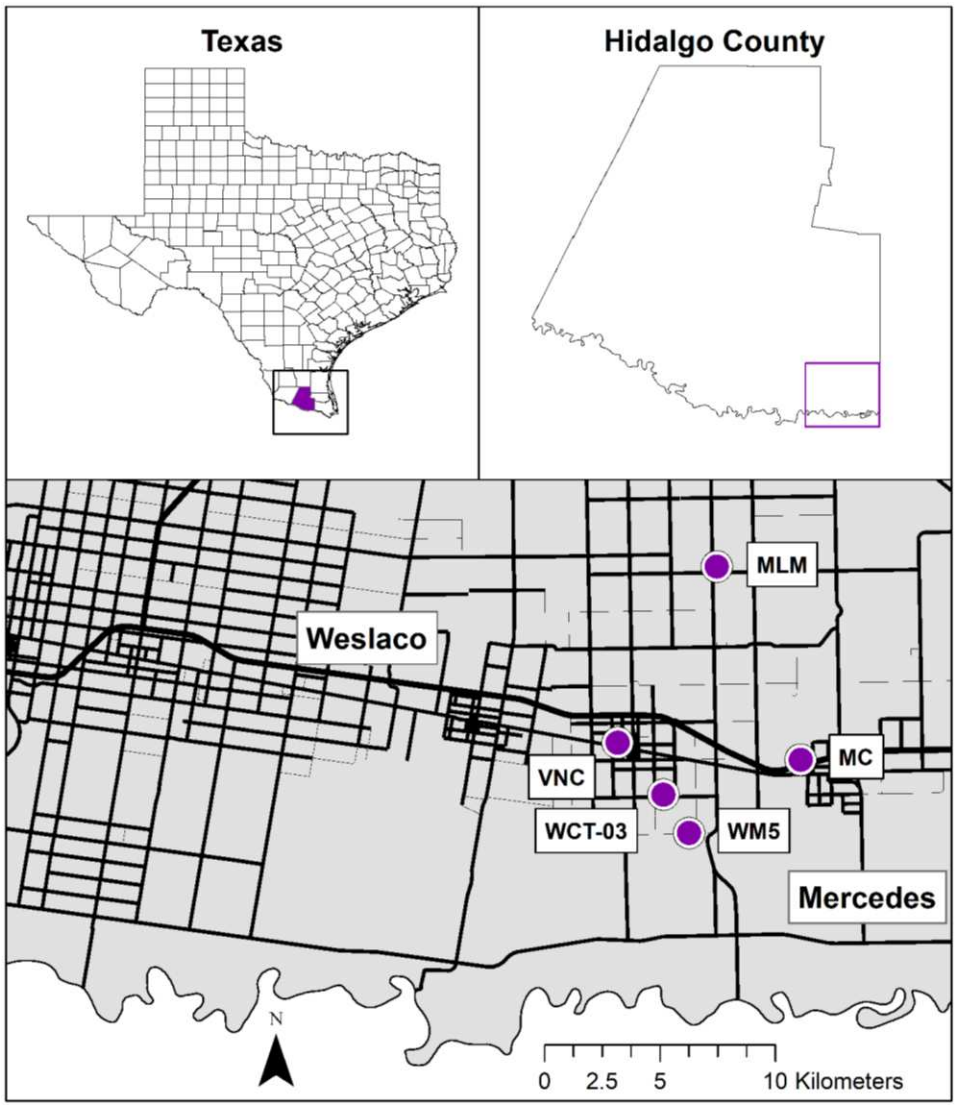

Figure 3. Map of *Ae. aegypti* collection sites in Hidalgo County, TX in the spring of 2018.

Table 1. Location and setting of the collection sites in Hidalgo County, TX in the Spring of 2018.

ID	City	Location	Setting
MC	Mercedes	Mercedes Cemetery	Cemetery
MLM	Mercedes	Mercedes La Meza	Colonia
VNC	Weslaco	Valley Nature Center	Natural Area
WCT-03	Weslaco	Weslaco Christian Court	Neighborhood
WM5	Weslaco	Weslaco Mile 5	Farm/Natural Area

Rearing

Filter papers containing *Ae. aegypti* eggs were placed in clear plastic bins (L 17.0 cm x W 30.25 cm x H 9.50 cm) filled with 2 liters of water. To ensure temperature equilibrium, de-oxygenation and de-chlorination, the water was placed in the incubator 24 hours prior to the introduction of eggs. Approximately 0.100 g of Brewer's Yeast dietary supplement (Lewis Labs International Limited, Westport, CT) was added to the bins before placing them into the incubator. These steps were repeated for each collection site.

After eclosion, between 24 - 48 hours, larvae were separated into multiple bins (~ 50 larvae per bin). Several plastic bins were prepared by the method described above to standardize larval density requirements. The larvae were fed 2 - 3 mL of a 10 % liver powder solution as needed (Catalog number: 900396, Lot number: Q4478, MP Biomedicals, Solon, OH). The larvae were kept in Forma Environmental Chamber incubators (Thermo: Electron Corporation, Waltham, MA) at 26.5°C, 70 – 80 % humidity with a 12:12 photoperiod.

Pupae were picked with a 5 mL plastic transfer pipette every 24 hours after the commencement of pupation and placed into a Bug Dorm-1 (MegaView Science Company, Limited, Taichung, Taiwan) (Dimension: L 30 cm x W 30 cm x H 30 cm). Adult females were separated by age based on time of emergence (**Figure 4**). Adults

were fed *ad libitum* by soaking a cut cotton swab in a 10 % sugar solution. They were housed in the incubator until they were separated for deoxyribonucleic acid (DNA) extraction or reached the appropriate age for bottle bioassays (3 - 4 days old).

This rearing protocol deviated during the temephos larval bioassays. Prior to pupation, late-second and early-third instar larvae were removed and placed into a small cup of water. A mesh screen was placed over the cup and the cup was placed into the incubator.

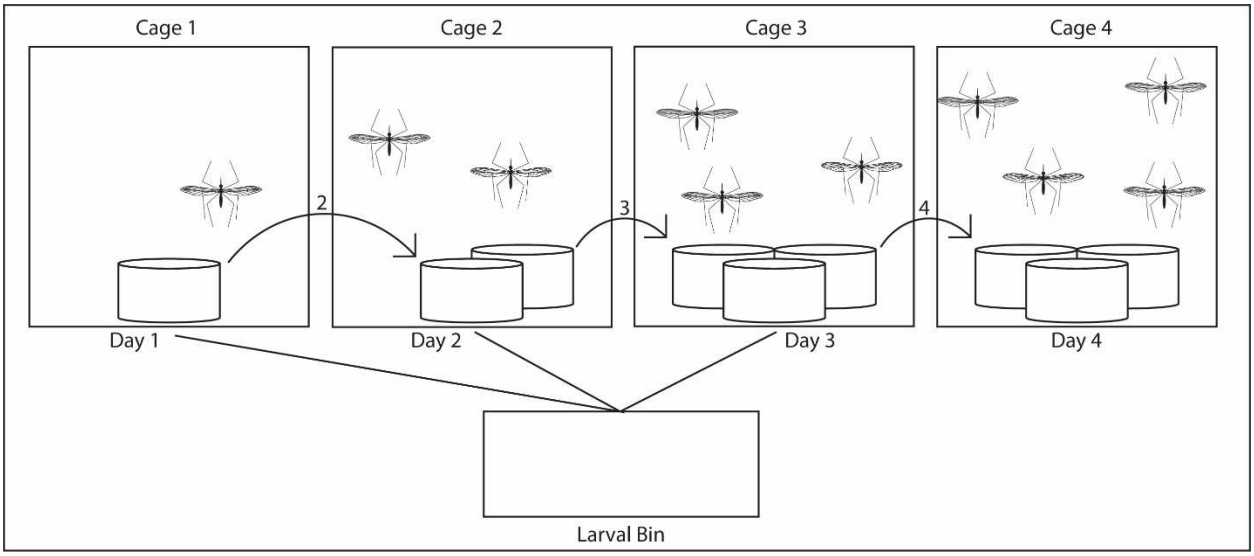

Figure 4. Cage setup for female age separation. Day 1) Pupae were selected from the larval bin and placed in Cage 1. **Day 2**) Pupa were selected from the larval bin and placed in Cage 2. Then, the remaining pupae from the container from Cage 1 was placed in Cage 2. **Day 3**) Pupa were selected from the larval bin and placed within Cage 3. Pupal containers from Cage 2 were placed within Cage 3. **Day 4**) Pupal containers were placed from Cage 3 to Cage 4.

Solution Preparation, Bottle Coating, and Set-up

Two stocks were prepared for the bottle bioassays. The first stock contained 100 mg of permethrin (technical grade; Chem Services, West Chester, PA) with 1.0 mL of acetone as a diluent (final concentration: 0.1 mg/µl) (CDC, 2012). This stock was stored at 4°C and used to prepare the second stock. The second stock contained 10 µl of stock one and 990 µl of acetone as a diluent (final concentration: 1.0 µg/µl).

Bottles were prepared by coating 250 mL Wheaton bottles (Wheaton Industries Incorporated, Millville, NJ) with a desired concentration of permethrin (Brogdon & McAllister, 1998). Control bottles were prepared with 1.0 mL of acetone only. The bottles were capped and rotated to ensure an even coating of insecticide within the bottle. The bottles were then stored for 24 hours in a dark environment with the caps removed to ensure all the acetone evaporated.

Approximately 25 3-to-4-day old female mosquitoes from each collection site were aspirated into a tube using a mouth aspirator with a built-in high efficiency particulate air (HEPA) filter (Model number: 612, John W. Hock Company, Gainesville, FL) (**Figure 5**). This process was continued until every bottle used in the assay had a tube with ~ 25 mosquitoes prepared. Mosquitoes were placed into each bottle by lightly tapping the inverted tube positioned at the opening of the bottle.

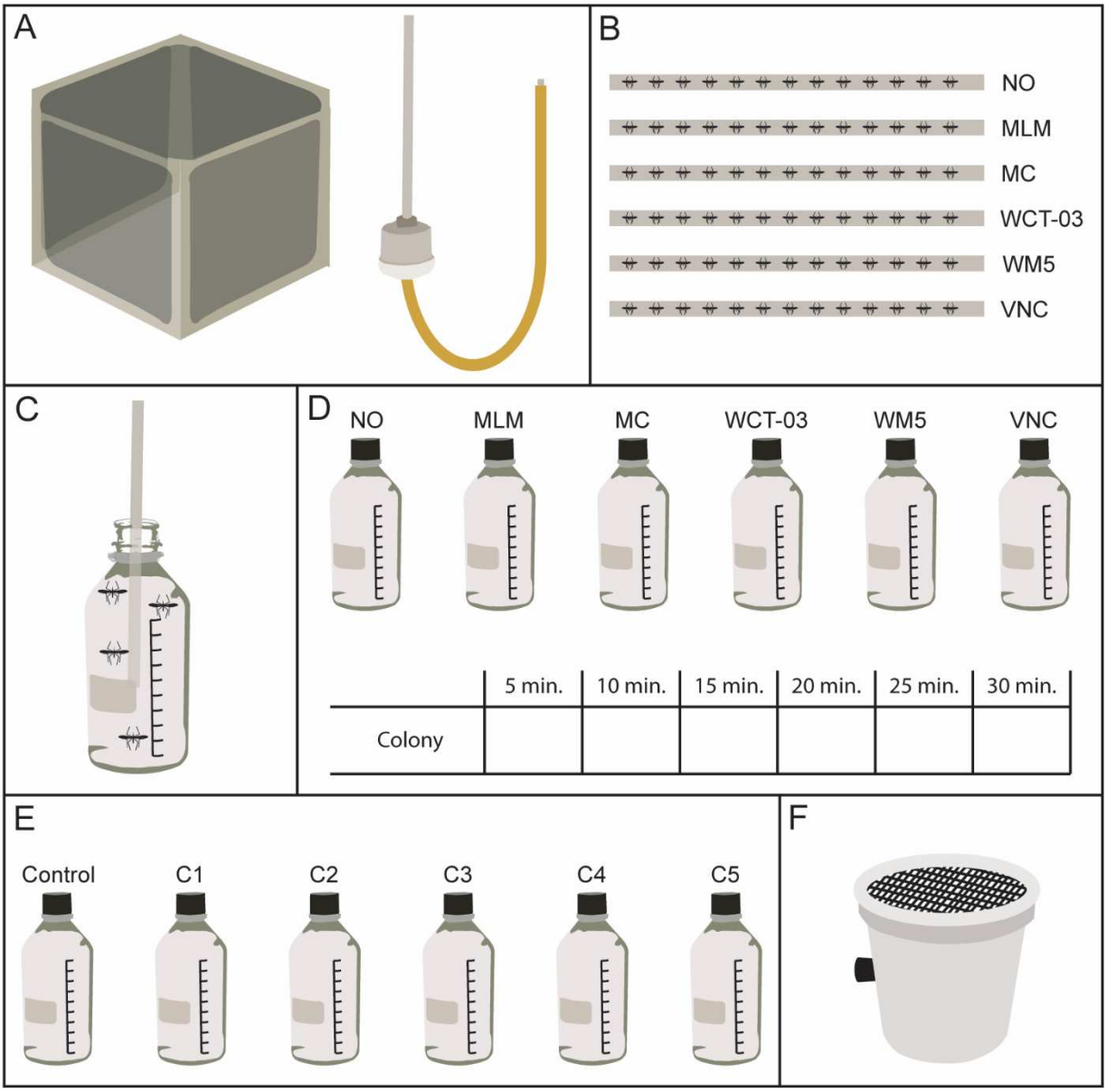

Figure 5. Bottle bioassay protocols. A) 25 individuals were aspirated from each colony into aspiration tubes. **B**) Aspiration tubes were gathered for each colony. **C**) Bottle caps were removed, and the aspiration tubes were inserted into the bottle opening and lightly tapped. **D**) Set up for the Diagnostic time (DT) and initial susceptibility test: knockdown rates were recorded every 5 minutes for each bottle which contained a consistent dose (15 µg/bottle). **E**) Lethal concentration 50 % (LC_{50}) setup for one colony (C indicated concentration). Concentration ranges were selected to produce 5 to 99 % mortality. **F**) One-pint storage cups were used to house mosquitoes for 24 hours after 1 hour of exposure.

Initial Susceptibility Test

Bottle bioassays were utilized to determine the DT for the known susceptible New Orleans (NO) colony using the CDC recommended DD (15 µg/bottle). The DT was defined as the time required to observe 100 % knockdown in NO. Knockdown was defined as immobility or the inability to stand/take off. Knockdown values were recorded every 5 minutes until the DT was determined. The knockdown percentages of the suspected resistant field collections were calculated at the DT. The results from the initial susceptibility test were interpreted using the WHO criteria which indicated resistance status. The criteria compared knockdown percentages to the DT and are as follows: if 98 to 100 % knockdown was observed, the collection is suspected to be susceptible. If 80 to 97 % knockdown was observed, resistance is suggested, but confirmation is needed. Lastly, resistance is indicated by less than 80 % knockdown (CDC, 2012). Four replicates were performed with the addition of a control bottle for each field collection site. Three replicates for the NO colony were sufficient to produce the DT. If less than 3 % knockdown was recorded in the control bottles, then no correction was needed. If greater than 10 % knockdown was recorded, then the assay was discarded. If there was 3 to 10 % knockdown, then Abbott's formula (depicted below) was used to produce a corrected knockdown value.

$$Corrected\ Knockdown = \frac{(Kd\ in\ Test\ Bottles\ [\%] - Kd\ in\ Control\ Bottle\ [\%])}{(100\% - Kd\ in\ Control\ Bottle\ [\%])} \times 100$$

Where Kd = Knockdown

Permethrin Resistance Confirmation

Confirmation of permethrin resistance was achieved by producing LC_{50} and RR_{50} values. LC_{50} values were calculated by exposing the suspected resistant collections to

five concentrations of permethrin, selected to achieve mortality percentages that ranged from 5 – 99 %. Control bottles, described above, were added to each replicate. The methodology for bottle preparation and the number of individuals were previously described. The number of mosquitoes knocked down was recorded every five minutes for 1 hour. To ensure that no mosquitoes escaped, both inactive and active mosquitoes were transferred into one-pint cups with mesh screens. The mosquitoes were fed 10 % sugar water solution *ad libitum* and placed into an incubator at 26.5°C with 70 – 80 % humidity with a 12:12 photoperiod for 24 hours, at which point mortality rates were documented. This process was repeated in triplicate for NO and the collection sites. Only LC_{50} values for NO, WCT-03, and WM5 were produced due to availability of F_1 mosquitoes. The RR_{50} values were calculated by dividing the LC_{50} values of the collection sites by the LC_{50} value of the NO colony. RR_{50} values greater than 1 indicated that the collection sites were more resistant to permethrin than the reference colony.

Resistance confirmation via identification of kdr alleles was achieved by performing AS-PCR assays. Remaining F_1 adult females from each collection site were aspirated into 1-pint cups affixed with mesh screens and placed into a -20°C freezer. Once anesthetized the individuals were transferred into Falcon® tubes and stored in a -80°C freezer. These individuals were designated for DNA extraction and AS-PCR analysis. The mosquito DNA was isolated using a single-tube extraction technique (Black & DuTeau, 1997). Once extracted, the DNA was resuspended in 150 µl of TE buffer (10 mM of Tris-HCl, 1 mM of EDTA, pH 8.0) and stored at -80°C. A ThermoScientific NanoDrop ND-1000® spectrophotometer was used to ensure that the DNA concentration and purity levels were appropriate for AS-PCR analysis.

The presence of non-synonymous point mutations at the 410, 1,016, and 1,534 loci were identified by measuring denaturation curves using AS-PCR with a CFX96™ Real Time-System, C1000™ thermocycler (BioRad, Hercules, CA) (**Figure 6**). The AS-PCR methodologies were described in Saavedra-Rodriguez et al. (2007, 2018) and Yanola et al. (2011). The primers used for AS-PCR were described in **Table 2.** Each reaction for V410L identification contained 50 µM of each forward primer, 100 µM of reverse primer, 9.7 µl of ddH2O, 10 µl of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and 1 µl of DNA template. The reaction followed conditions of 3 min. at 95°C, 39 cycles of 10 sec. at 95°C, 10 sec. at 60°C, 30 sec. at 72°C followed by a melting curve from 65°C to 95°C with increments of 0.2°C during 10 sec. Each reaction for V1,016I contained 500 µM of each primer, 9.88 µl of ddH2O, 10 µl of iQ SYBR Green Supermix, 1 µl of DNA template. The reaction followed the same thermocycler conditions as V410L. Each reaction for F1,534C contained 0.165 µM of C1,534fw primer, 0.5 µM of F1,534fw and 1,534rev, 9.53 µl of ddH2O, 10 µl of iQ SYBR Green Supermix, and 1 µl of DNA template. The reaction followed conditions of 3 min. at 95°C, 39 cycles of 10 sec. at 95°C, 10 sec. at 57°C, 30 sec. at 72°C followed by a melting curve from 65°C to 95°C with increments of 0.5°C during 5 sec. AS-PCR was performed on 48 females from MC, MLM, VNC, and 24 females from WCT-03 and WM5.

Table 2. Primers used for AS-PCR.

V410L	
V410fw	(5′-GCGGGCAGGGCGGCGGGGGCGGGGCCATCTTCTTGGGTTCGTTCTACCGTG-3′)
L410fw	(5′-GCGGGCATCTTCTTGGGTTCGTTCTACCATT-3′)
410rev	(5′-TTCTTCCTCGGCGGCCTCTT-3′)
V1,016I	
V1016fw	(5’-GCGGGCAGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCACCCCGCACCGG-3’)
I1016fw	(5’-GCGGGCACAAATTGTTTCCCACCCGCACTGA-3’)
1016rev	(5’-TGATGAACCSGAATTGGACAAAAGC3’)
F1,534C	
C1534fw	(5′-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3’)
F1534fw	(5’-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3’)
1534rev	(5’-TCTGCTCGTTGAAGTTGTCGAT-3’)

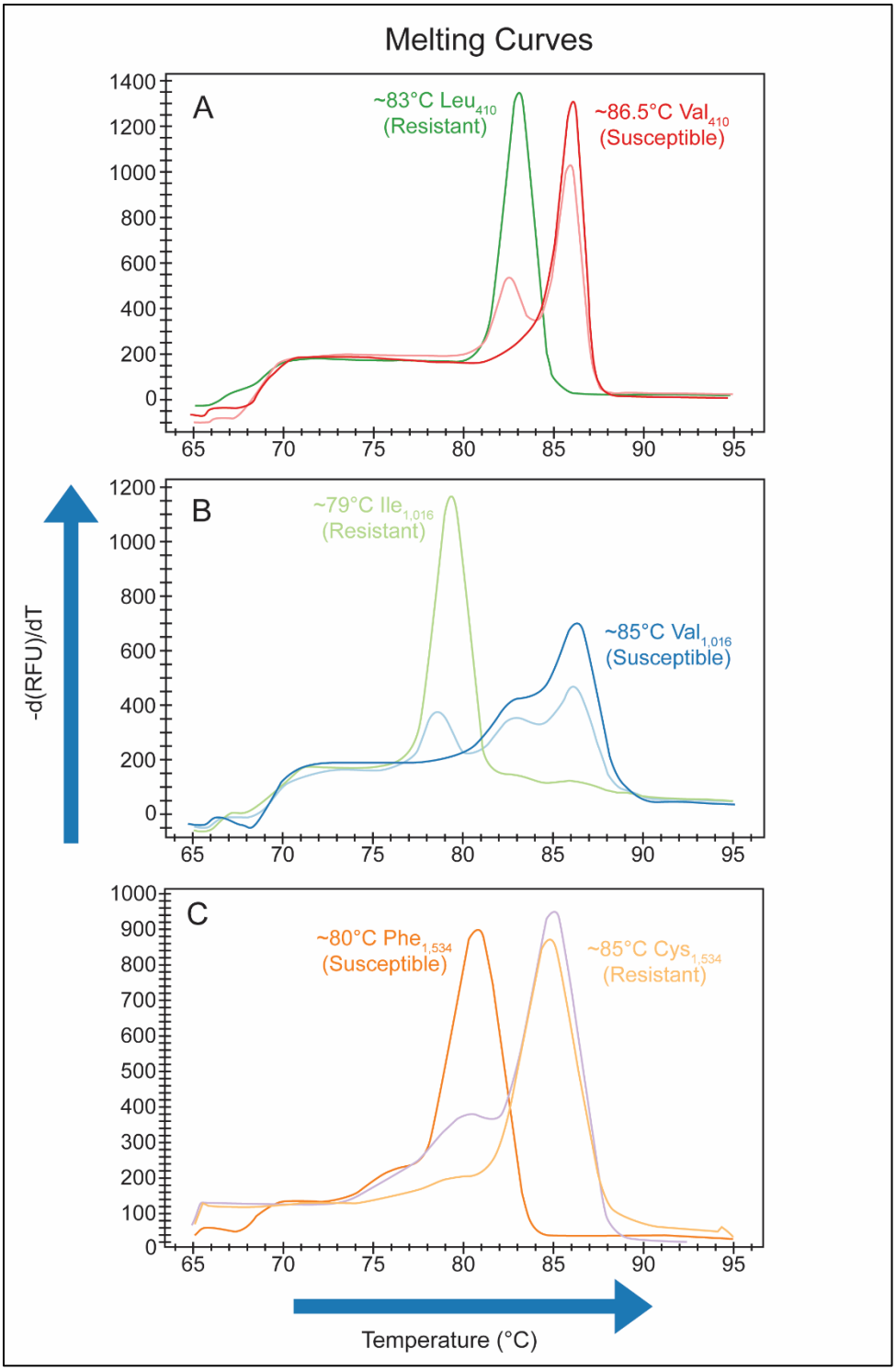

Figure 6. Melting curves for AS-PCR. Susceptible and resistant genotypes for: **A**) V410L: LL_{410} single peak at 83°C (resistant), VL_{410} peaks at both 83°C and 86.5°C, VV_{410} single peak at 86.5°C (susceptible). **B**) V1,016I: $II_{1,016}$ single peak at 79°C (resistant), $VI_{1,016}$ peaks at both 79°C and 85°C, $VV_{1,016}$ single peak at 85°C (susceptible). **C**) F1,534C: $FF_{1,534}$ single peak at 80°C (susceptible), $FC_{1,534}$ peaks at both 80°C and 85°C, $CC_{1,534}$ single peak at 85°C (resistant).

Temephos Resistance

A dose-mortality bioassay was performed to screen the collection sites for resistance by exposing late-second and/or early-third instar larvae to varying concentrations of temephos. Mortality levels were recorded at 24 hours of exposure. Five concentrations were selected to achieve mortality percentages that ranged from 5 - 99 %. No larvae past the third instar stage were used. This ensured that pupation did not occur during the 24-hour exposure period.

Three stocks were prepared for the larval bioassays. The first stock contained 100 mg of temephos (PS-655, Chem Service) with 1.0 mL of acetone as a diluent (final concentration: 0.1 mg/µl). This stock was stored at 4°C and used to prepare the second stock. The second stock contained 10 µl of stock one and 990 µl of acetone as a diluent (final concentration: 1.0 µg/µl). Stock three (ethanol stock) contained varying volumes of stock two and ethanol based on replicate amount.

To prepare individual bioassay cups, 95 mL of autoclaved water was added to a 200 mL plastic cup. A volume of 1.0 mL of ethanol stock (of appropriate temephos concentration) was then added to the plastic cup. The ethanol from the stock evaporated after fifteen to thirty minutes (WHO, 1981). While the ethanol evaporated, approximately 20 to 30 late-second and early-third instar larvae were picked and placed in a 5.0 mL dixie cup. After fifteen to thirty minutes the selected larvae were then placed in the 200 mL plastic cups and brought to a total volume of 100 mL with autoclaved water. This process was repeated for the control cups, which contained 95 mL of autoclaved water and 1 mL of ethanol, before being brought to a total volume of 100 mL. Each temephos concentration was tested three times for each collection site, as

well as for the NO colony. After 24 hours of continuous exposure, each cup was assessed for mortality proportions. The LC_{50} and RR_{50} values were calculated as previously described in the permethrin resistance confirmation methodology section.

Analysis and Statistics

Knockdown percentages and allele frequencies with 95 % confidence intervals were calculated in R (Version 4.3.2) using the *binconf* function from the *Hmisc* package (Harrell, 2023; R Core Team, 2023). Mortality proportions were analyzed using IRMA W Qcal software (http://sourceforge.net/projects/irmaproj/files/) to determine LC_{50} values for permethrin and temephos using a binomial logistic regression model (Lozano-Fuentes et al., 2012). The RR_{50} values for permethrin and temephos were produced by dividing the LC_{50} of the suspected resistant collection by the LC_{50} of the known susceptible colony. RR_{50}, allele frequency, and trilocus genotype combination plots were produced in R using the *ggplot2* (Wickham, 2016).

The collection sites were tested for HW equilibrium by calculating the expected genotype frequencies using the following series of equations (**Figure S1**):

$$R_{freq} = \frac{2O_{RR} + O_{RS}}{2O_{Tot}}$$

Where R_{freq} = Resistant allele frequency, O_{RR} = Observed number homozygous resistant individuals, O_{RS} = Observed number heterozygous individuals, O_{Tot} = Total number of individuals observed

$$S_{freq} = 1 - R_{freq}$$

Where S_{freq} = Susceptible allele frequency

$$E_{RR} = {R_{freq}}^2 \times O_{Tot}$$

Where E_{RR} = Expected number homozygous resistant individuals

$$E_{RS} = 2R_{freq} \times S_{freq} \times O_{Tot}$$

Where E_{RS} = Expected number heterozygous individuals

$$E_{SS} = {S_{freq}}^2 \times O_{Tot}$$

Where E_{SS} = Expected number homozygous susceptible individuals

The chi-squared statistic was calculated to test for significant deviations from HW using the following equation:

$$X^2_{[1\ d.f.]} = \frac{(O_{RR} - E_{RR})^2}{E_{RR}} + \frac{(O_{RS} - E_{RS})^2}{E_{RS}} + \frac{(O_{SS} - E_{SS})^2}{E_{SS}}$$

Deviations from HW equilibrium were measured using the F_{IS} inbreeding coefficient. This was calculated using the following equation:

$$F_{IS} = 1 - \frac{O_{RS}}{E_{RS}}$$

The collection sites were also grouped for HW equilibrium analysis due to close spatial distances between sites and to increase sample size. I suspected this would more accurately reflect conditions that would cause the population to deviate from HW equilibrium.

RESULTS

Initial Susceptibility Test

One hundred percent knockdown was observed in the known susceptible New Orleans (NO) colony after twenty minutes exposure of adult mosquitoes to permethrin (**Table 3**). The use of Abbott's formula to correct knockdown percentages was not required because < 3 % knockdown was observed in the control groups for the duration of the study. All sites had < 10 % knockdown at the DT. Knockdown percentages, from

largest to smallest, for the collection sites at the DT were as follows: WCT-03 (9 %), MC (5 %), WM5 (5 %), VNC (4 %), and MLM (2 %). The initial susceptibility test was concluded at 30 minutes.

Table 3. Knockdown percentages at the diagnostic time. The number of individuals tested, N, the number of knockdown individuals, and the knockdown percentages of the collection sites at the diagnostic time (DT) is shown with a 95 % confidence interval.

Site	N	Number of knockdown individuals	Knockdown percentage at DT (20 Min.)	95 % CI (%)
NO	75	75	100	(95 – 100)
MC	100	5	5	(2 – 11)
MLM	100	2	2	(1 – 7)
VNC	99	4	4	(2 – 10)
WCT-03	100	9	9	(5 – 16)
WM5	100	5	5	(2 – 11)

Permethrin Resistance Confirmation

The permethrin LC_{50} for NO was 0.6 µg/bottle (**Table 4**). WM5 had the largest permethrin LC_{50} value (43.16 µg/bottle) and a RR_{50} of 71.55, which was significantly different than the LC_{50} and RR_{50} values for WCT-03, 25.70 µg/bottle and 42.59, respectively (**Figure 7**). Permethrin LC_{50} and subsequent RR_{50} values for MC, MLM, and VNC were not calculated.

Table 4. Permethrin LC_{50} bottle bioassay calculations. Summary of LC_{50} values with 95 % confidence intervals (CI). Dose-response was adjusted to a binomial logistic regression model. The p-value, slope, intercept, and RR_{50} values for each collection are included. A p-value > 0.05 for the goodness-of-fit indicated that the observed data fits the binomial logistic regression model.

Site	LC_{50} (µg/bottle)	95 % CI (µg/bottle)	p-value	Slope	Intercept	RR_{50}
NO	0.60	0.54-0.67	0.66	3.39	1.71	-
WCT-03	25.70	24.51-26.94	0.75	8.14	-26.43	42.59
WM5	43.16	38.27-48.69	0.81	2.43	-9.15	71.55

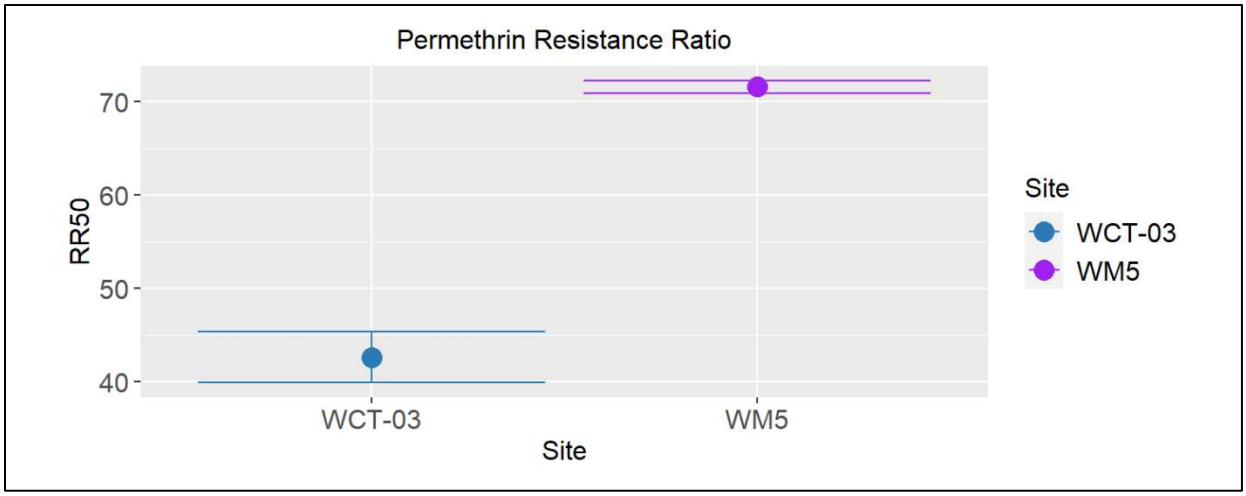

Figure 7. Plot of permethrin resistance ratios (RR_{50}). RR_{50} values for permethrin were calculated by dividing the LC_{50} values of the collection sites by the LC_{50} value of the susceptible (NO) colony. WCT-03 is shown in dark blue and WM5 in violet. RR_{50} values above 1 indicated that the collection site is more resistant to permethrin than the reference colony.

To confirm the presence of knockdown resistance in our collection sites, I determined the resistant allele frequencies at three kdr sites (V410L, V1,016I, and F1,534C). Resistant allele $C_{1,534}$ reached fixation in all 5 collection sites (**Figure 8**). Resistant alleles L_{410} and $I_{1,016}$ were present at the same frequency for MC (0.54), VNC (0.46), and WM5 (0.85). The frequency of resistant allele $I_{1,016}$ was slightly higher than L_{410}, though not significant, for MLM (0.61, 0.60, respectively) and WCT-03 (0.77, 0.73, respectively). Dilocus genotypes V410L and V1,016I were linked for MC, VNC, and WM5. Dilocus genotypes V410L and V1,016I were linked for MLM, except for one individual that was (VV_{410}, $VI_{1,016}$) (**Figure 9**). Dilocus genotypes were linked for WCT-03, except for two individuals that both had (VL_{410}, $II_{1,016}$) genotypes. WM5 had the largest proportion of the most resistant trilocus genotype combination (LL/II/CC) with 71 %, followed by WCT-03 (54 %), MLM (44 %), MC (33 %), and VNC (19 %).

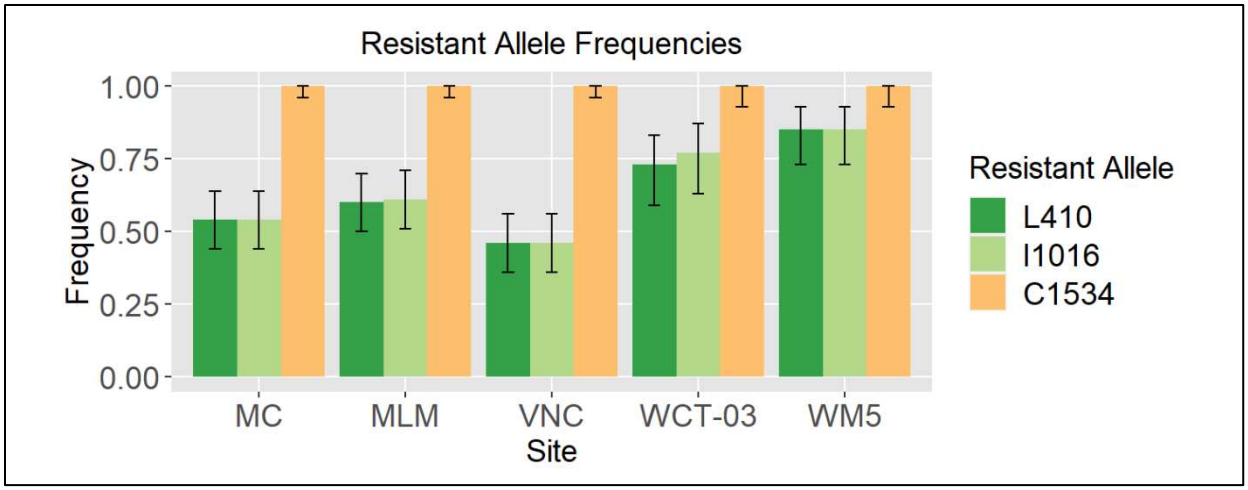

Figure 8. Kdr allele frequencies for the collection sites. The resistant allele frequency for L_{410} (dark green), $I_{1,016}$ (light green), and $C_{1,534}$ (light orange) at each of the five collection sites. Error bars indicate 95 % confidence intervals.

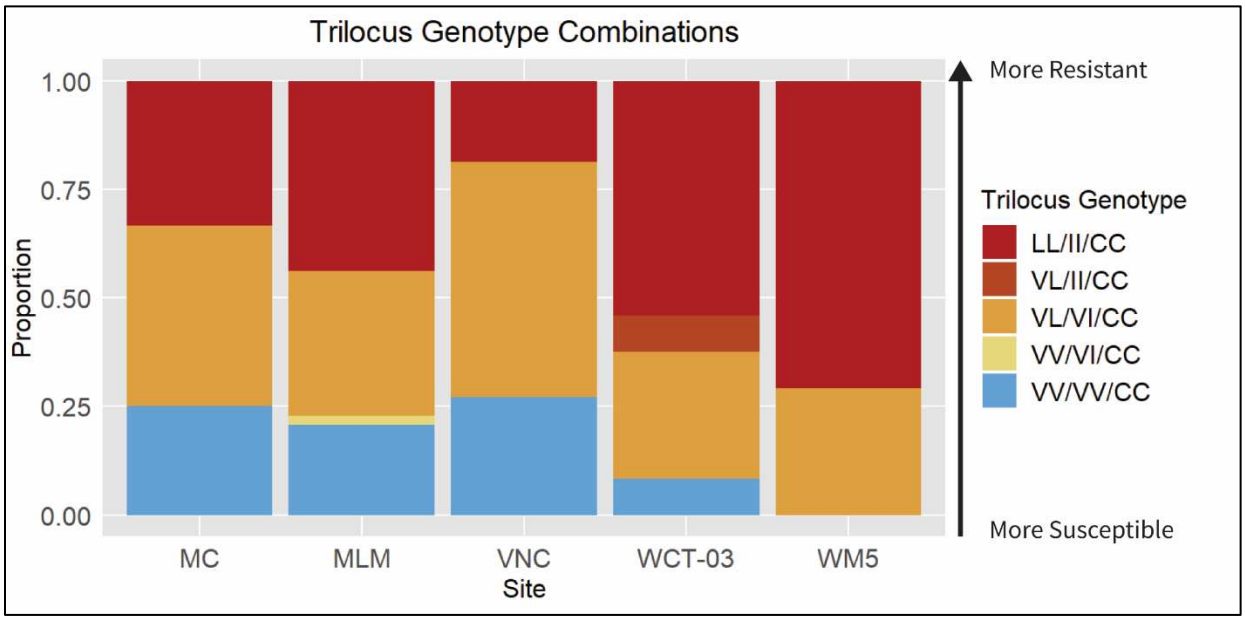

Figure 9. Proportion of trilocus genotype combinations. Proportions of trilocus genotypes appearing at each collection site. The first two letters indicate the V410L mutation where V = susceptible allele and L = resistant allele. The middle two letters indicate the V1,016I mutation where V = susceptible allele and I = resistant allele. The last two letters indicate the F1,534C mutation where F = susceptible allele and C = resistant allele. Combinations are depicted such that cooler colors represent more susceptible individuals and warmer colors represent more resistant individuals.

AS-PCR was also used to test the collections for HW equilibrium at each of the three kdr sites (**Table S1**). There was an excess of homozygotes for V410L and V1,016I for MC, MLM, and WCT-03 (**Table 5**). There was an excess of heterozygotes at VNC

and WM5. When analyzing sites independently, MLM had the only significant deviation (p-value < 0.05) from HW equilibrium, which occurred at the V410L site (F_{is} = 0.30). When analyzing sites collectively, significant deviations from HW equilibrium were observed at both the V410L and V1,016I kdr sites (F_{is} = 0.15 and 0.16, respectively).

Table 5. F_{IS} coefficients for the field collection sites. N indicates the number of individuals sampled for allele-specific polymerase chain reaction (AS-PCR). Bold values indicate significant deviations from Hardy-Weinberg equilibrium (p-value < 0.05). A positive F_{IS} value indicates an excess of homozygotes. A negative F_{IS} indicates an excess of heterozygotes. (-) indicates that the resistant allele has reached fixation.

Site	N	V410L	V1,016I	F1,534C
MC	48	0.16	0.16	-
MLM	48	**0.30**	0.25	-
VNC	48	-0.09	-0.09	-
WCT-03	24	0.05	0.17	-
WM5	24	-0.17	-0.17	-
COMBINED	192	**0.15**	**0.16**	-

Temephos Resistance

The temephos LC_{50} for NO was 0.011 µg/mL. VNC had the largest temephos LC_{50} (0.132 µg/mL), which was statistically different from the rest of the collection sites (**Table 6**). The second largest LC_{50} value was WM5 (0.094 µg/mL), followed by MLM (0.091 µg/mL), and MC (0.087 µg/mL). WCT-03 had the lowest LC_{50} at 0.062 µg/mL, which was statistically different from the rest of the collection sites.

Table 6. Temephos LC_{50} larval bioassay calculations. Summary of LC_{50} values with 95 % confidence intervals (CI). Dose-response was adjusted to a binomial logistic regression model. The p-value, slope, intercept, and RR_{50} values for each collection are included. A p-value > 0.05 for the goodness-of-fit indicated that the observed data fits the binomial logistic regression model.

Site	LC_{50} (µg/ml)	95 % CI (µg/ml)	p-value	Slope	Intercept	RR_{50}
NO	0.011	0.010-0.012	0.08	3.44	15.50	-
MC	0.087	0.078-0.097	0.53	3.00	7.31	7.86
MLM	0.091	0.083-0.100	0.90	3.90	9.33	8.22
VNC	0.132	0.124-0.140	0.06	4.90	9.93	11.86
WCT-03	0.062	0.056-0.068	0.23	4.00	11.13	5.58
WM5	0.094	0.088-0.100	0.24	4.68	11.07	8.45

VNC had the largest temephos RR_{50} (11.86), which was statistically different from the rest of the collection sites (**Figure 10**). The second largest RR_{50} value was WM5 (8.45), followed by MLM (8.22), and MC (7.86). The lowest temephos RR_{50} value, WCT-03 (5.58), was statistically different from the rest of the collection sites.

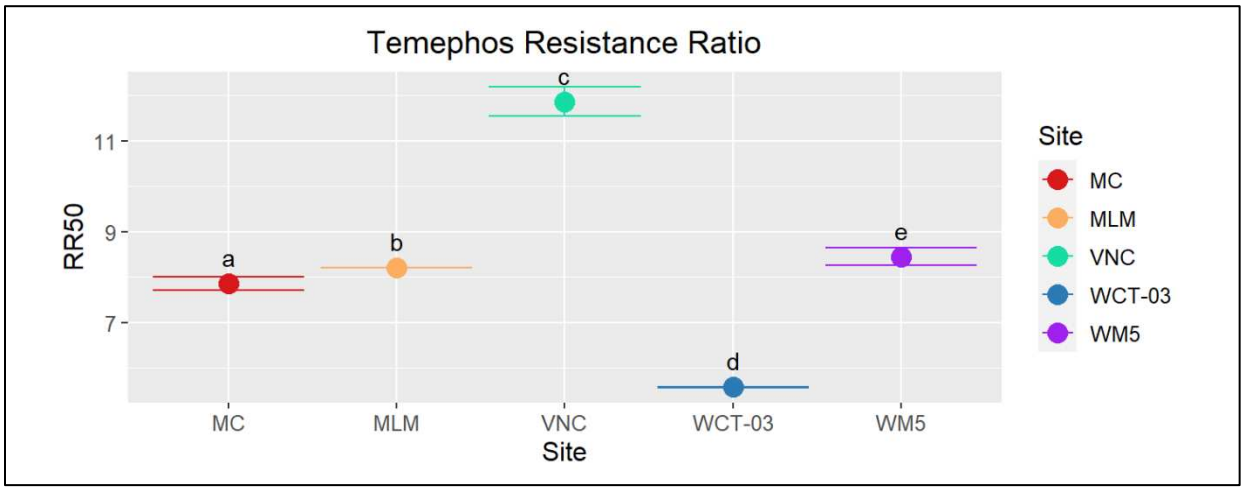

Figure 10. Plot of temephos resistance ratios (RR_{50}). RR_{50} values for temephos were calculated by dividing the LC_{50} values of the collection sites by the LC_{50} value of the susceptible (NO) colony. Sites appear in alphabetical order and are as follows MC (red), MLM (orange), VNC (green), WCT-03 (dark blue), and WM5 (violet). RR_{50} values above 1 indicated that the collection site is more resistant to temephos than the reference colony. Letters (a,b,c,d,e) indicated that the RR_{50} values were statistically different from one another.

DISCUSSION

Investigating the resistance status is critical because it can influence routine application strategies while maintaining outbreak response preparedness. The goal of my study was to gather preliminary data on permethrin and temephos resistance in *Ae. aegypti* populations collected in Mercedes and Weslaco, TX. Conducting resistance status studies is paramount in areas which have characteristics that facilitate the anthropophilic nature of *Ae. aegypti*, as it relates to controlling the spread of vector-borne diseases (VBD).

Initial Susceptibility Test

In 2012, the CDC provided a sample DT and DD, resulting in 100 % knockdown of susceptible individuals within 30 minutes when exposed to 15 µg/bottle. However, I observed a faster DT (20 minutes) using the known susceptible New Orleans (NO) colony (**Table 3**) (CDC, 2012). While it is important to note this discrepancy, the values provided by the CDC are a suggested starting point. The purpose of the initial susceptibility test was to screen for permethrin resistance by comparing knockdown percentages between the field collections and a known susceptible colony. In my study, NO was selected as the known susceptible colony because it has maintained permethrin sensitivity and has been free of kdr genes since 2005. The subsequent LC_{50} permethrin determination for NO at 0.6 µg/bottle is consistent with resistance status routinely observed within the laboratory (**Table 4**) (Vera-Maloof et al., 2020). Consistency between these results further validates the use of the NO colony as a reference for permethrin susceptibility. The CDC noted the DT is the most important value, as it will serve as a threshold in determining insecticide resistance within field

collections (CDC, 2012). It is crucial to validate the use of the known susceptible colony because mosquito control entities utilize different colonies as a reference for insecticide sensitivity. This will ensure that the quality of the comparison made between the suspected resistant field collections and the known susceptible colony are reliable (Moyes et al., 2017).

The presence of resistance was expected due to previously documented reports of resistance in the region (Bolling, 2019). In 2022, the CDC released updates to the susceptibility test in which the suggested DD for *Ae. aegypti* increased from 15 µg/bottle to 43 µg/bottle and the DT was reduced from 30 minutes to 10 minutes (CDC, 2022). Future studies using NO as a reference colony would likely observe a reduction in the DT using this DD value. Additionally, while this DD was not explicitly tested, the permethrin LC_{50} value for WM5 (43.16 µg/bottle) for one hour of exposure, suggests that less than 80 % knockdown would be observed if the updated DD was used. While a test duration of 2 hours was outlined by the CDC, knockdown percentages far below the 80 % threshold were justification for stopping the initial susceptibility test at 30 minutes, in favor of testing additional permethrin concentrations. While resistance confirmation under the WHO standards is not a requirement when knockdown percentages are below 80 %, the low knockdown percentages observed in my study warranted further investigation.

Permethrin Resistance Confirmation

The permethrin RR_{50} value was slightly over 40-fold in the WCT-03 collection and slightly over 70-fold in the WM5 collection (**Figure 7**). Both permethrin RR_{50} values depict a relatively high magnitude of resistance (Estep et al., 2018).

The most notable limitation of this study was the inability to produce a wide enough concentration range due to a lack of F_1 mosquito availability. This ultimately prohibited the permethrin LC_{50} determinations for MC, MLM, and VNC. In future studies, if F_1 mosquito availability is low, an option would be to rear subsequent generations until there is an adaquate number of individuals to perform all of the desired assays. This methodology has been previously described in a similar resistance study, with the caveat being that the number of generations reared remained well below 10, in order to reduce any resistance reversion (Kandel et al., 2019). Regardless, both WCT-03 and WM5 exhibited a high magnitude of resistance to permethrin and were similar to recent studies conducted in Florida and California (Cornel et al., 2016; Estep et al., 2018). Given the close proximity of the other sites, I hypothesized that similar resistance levels would be observed if LC_{50} values were determined.

Resistance confirmation via AS-PCR revealed that $C_{1,534}$ reached fixation in all 5 collection sites (**Figure 8**). This is not surprising as $C_{1,534}$ fixation has been documented in multiple locations in southern Mexico (Vera-Maloof et al., 2015). Additionally, resistant allele $C_{1,534}$ has been documented in high frequencies in Florida, New Mexico, and within the state of Texas (Estep et al., 2018; Hernandez et al., 2023; Kandel et al., 2019). Associations between high frequencies of the $C_{1,534}$ allele and large permethrin RR_{50} values (WCT-03 and WM5) are consistent with the findings by Hu et al. (2011), which suggested that the F1,534C mutation provides protection against type I pyrethroids (Hernandez et al., 2023; Hu et al., 2011). My study observed high frequencies of both L_{410} and $I_{1,016}$ resistant alleles at all the collection sites. Haddi et al. (2017) and Saavedra-Rodriguez et al. (2018) suggested that there is a strong

correlation between genotype and phenotype for V410L, either alone or accompanied by F1,534C. Both would confer resistance to type I pyrethroids. Additionally, binding site sensitivity is impacted when V1,016I and F1,534C are linked (Dong et al., 2014; Du et al., 2013). I found that the dilocus genotypes V410L and V1,016I were almost always linked and F1,534C reached fixation. This is consistent with previous reports (Mack et al., 2021).

My study observed a large proportion of homozygous resistant trilocus genotype combinations at each collection site, which explains the low levels of knockdown observed in the initial susceptibility test (**Figure 9**). Even though the collection sites are geographically close, differences in the proportion of resistant trilocus genotypes were observed. However, this result is not surprising as Estep et al. (2018) described collections from opposite ends of a neighborhood differing in the frequency of homozygous resistant $II_{1,016}$ individuals. I grouped the sites for the HW equilibrium analysis because I believed it would more accurately represent the population within Weslaco and Mercedes, TX. The significant deviations from the HW equilibrium for the V410L and V1,016I kdr sites were likely due to selection pressures from permethrin.

Temephos Resistance

The presence and magnitude of temephos resistance was confirmed by conducting larval bioassays to produce RR_{50} values. The temephos LC_{50} for NO in my study was determined to be 0.011µg/ml, which is consistent with resistance routinely observed within the laboratory (**Table 6**) (Saavedra-Rodriguez et al., 2014). Consistency between these results validates the use of the NO colony as a reference for temephos susceptibility. All temephos RR_{50} values were statistically distinct from one

another. They ranged from approximately 6-fold resistance in the WCT-03 collection and nearly reached 12-fold resistance in the VNC collection (**Figure 10**). The lower resistance magnitude shown for temephos, compared to permethrin, may be attributed to cessation of temephos production since 2016 in the CONUS. While the resistance magnitudes for temephos and permethrin appear to be associated with one another when comparing WCT-03 and WM5 (**Figure 7** and **Figure 10**), previous evidence suggests that there is no cross-resistance between the two insecticides and that independent mechanisms are likely responsible (Saavedra-Rodriguez et al., 2014). While outside the scope of this study, future studies would benefit from performing biochemical assays to confirm that detoxification enzymes are the primary mechanism of temephos resistance (Saavedra-Rodriguez et al., 2014).

CHAPTER 3: FITNESS COSTS ASSOCIATED WITH MUTATIONS IN THE VOLTAGE-GATED SODIUM CHANNEL

SUMMARY

Insecticides remain the most utilized method to reduce mosquito populations which transmit viruses of high public health importance. However, consistent use of the same insecticides has produced strong resistance mechanisms. When the use of these insecticides is halted, resistant allele frequencies reduce, and populations trend towards susceptibility. This suggests that there are fitness costs acting as drivers during this reversion process. Studying the factors that drive fitness costs is crucial for public health because it provides data that can be used to develop effective strategic control plans. Wing length and egg production are two metrics that have been described as fitness costs in resistant *Aedes aegypti* (*Ae. aegypti*). These fitness costs have been associated with resistant alleles within the voltage-gated sodium channel (VGSC). I examined two resistant populations, largely free of pyrethroids since 2013, to determine if two resistant alleles at the 410 and 1,016 VGSC sites were associated with wing length and egg production. I also examined the appropriateness of using wing length as a covariate in egg production analyses. I found a significant difference in mean wing length between the homozygous resistant (RR) and homozygous susceptible (SS) genotypes at the 410 and 1,016 loci. The interaction between wing length and genotype had no significant effect on egg production. Genotype and wing length, as individual variables, had no significant effect on egg production.

INTRODUCTION

Vector management programs rely heavily on the use of insecticides to reduce mosquito populations which are responsible for transmitting viruses known to cause human diseases (Van Den Berg et al., 2021). As discussed in previous chapters, insecticide applications act as a selection pressure on mosquito populations, which leads to the development of resistance mechanisms (e.g. overexpression of detoxifying enzymes or target site modifications) (Liu, 2015). It has been shown that in the absence of these intense selection pressures, resistant allele frequencies reduce, and populations trend towards susceptibility (Brito et al., 2013; Parker-Crockett et al., 2022; Penilla-Navarro et al., 2024). This suggests that an association exists between fitness costs and the mechanisms responsible for resistance (Crow, 1957). The relationship between fitness and resistance is important because Integrated Resistance Management (IRM) practices can utilize association studies to describe how populations may revert to susceptibility. This can help develop effective strategies like rotational applications, creating zones left intentionally untreated, or suspending applications of specific insecticides for a calculated amount of time. Additionally, the magnitude of a fitness cost can impact the rate of reversion (Schechtman & Souza, 2015). In this study, I examined two populations in Southern Mexico, which have largely been free of pyrethroid exposure since 2013, to determine if two knockdown-resistant (kdr) alleles at the 410 and 1,016 voltage-gated sodium channel (VGSC) sites had an association with two fitness cost metrics: wing length and egg production.

Pyrethroids were used intensively in Tapachula, Mexico from 2000 until 2013 when they were phased out and replaced with organophosphates (Aponte et al., 2013;

Penilla-Navarro et al., 2024). Intense pyrethroid use during this time selected for kdr alleles and high levels of resistance developed. These kdr alleles are the result of non-synonymous point mutations that encode for an amino acid replacement within the VGSC (Saavedra-Rodriguez et al., 2021). In Mexico, the amino acid replacements associated with varying levels of pyrethroid resistance are V410L, V1,016I, and F1,534C (Saavedra-Rodriguez et al., 2018). Saavedra-Rodriguez et al. (2018) found that the resistant allele frequency of L_{410}, $I_{1,016}$, and $C_{1,534}$ rapidly increased from 2000 to 2016. A recent study monitored pyrethroid resistance levels and resistant allele frequencies in 24 mosquito populations in Tapachula between 2016 and 2020. These findings indicated *Ae. aegypti* populations had reversion towards susceptibility in the absence of pyrethroid use, shown by a reduction in resistant allele frequencies and LC_{50} (lethal concentration 50 %) values (Penilla-Navarro et al., 2024). In 2018, collections were made within Tapachula to screen for resistant alleles and determine the magnitude of resistance to type I and type II pyrethroids (Solis-Santoyo et al., 2021). Colinas del Rey (COL) and Raymundo Enriquez (RAY) were two sites that mosquitoes were collected from in 2018. Solis-Santoyo et al. (2021) reported the resistant allele $I_{1,016}$ in the following frequencies: 0.32 for COL and 0.39 for RAY. Within the same collection, COL had a $C_{1,534}$ frequency of 0.99, and RAY reached fixation (Solis-Santoyo et al., 2021). Based on previous reports made by Mack et al. (2021) on V410L and V1,016I linkage, the L_{410} resistant allele frequencies for COL and RAY would likely be similar to that of $I_{1,016}$. I used mosquitoes from COL and RAY, which were part of the same collections made in 2018, to perform my fitness cost study. I selected these localities and mosquitoes because the region was subjected to intense pyrethroid

exposure from 2000 to 2013, and resistant alleles were still present years after discontinuing pyrethroid use (Aponte et al., 2013; Solis-Santoyo et al., 2021).

A variety of fitness costs have been associated with insecticide resistance across pest species, and specific resistance mechanisms can dictate the fitness metric (Kliot & Ghanim, 2012). For instance, it was observed in *Culex pipiens* that the overexpression of detoxifying enzymes impacted energetic resource allocation (resource-based trade-offs) which reduced adult longevity (Rivero et al., 2011). Rivero et al. (2011) also found significantly less energetic reserve in individuals that had acetylcholinesterase modifications, likely caused by nervous system hyperactivation. In comparison, in the absence of insecticides, target site modifications have been shown to alter VGSC functions such as gating properties. For instance, both the voltage-dependent activation and the steady state inactivation curves positively shifted in the pyrethroid-resistant VGSC (D. Lee et al., 1999). Lee et al. (1999) also observed behavioral changes in pyrethroid-resistant individuals which was attributed to an increase in the action potential threshold. Several mutations within the VGSC of *Ae. aegypti* mosquitoes have been associated with fitness costs. Brito et al. (2013) observed that $I_{1,016}$ and $C_{1,534}$ resistant alleles were associated with an increase in locomotive activity and repressed fecundity. Associations between other kdr alleles and an increase in larval developmental time, smaller wings, and a reduction in adult longevity have also been described (Rigby et al., 2020). However, Rigby et al. (2020) suggested that the lower fecundity levels were not attributed to the pleiotropic effects of kdr. Variation in study designs may explain the variety of contradictory fitness results.

Making comparisons between two unrelated mosquito strains has been a weakness in fitness cost studies (Ffrench-Constant & Bass, 2017). To obtain a similar genetic background between the susceptible and resistant strains, studies have performed backcrossing or selection processes (Argentine et al., 1989). In this study, similar to Bourguet et al. (2004), I compared fitness cost metrics between individuals with different kdr genotypes from the same geographical area. I achieved this by selecting females and segregating them into individual habitation cups, so that egg production could be linked with a specific genotype retrospectively. This experimental design would encompass homozygous susceptible (SS), heterozygous (RS), or homozygous resistant (RR) individuals at the 410 and 1,016 kdr sites. Ffrench-Constant and Bass (2017) highlighted the importance of conducting fitness cost studies using field-collected mosquitoes in the field, however my study was performed in a laboratory with field-collected mosquitoes. While this may be considered a weakness to Ffrench-Constant and Bass (2017), I aimed to associate specific kdr mutations with egg production, under controlled parameters, while accounting for wing length as a potential second fitness cost.

Wing length was an important measurement to include in my study for two reasons. The first reason is that it has been described as a fitness cost, and the second is that it can be utilized as a proxy for body size (Briegel, 1990; Nasci, 1987; Rigby et al., 2020). Body size is an important factor influencing egg production. Briegel et al. (1990) observed larger females imbibed two times as much blood and produced nearly four times as many eggs. In their study, larger females were described to have a mean wing length of 3.4 mm, while smaller females had a mean wing length of 2.4 mm.

GOAL

The goal of my study was to identify if an association exists between the V410L and V1,016I genotypes and egg production. This was achieved by segregating individuals into habitation cups to link genotypes with egg production, while accounting for wing length as both a covariate and a potential fitness cost. It was hypothesized that L_{410} and $I_{1,016}$ resistant alleles would be associated with decreased egg production ($\mathbf{H_1}$). This was hypothesized based upon previous studies identifying associations between VGSC mutations and decreased egg production.

METHODOLOGY

Mosquito Collections

Ae. aegypti mosquitoes used in this study were collected by the Centro Regional de Investigación en Salud Pública / Instituto Nacional de Salud Pública (CRISP/INSP) in 2018, between January and April. The two collection sites in Tapachula, Mexico included Colinas del Rey (COL, 14°55’50.9’’N, 92°14’50.2”W), and Raymundo Enriquez (RAY, 14°53′1.4″N, 92°18’48.8″W). Twelve oviposition cups (1-liter plastic cups lined with 15 cm of # 615 filter paper) were placed at each collection site, with weekly collections taking place between January and April. F_0 eggs were reared at 27 ±2°C, 70 – 80 % humidity with a 12:12 photoperiod. Upon emergence, they were identified to species by CRISP/INSP (Rueda, 2004). F_0 adult *Ae. aegypti* females were blood fed following procedures outlined in Penilla-Navarro et al. (2024). F_1 eggs were preserved and shipped to Colorado State University (CSU), Fort Collins, CO. Refer to **Figure 11**

for the geographical locations of the collection sites. My study was conducted in 2018 at CSU and used male and female adults reared from F_1 eggs sent by CRISP/INSP.

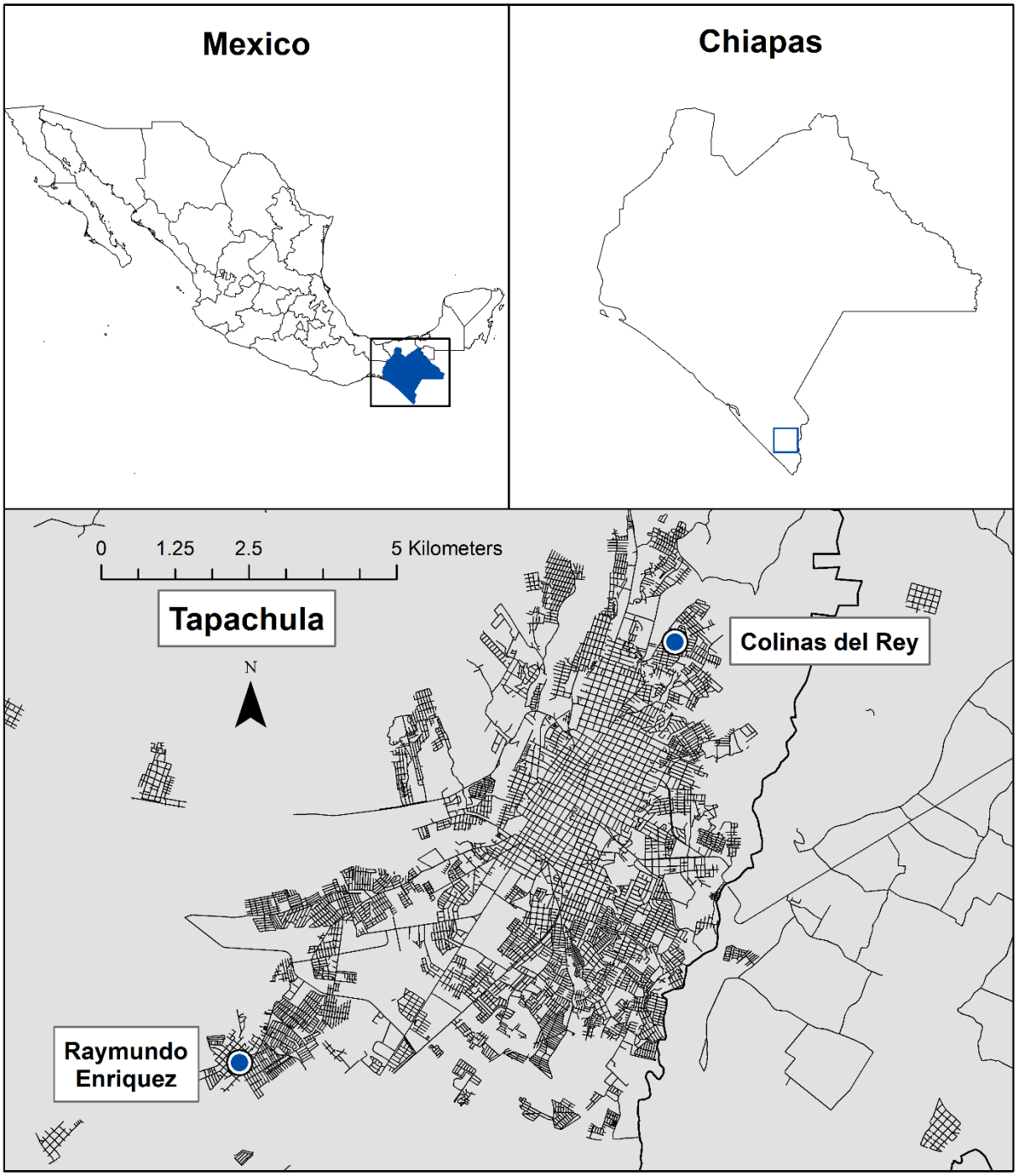

Figure 11. Map of *Ae. aegypti* collection sites in Tapachula, Mexico in 2018

Biological Replicates

One biological replicate is represented in **Figure 12**. Fifty biological replicates were performed for each collection site at one time equaling one round. This process was repeated for a second round which provided the study with a total of 100 biological replicates per collection site.

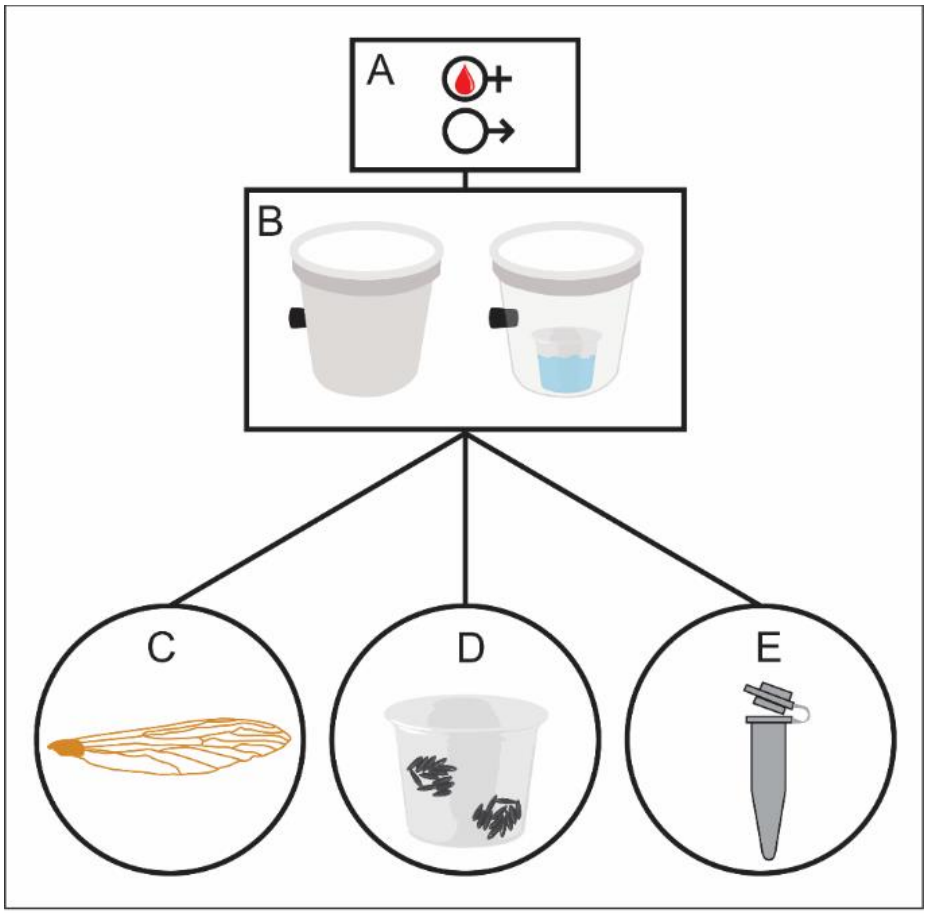

Figure 12. Overview of one biological replicate. A) One 3-day old blood-fed female and one male were aspirated using a mouth aspirator with a built-in high efficiency particulate air (HEPA) filter into a 1-pint paper habitation cup. **B**) To facilitate oviposition, a 5 mL cup (lined with filter paper and filled with 1 mL of water) was placed inside of the 1-pint cup. Mosquitoes were fed *ad libitum* with a 10 % sucrose solution. After five days the female was anesthetized (fully described in the mosquito anesthetizing subsection). **C**) The left wing was removed and measured. **D**) The egg paper was removed, and the eggs were counted. **E**) Deoxyribonucleic acid (DNA) from the female was extracted to identify V410L, V1,016I, and F1,534C genotypes via allele-specific polymerase chain reaction (AS-PCR) analysis.

Rearing

Details regarding the rearing process are outlined in the Rearing subsection within Chapter 2.

Egg Production

Adult females were housed for three days in a cage with an even sex ratio to ensure insemination (Rigby et al., 2020). Adult age separation was achieved by transferring pupae cups between cages daily (**Figure 13**). An even sex ratio was achieved by visually inspecting cages daily and transferring male mosquitoes between cages using a mouth aspirator with a built-in high efficiency particulate air (HEPA) filter (Model number: 612, John W. Hock Company, Gainesville, FL) as needed. Three-day old females were provided an artificial blood meal using a glass membrane feeder (Lillie Glass Blowers, Smyrna, GA) with heated citrated sheep blood and hog gut (Colorado Serum Company, Denver, CO). Following the blood meal, one engorged female and one male were aspirated into a one-pint paper habitation cup. Insemination typically occurs prior to a blood meal, however a recent study suggested that it also occurs after (Dieng et al., 2019). For this reason, the male mosquito was added to the habitation cup for the length of the study.

The habitation cup was labeled with the appropriate identifiers (round, collection site, and biological replicate number). An oviposition cup (5 mL plastic opaque dixie-cup lined with filter paper and filled with 1 mL of water), with corresponding identifiers, was placed inside the habitation cup prior to the aspiration of mosquitoes. A fine mesh screen was affixed to the top of the habitation cup, which allowed the adults to feed *ad libitum* on a 10 % sucrose solution. The habitation cup was maintained at 26.5°C, 70 –

80 % humidity with a 12:12 photoperiod for five days to allow egg oviposition (Gusmão et al., 2010).

On the fifth day, females were anesthetized (fully described in the next subsection), and the oviposition cup was then removed, placed on a tray, and stored in the incubator. Egg counts for all biological replicates were made after obtaining the wing length measurements and the females were stored for eventual DNA analysis. The filter paper inside the oviposition cup was sprayed with tap water to reduce the possibility of losing eggs when transferring to the dissecting microscope. The eggs for each biological replicate were manually counted using a dissecting microscope with the aid of a tally counter.

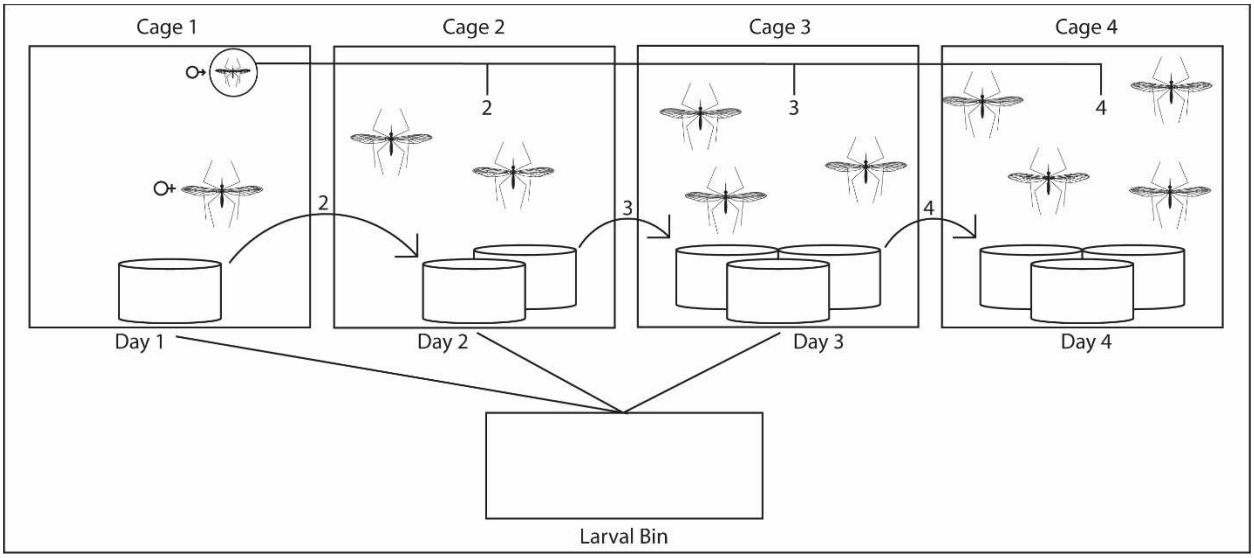

Figure 13. Female age separation and even sex ratio. Day 1) Pupae were selected from the larval bin and placed in Cage 1. **Day 2**) Pupae were selected from the larval bin and placed in Cage 2. Then, the pupal container from Cage 1 was placed in Cage 2. To achieve an even sex ratio, male mosquitoes from Cage 1 were aspirated to Cage 2. **Day 3**) Pupae were selected from the larval bin and placed within Cage 3. Pupal containers from Cage 2 were placed within Cage 3. To achieve an even sex ratio, male mosquitoes were aspirated from Cage 2 into Cage 3. **Day 4**) Pupal containers were placed from Cage 3 to Cage 4. Male mosquitoes were aspirated from the previous cages into Cage 4 to achieve an adequate sex ratio**.**

Mosquito Anesthetizing

The mosquitoes were anesthetized using FlyNap® (FlyNap® Anesthetic Kit, Item # 173010, Carolina Biological Supply Company, Burlington, NC) (**Figure 14**). To anesthetize the female and male mosquito, a plastic cup was inverted and placed over the habitation cup creating an airtight seal. The rubber stopper plugging the aspiration hole was removed, and the bent FlyNap® applicator was inserted into this hole and surrounded by a cotton swab to ensure the mosquitoes could not escape. After approximately five minutes, both male and female mosquitoes were anesthetized. Once anesthetized, the inverted cup, the habitation cup cover, and the FlyNap® applicator were removed. The male mosquito was then discarded and, using forceps, the female mosquito was removed for wing length measurement and DNA analysis. The oviposition cup was removed and stored.

Wing Length

To obtain the wing length measurement, the anesthetized female was first positioned under a dissecting microscope. Pressure was applied to the dorsal portion of the thorax with forceps to make the wing erect without damaging its structure. The left wing was then removed using a scalpel and transferred under a binocular Carl Zeiss™ (Oberkochen, Baden-Wurttemberg, Germany) AxioLab™ A1 light microscope, fitted with an ocular micrometer. Once positioned under the microscope, the wing was measured from the axillary incision to the apical margin, excluding the fringe hairs (**Figure 15**) (Nasci, 1987). Wing length measurements were converted from ocular micrometer units to millimeters.

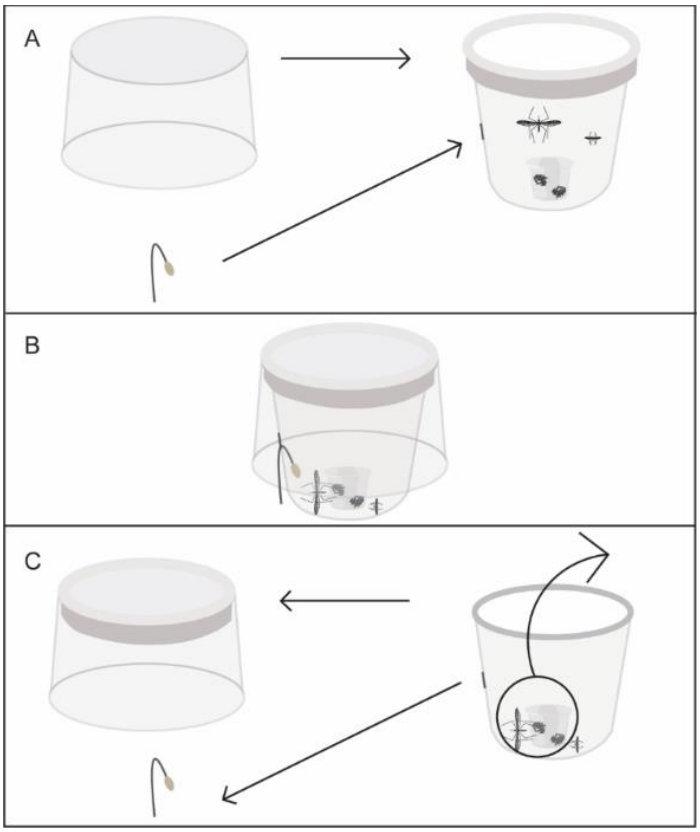

Figure 14. Overview of anesthetizing protocol. A) The bent FlyNap® applicator was placed into the aspiration hole, and an airtight seal was achieved by placing an inverted plastic cup on top of the habitation cup. **B**) The FlyNap® took approximately 5 minutes to anesthetize the mosquitoes. **C**) Once anesthetized, the plastic cup, the habitation cup lid, and the FlyNap® applicator were removed and placed aside. The female mosquito was removed with a forceps, and the oviposition cup was removed and placed on a tray.

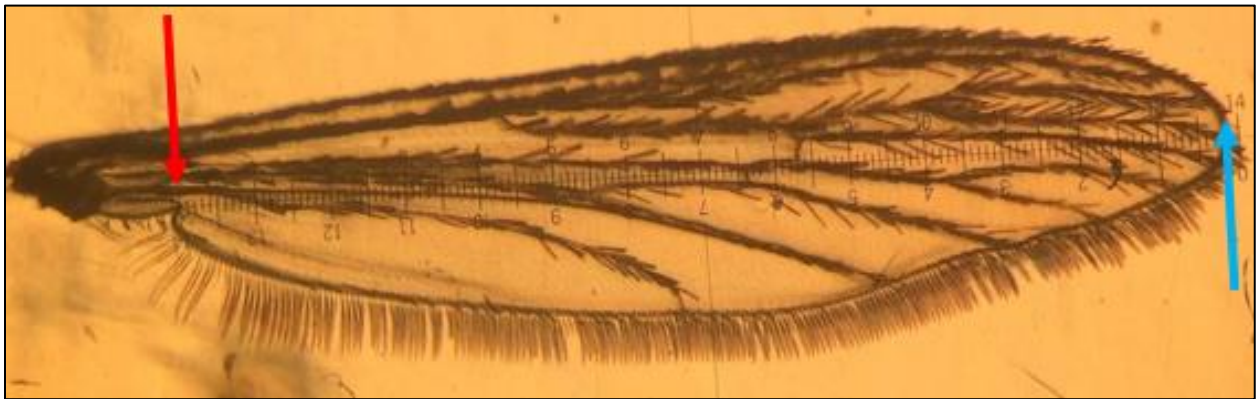

Figure 15. **Wing length measurement.** The left wing of the female was measured in ocular micrometer units and converted to millimeters. Wing length was described as the measurement between the axillary incision (red arrow) and the apical margin (blue arrow), excluding the fringe hairs.

Trilocus Genotyping

Following wing removal and measurement, the female was placed into a microcentrifuge tube and stored at -80°C. For protocols on DNA extraction and AS-PCR analysis, refer to the Permethrin Resistance Confirmation subsection in the Methodology section within Chapter 2. The AS-PCR results were displayed as genotypes, which represent non-synonymous point mutations that encode for an amino acid replacement within the VGSC (Saavedra-Rodriguez et al., 2021). The genotype combinations were arranged by kdr site: (V410L / V1,016I / F1,534C) where (410: VV = homozygous susceptible, VL = heterozygous, and LL = homozygous resistant), (1,016: VV = homozygous susceptible, VI = heterozygous, and II = homozygous resistant), (1,534: FF = homozygous susceptible, FC = heterozygous, and CC = homozygous resistant).

Analysis and Statistics

Initial selection criteria removed individuals that died prior to allotted oviposition time, had damaged wings preventing measurement, or had unassigned genotypes via AS-PCR. Fifty out of 200 biological replicates were removed based on initial criteria, leaving 150 females left for analyses. Allele frequencies with 95 % confidence intervals were calculated in R (Version 4.3.2) using the *binconf* function from the *Hmisc* package (Harrell, 2023; R Core Team, 2023). The collection sites were tested for Hardy-Weinberg (HW) equilibrium by calculating the expected genotype frequencies (**Table S2**). Significant deviations from HW equilibrium were calculated by performing a chi-square test. These data were displayed as a table comparing F_{is} inbreeding coefficients (for HW equilibrium and F_{is} equations, see the Analysis and Statistics subsection in the

Methodology section within Chapter 2). Based on trilocus genotype combinations, three additional individuals were removed, leaving 147 individual females for the remainder of the analyses (**Table S3A - D**). I compared resistant allele frequencies, deviations from HW equilibrium, and trilocus genotype combinations to determine if it was appropriate to group the collection sites for further analyses.

A one-way analysis of variance (ANOVA) was constructed using the *lm* function in R to test whether there was a significant difference in wing length between the three genotypes. Genotype was used as the independent variable, and wing length was used as the dependent variable. The assumption of normality was visually checked using the normal Q-Q plot, and a Shapiro-Wilks test was conducted on the residuals of the ANOVA model using the shapiro.test function from the base *stats* package in R. This assumption was met (**Figure S1**, p-value: 0.64). The assumption of homogeneity of variance was visually checked using the residuals vs. fitted plot, and a Levene's test was conducted on the residuals of the model using the *leveneTest* function from the *car* package in R (Fox & Weisberg, 2019). This assumption was met (**Figure S1**, p-value: 0.47). The results from this analysis were displayed in a type III ANOVA table (**Table S4**). Using the Tukey's Honestly Significant Difference (HSD) method as a post-hoc test, I performed pairwise comparisons by calculating Tukey adjusted p-values with the *emmeans* package in R (**Table S5A – B**) (Russell, 2024).

A one-way analysis of covariance (ANCOVA) was constructed using the *lm* function in R to test whether there was a significant difference in egg production between the three genotypes, while accounting for wing length. This method allowed for the inclusion of the wing length and genotype (wing length*genotype) interaction term.

Egg production was the dependent continuous variable, genotype was the independent categorical variable, and wing length was the continuous covariate. First, the assumption of homogeneity of regression slopes was checked by including wing length*genotype as an interaction term. The scatter plot comparing egg production and wing length was visually checked for parallelism between regression slopes. The assumption of homogeneity of regression slopes was met (F-value: 0.44, p-value: 0.65), and the ANCOVA model was simplified by excluding the wing length*genotype interaction term. The assumption of normality was not met (**Figure S2**, p-value = 0.001), however the assumption of homogeneity of variance was (**Figure S2**, p-value = 0.82). The normality and homogeneity of variance assumptions were checked using the methods described above. Additionally, “rounds” were treated as a blocking variable due to two independent cycles of biological replicate observations. The results from this analysis were displayed in a type III ANOVA table (**Table S6**). All the figures in this study were produced using the *ggplot2* package in R (Wickham, 2016).

RESULTS

Fifty of the 200 individuals were removed from the analysis based on the initial selection criteria. Resistant allele frequencies, deviations from HW equilibrium, and trilocus genotype combinations were compared to determine the appropriateness of grouping the collection sites for the ANOVA and ANCOVA analyses.

Resistant allele frequencies for L_{410}, $I_{1,016}$, and $C_{1,534}$ were determined by screening all biological replicates at the three kdr sites via AS-PCR (**Figure 16**). There were no significant differences in the resistant allele frequencies between the two collections for any of the kdr sites. However, COL had higher L_{410} and $I_{1,016}$ frequencies

than RAY (COL: 0.41, RAY: 0.32) and (COL: 0.41, RAY: 0.34), respectively. RAY reached $C_{1,534}$ fixation, and COL was just below fixation (0.97).

There were no significant deviations from HW equilibrium for either collection at the V410L and V1,016I sites (**Table 7**). The inbreeding coefficients (F_{is}) for COL indicated a slight excess of homozygotes at the V410L and V1,016I sites (0.05, 0.05, respectively). The F_{is} for RAY indicated a slight excess of heterozygotes at the V410L and V1,016I sites (- 0.05, - 0.03, respectively). A significant deviation from HW equilibrium was observed at the F1,534C site for COL (p-value < 0.05, $F_{is} = 1$), and RAY reached resistant allele fixation. See **Table S2** for chi-square analysis, and inbreeding coefficient data.

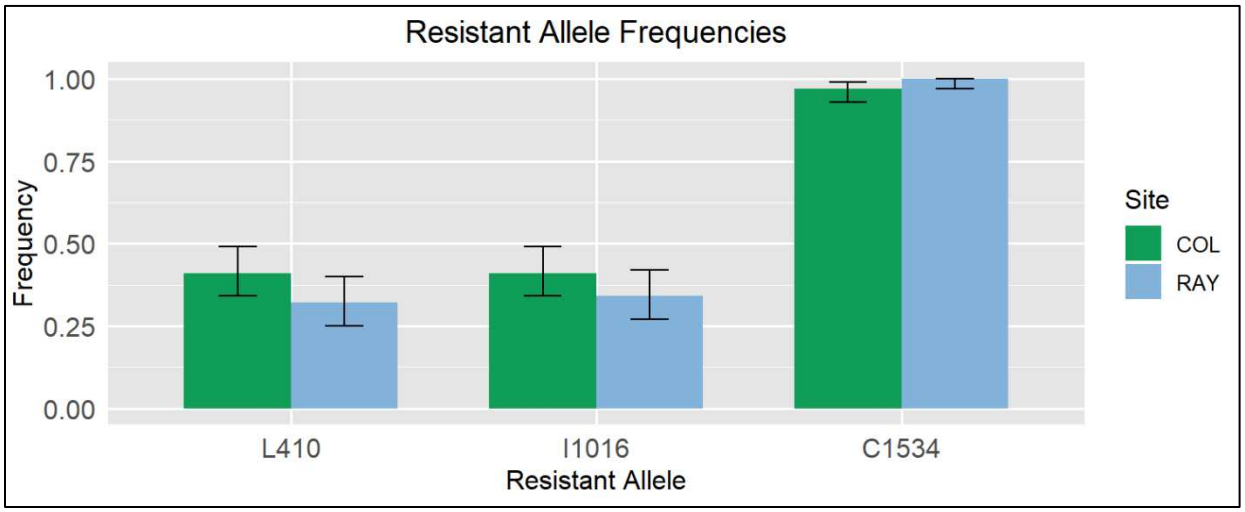

Figure 16. Kdr allele frequencies for the collection sites. Displayed are the resistant allele frequencies for L_{410}, $I_{1,016}$, and $C_{1,534}$ for COL (green) and RAY (blue). Error bars indicate 95 % confidence intervals.

Table 7. F_{IS} coefficients for the two populations sampled. N indicates the number of individuals sampled for allele-specific polymerase chain reaction (AS-PCR). The bold value indicates a significant deviation from Hardy-Weinberg equilibrium (p-value < 0.05). A positive F_{IS} value indicates an excess of homozygotes. A negative F_{IS} indicates an excess of heterozygotes. (-) indicates that the resistant allele has reached fixation.

Site	N	V410L	V1,016I	F1,534C
COL	76	0.05	0.05	**1**
RAY	74	-0.05	-0.03	-

The breakdown of genotype combinations included in the analyses for each site were as follows: COL (VV/VV/CC, n = 27), (VL/VI/CC, n = 33), (LL/II/CC, n = 14), and RAY (VV/VV/CC, n = 32), (VL/VI/CC, n = 34), and (LL/II/CC, n = 7) (**Figure 17**). Two trilocus genotype (VL/VI/FF) individuals were removed from further analysis because this combination was only found at COL, and they were homozygous susceptible at the F1,534C kdr site. One trilocus genotype (VV/II/CC) individual was removed because it was only found at RAY, and the genotypes did not match at the V410L and V1,016I kdr sites (homozygous susceptible/homozygous resistant). All remaining individuals (n = 147) were linked at the V410L and V1,016I kdr sites (**Table S3A – D**). The remaining individuals all carried the homozygous resistant genotype (CC) at the F1,534C kdr site.

Data from COL and RAY were combined for the remaining analyses based on: (I) a lack of significant difference in resistant allele frequencies between the two sites for all kdr mutations (**Figure 16**), (II) similarities in HW equilibrium analysis (**Table 7**) and (III) similarities in trilocus genotype combinations (**Figure 17**). Previous studies conducted in Tapachula in 2018 reported that COL and RAY had similar permethrin and deltamethrin LC_{50} values (Solis-Santoyo et al., 2021). For this reason, the biological replicate results from COL and RAY were combined and assigned into one of three categorical genotypes (LL/II/CC = RR, VL/VI/CC = RS, and VV/VV/CC = SS). This nomenclature was used for the remainder of the analyses.

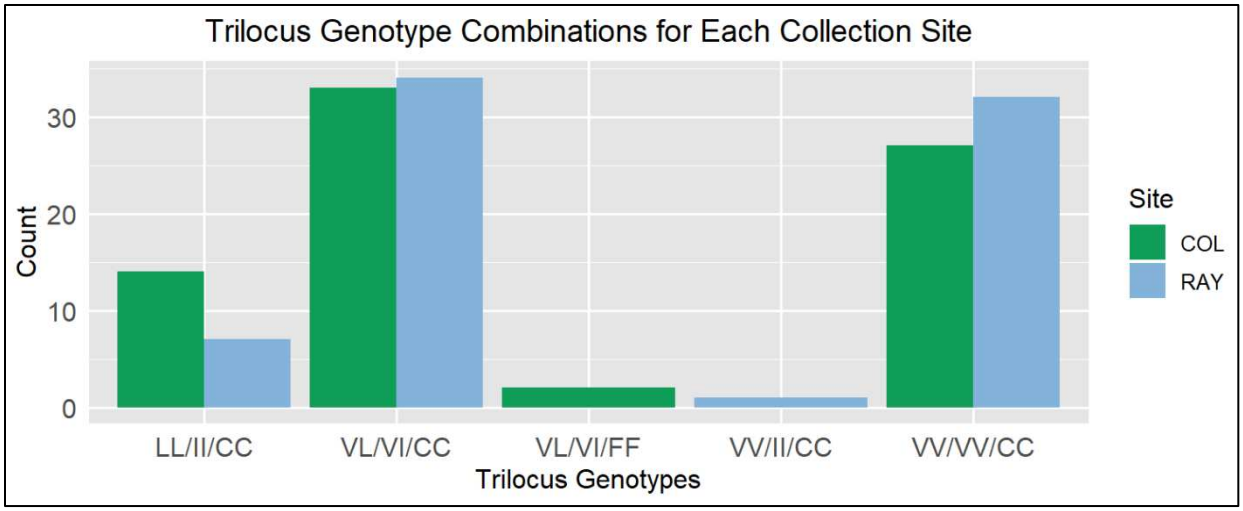

Figure 17. Trilocus genotype combinations. Depiction of all the trilocus genotype combinations found in the study, separated by collection site. COL is depicted in green and RAY in blue. A wide color bar indicates the trilocus genotype combination was only found at that site.

The summary statistics for egg production and wing length are shown in **Table 8**, **Figure 18**, and **Figure 19.** Of the 147 individuals analyzed, 21 were RR genotype with a mean egg production of 90.8, 67 were RS genotype with 91.7 eggs, and 59 were SS with 84.5 eggs.

Table 8. Egg production and wing length summary statistics. Mean egg production and wing length (mm) are depicted for each genotype. N indicates the number of individuals observed, and ME indicates margin of error. Same letters in the mean column are not statistically different (0.05 level of significance).

Genotype	N	Egg Production		Wing Length	
		Mean	ME	Mean	ME
RR	21	90.8[a]	8.30	2.65[a]	0.01
RS	67	91.7[a]	4.58	2.68[ab]	0.01
SS	59	84.5[a]	4.89	2.70[b]	0.01

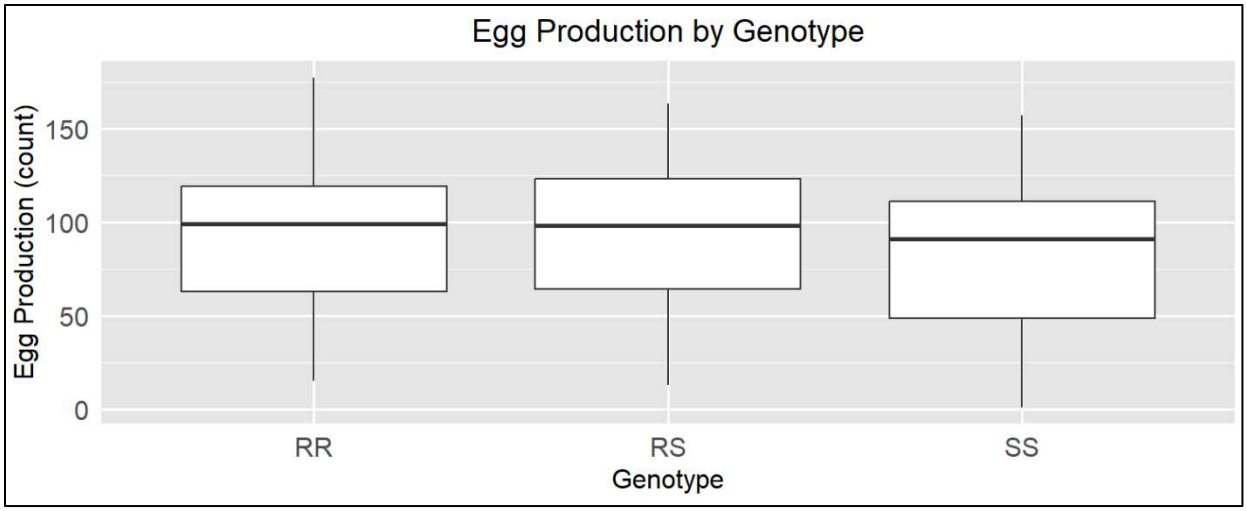

Figure 18. Boxplot of egg production by genotype. Each box and whisker summarize observed egg production by genotype group. (RR = LL/II/CC, RS = VL/VI/CC, and SS = VV/VV/CC). The thick line within the box represents the median, and the box represents the interquartile range for the genotype group. The whiskers represent the 1st and 4th quartiles.

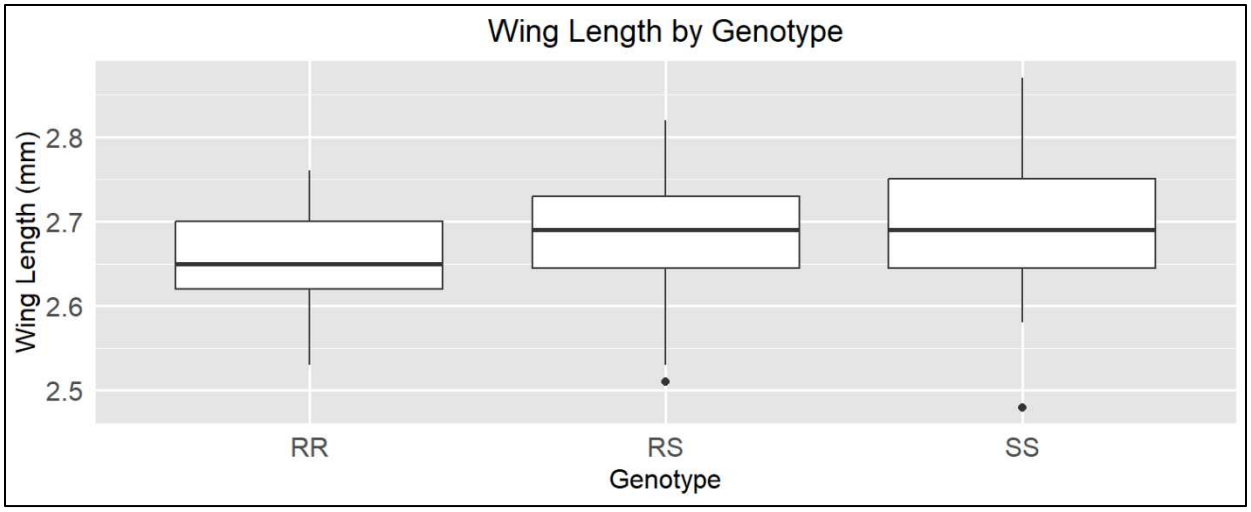

Figure 19. Boxplot of wing length by genotype. Each box and whisker summarize the observed wing lengths (mm) by genotype group. (RR = LL/II/CC, RS = VL/VI/CC, and SS = VV/VV/CC). The thick line within the box represents the median, and the box represents the interquartile range for the genotype group. The whiskers represent the1st and 4th quartiles. Outliers are represented by black circles and are calculated as 1.5 times the interquartile range from either end of the box.

The ANOVA model suggested there was not a significant effect of genotype on wing length (F-value: 3.02, p-value: 0.05) (**Table S4**). However, post-hoc testing using the Tukey's (HSD) method suggested there was a significant difference in wing length

between the RR and SS genotypes (estimated difference: 0.04 mm, p-value: 0.04) (**Table S5A – B**). The remaining pairwise comparisons were not significant (RR-RS, estimated difference: 0.03 mm, p-value: 0.15) and (RS-SS, estimated difference: 0.01 mm, p-value: 0.66).

Figure 20 depicts all observational results of the study. Wing length was plotted against egg production and separated by genotype. The widest wing length range was observed for SS, shown in blue (2.48 to 2.87 mm), followed by RS in orange (2.51 to 2.82 mm), and then RR in green (2.53 to 2.76 mm).

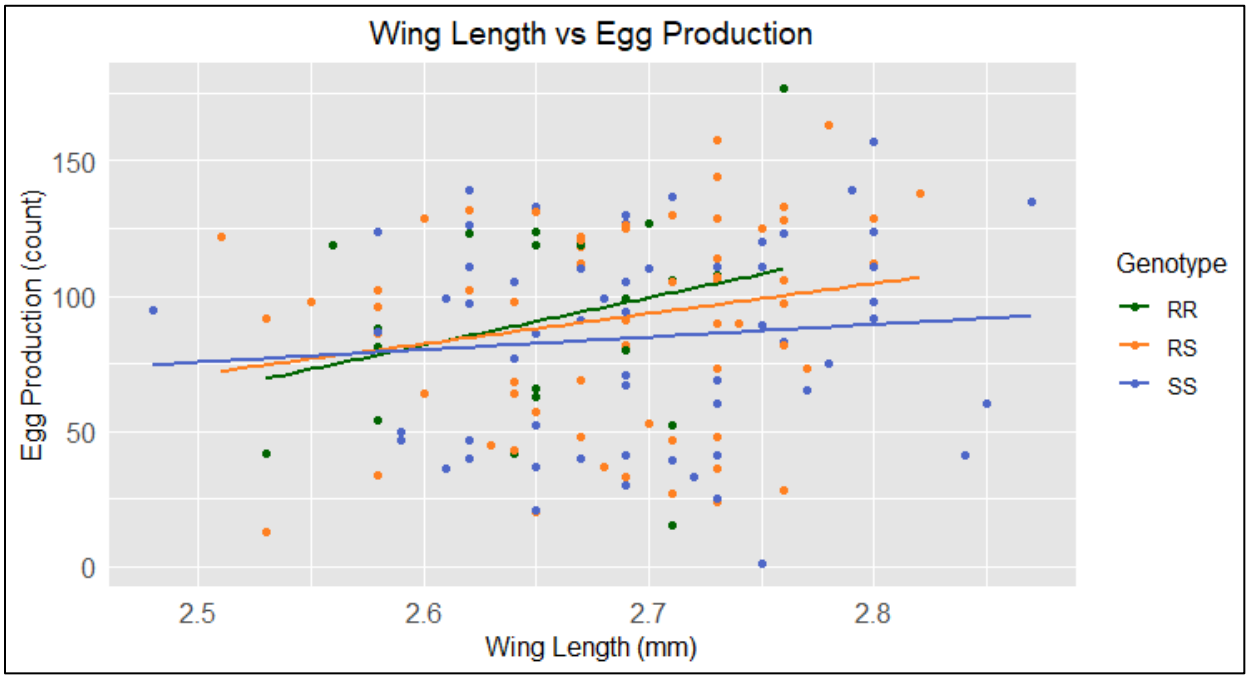

Figure 20. Wing length and egg count for individuals of each genotype. Wing length (mm) was plotted against egg production (count) for each biological replicate with distinctions of the individual's genotype. Green data points and its linear regression is representative of RR individuals, orange is representative of RS individuals, and blue is representative of SS individuals. The length of the regression slope indicates the range of wing lengths observed.

Wing length was included in a one-way ANCOVA model as a covariate despite post-hoc testing from the ANOVA model which suggested a significant wing length difference between the RR and SS genotypes. The ANCOVA model was simplified by removing the non-significant wing length*genotype interaction term (F-value: 0.44, p-value: 0.65). The F-test was deemed valid in determining potential effects of genotype, wing length, and round on egg production due to its robustness to non-normality when the assumption of homogeneity of variance was met (Blanca et al., 2017). Initially, collection sites were included as a categorical group in the model. The collection site having no effect (F-value: 0.01, p-value: 0.92) was justification for removing it for the remainder of the analysis. There was no significant effect of wing length on egg production (F-value: 3.64, p-value: 0.06) (**Table S6**). There was no effect of the round on egg production (F-value: 0.20, p-value: 0.65) and most importantly, the genotype had no significant effect (F-value: 0.97, p-value: 0.38).

DISCUSSION

I suspected that the number of homozygous resistant individuals would likely represent a small proportion of the total if the collection sites exhibited similar resistant allele frequencies to those reported by Solis-Santoyo et al. (2021). As suspected, I observed small sample sizes for the RR genotype for both collection sites. While the F-test has been described by Blanca et al. (2017) as robust to small, unequal sample sizes and non-normality, the collection sites were grouped for further analyses. I also highlighted the importance of investigating fitness costs using individuals with similar genetic backgrounds to limit potential confounding variables. To justify grouping the collection sites, I compared the results from the AS-PCR analysis to the resistant allele

frequencies and LC_{50} values reported by Solis-Santoyo et al. (2021). I referenced my study to theirs because the mosquitoes used in both experiments were part of the same collections.

Solis-Santoyo et al. (2021) reported similar LC_{50} values between the two collection sites for permethrin and deltamethrin. COL had a permethrin LC_{50} value of 19.15 µg/bottle (17.29 − 21.20), and RAY had a value of 28.34 µg/bottle (26.25 − 30.61). COL had a deltamethrin LC_{50} value of 4.56 µg/bottle (3.84 − 5.41), and RAY had a value of 4.25 µg/bottle (3.65 − 4.95). Grouping individuals for bottle bioassays in my study was not possible, however given the similarity in resistant allele frequencies between the two studies, the LC_{50} values reported by Solis-Santoyo et al. (2021) were used as a reference for pyrethroid sensitivity. The lack of significant differences between COL and RAY for L_{410}, $I_{1,016}$, and $C_{1,534}$ frequencies, coupled with their reported LC_{50} values, supported that the collection sites were likely experiencing comparable levels of selection pressures or gene flow.

A potential limitation in my study was not investigating differences in the expression of detoxifying enzyme families, such as cytochrome P450 monooxygenases (CYP) and glutathione S-transferases (GST), which have been associated with pyrethroid resistance in *Ae. aegypti* (Marcombe et al., 2009). While an inverse relationship has been described between the $I_{1,016}$ frequency and the number of genes differentially transcribed (both up and down regulated), proximate collections have been shown to exhibit different transcription profiles (Saavedra-Rodriguez et al., 2014).

The goal of my study was to identify if an association exists between the V410L and V1,016I genotypes and egg production. My study accounted for wing length as both

a covariate and a potential fitness cost. The results provided no evidence of such an association (**Table S6**, p-value: 0.38). It is important to note that all individuals used in the analyses were both homozygous resistant for $C_{1,534}$, and were linked at the V410L and V1,016I sites. Therefore, assigning the individuals into one of three categorical genotypes (LL/II/CC = RR, VL/VI/CC = RS, VV/VV/CC = SS) was practical, but the potential effect of F1,534C genotype combinations on egg production could not be determined. This is important because the assigning of VV/VV/CC as SS only describes susceptibility at the 410 and 1,016 loci, and the homozygous resistant genotype CC at the 1,534 locus has been linked with resistance to type I pyrethroids (Hu et al., 2011). Brito et al. (2013) attempted to observe differences in egg production between a susceptible reference strain (Rock), free of kdr mutations, and a strain selected for the V1,016I mutation (Rock-kdr). However, they discovered that the F1,534C mutation was present in the original couple. Brito et al. (2013) observed a significant difference in egg production between Rock and Rock-kdr.

I examined the effect of genotype on wing length because it has been described as a potential fitness cost (Rigby et al., 2020; Smith et al., 2021). This also determined the appropriateness of using wing length as a covariate in the egg production analysis. Wing length is an important indicator of fitness as larger female mosquitoes have shown increased insecticide tolerance and host-seeking performance (Nasci, 1986; Oliver & Brooke, 2013). Rigby et al. (2020) and Smith et al. (2021) performed similar fitness cost studies, observing differences in egg production between susceptible and resistant individuals for the S989P and V1,016G kdr sites. Both observed that the mean wing length (mm) of the resistant individuals were statistically smaller than that of the

susceptible individuals. This was the case in my study (**Table S5B**, p-value: 0.04). I found nearly the same difference in mean wing length (0.05 mm) between the RR and SS genotypes as Smith et al. (2021). The wing length of heterozygous individuals were not measured in the Rigby et al. (2020) study, however they suspected an intermediate wing length value which would align closer to the susceptible individual. My study supports this, though my results were not significant (RR = 2.65 mm, RS = 2.68 mm, SS = 2.70 mm). I determined that wing length would best be utilized as a covariate in the egg production analysis, outweighing any potential effects that it would have as a fitness cost in a field setting. Wing length was used as a covariate because of its longstanding credibility as an indicator of body size, and body size has been correlated with egg production (Briegel, 1990; Nasci, 1987). However, I determined that wing length had no significant effect on egg production (**Table S6**, p-value = 0.06). Additionally, the interaction between genotype and wing length had no significant effect on egg production (p-value = 0.65).

A critique of my study could be that females were visually confirmed to have taken in a blood meal, but the size of the meal was not explicitly tested. A previous study observed larger females taking in twice as much blood and producing nearly four times as many eggs (Briegel, 1990). Due to the correlation between larval conditions and adult size, Briegel (1990) altered larval densities to produce three distinct size groups (2.4 mm, 2.9 mm, 3.4 mm). Briegel (1990) also observed that only larger females (wing length greater than 3 mm) have a maternal investment, whereby the individual utilizes body reserves to supplement resources that have not been acquired through a small blood meal. Due to consistencies in rearing methodology, the range of

wing lengths observed in my study was narrow (2.48 mm – 2.87 mm). For this reason, I suspected that the range of blood meal sizes would also be narrow. This may play a role in explaining why my study observed no statistically significant effect of wing length on egg production. While maternal investment cannot be ruled out, an average wing length of 2.68 mm suggests that it was not a factor in my study.

There was no significant difference in egg production from one round to the next as shown in **Table S6**. Because genotype had no effect on egg production, but wing length had a marginal effect, a lack of difference between the two rounds was likely due to the consistency of larval conditions. Therefore, future studies should place emphasis on rearing methodology, which dictates larval development and determines adult body size (Briegel, 1990).

CHAPTER 4: CONCLUDING REMARKS AND FUTURE CONSIDERATIONS

The study conducted on mosquitoes from Southern Texas provided evidence of resistance to two classes of insecticides which could aid in reducing vector populations within an area that is conducive for virus transmission. The observed resistance was not surprising due to previous reports of *Aedes aegypti* (*Ae. aegypti*) populations and knockdown resistance (kdr) genes in Texas, combined with routine spray applications in Weslaco. When mosquito control entities have limited funding, it is essential to supply resistance data for effective vector control, especially as only pyrethroids and organophosphates are available in the continental United States (CONUS) (Centers for Disease Control and Prevention [CDC], 2022). The data from this study suggests that the collection sites have differences in resistance levels between pyrethroids and organophosphates.

This study brought to light two challenges faced when conducting resistance status studies. First, decentralized mosquito abatement entities in the region face limited funding and access to high-complexity testing equipment. In the updated manual to evaluate resistance in the CONUS, the CDC recommends performing both molecular assays and intensity testing to confirm the presence of resistance. The results from the initial susceptibility test and subsequent lethal concentration 50 % (LC_{50}) values (intensity testing) were sufficient to conclude that there was permethrin resistance within the region. Therefore, selection and validation of a known susceptible colony is crucial in the absence of high-complexity testing equipment. When resources permit, allele-specific polymerase chain reaction (AS-PCR) and biochemical assays can be utilized to identify resistance mechanisms to their associated insecticides.

The second challenge is posed in instances when funding and equipment are available, but testing may be limited by the availability of *Ae. aegypti* F_1 mosquito eggs produced from field collections. I propose two solutions to address this challenge. The first is to rear subsequent generations while considering resistance reversion. The second is to conduct high-complexity testing on mosquitoes directly from bottle and larval assays. Not only would this be conducive when mosquito numbers are limited, but it would also correlate genotype and enzyme expression with phenotypic resistance.

To best strategize future vector control efforts, performing resistance tests for a variety of insecticide classes would be beneficial. Therefore, mosquito control entities can encompass a true resistance profile, allowing for either a rotational or a mosaic insecticide application scheme. Longitudinal studies using these techniques could determine the length of time required for populations of *Ae. aegypti* to return to insecticide susceptibility. Lower values of temephos resistance compared to permethrin suggest the potential to utilize an alternative organophosphate that is registered for use in the CONUS. Future studies would benefit by conducting resistance status assays on an alternative organophosphate to potentially be used rotationally with permethrin. However, this strategy should incorporate how quickly the population returns to a resistant status when exposed to a reintroduced insecticide class. Cessation of temephos and low levels of resistance suggest reversion to susceptibility. Determining the drivers of insecticide reversion is crucial, as it can impact rotational strategies in vector control.

The study conducted on mosquitoes from Tapachula, Mexico was meant to determine if two resistant alleles at the 410 and 1,016 voltage-gated sodium channel

(VGSC) sites were associated with egg production. Wing length has been regarded as both a fitness cost and a proxy for body size, which has been correlated with egg production. In this study, I attempted to determine how to view wing length, either as a fitness cost or as a covariate in the egg production analysis, aiming to identify potential drivers of insecticide reversion.

First, I incorporated wing length as a potential fitness cost and found that the wings of homozygous resistant (RR) individuals were marginally shorter than homozygous susceptible (SS) individuals. Strict larval conditions may have played a role in the marginal difference observed. Under natural conditions, larval habitats are likely to have a greater variation in nutrient availability and larval density. If resources permit, I suggest observing wing length differences between resistant and susceptible genotypes in a natural setting. If resources are limited, future laboratory studies can alter larval conditions in a controlled manner. Additionally, identifying the true effect of wing length as a fitness cost should be conducted in a natural setting.

I included wing length as a covariate in the egg production analysis, however no significant effect on egg production was observed. Future fitness cost studies may benefit from producing a wider adult size range, and including wing length as a covariate so long as the effect of genotype on wing length remains marginal. In the case of this study, the interaction between wing length and genotype had no significant effect on egg production, thereby supporting my decision to include wing length as a covariate. To increase efficiency, future studies may choose to incorporate open-source software like ImageJ, which would reduce the time required to measure wings and

increase accuracy (Collins, 2007), allowing for additional morphometric wing analyses to be conducted retrospectively.

I found no evidence to suggest that egg production was affected by the V410L and V1,016I genotypes. The biggest limitation of this study was that all individuals were homozygous resistant at the F1,534C kdr locus. Therefore, there were no true susceptible individuals used in this study. This is a limitation because I could not identify any potential effect that the F1,534C resistant alleles may have on egg production. Brito et al. (2013) observed a decrease in egg production in homozygous resistant ($I_{1,016}$ / $C_{1,534}$) individuals. If egg production is impacted by the $C_{1,534}$ allele, then this could potentially explain why my study did not observe any differences in egg production between genotypes. To determine any associations between the F1,534C mutation and egg production, future studies may benefit from obtaining susceptible individuals for all three kdr loci, by selecting geographical sites that are not at or nearing fixation for the $C_{1,534}$ allele. In the absence of pyrethroids, mosquito populations in Tapachula have exhibited reversion to susceptibility. The overall goal of my study was to determine if egg production, as a fitness cost, had any relevance to this reversion. For this reason, no attempt was made to alter the genetic background of the collected mosquitoes.

This study was designed such that all biological replicates had to be processed on the fifth day to standardize time allotted for oviposition. Only 100 biological replicates could be performed per round due to the lengthy processing times (anesthetization, wing removal, storage, and egg counting). As described above, incorporating ImageJ software would increase accuracy and efficiency as it relates to egg counts. As shown in this study, an increase in biological replicate number would encompass

underrepresented genotype combinations. Lastly, egg production is just one of many reproductive fitness costs that has been associated with resistance mechanisms. Preserving eggs after counts are made and expanding on the present methods may allow future studies to test for other reproductive parameters impacted by resistance mechanisms.

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APPENDIX

Table S1. Chi-square analysis and inbreeding coefficient data for Texas collections. Individuals for each collection, displayed in the total column, were screened via allele-specific polymerase chain reaction (AS-PCR) analysis. The genotype results for each of the three knockdown-resistant (kdr) sites (V410L, V1,016I, and F1,534C, respectively) are displayed in the observed columns. Resistant allele frequencies (L_{freq}, I_{freq}, and C_{freq}, respectively) are displayed with 95 % confidence intervals (CI). Susceptible allele frequencies (V_{freq}, V_{freq}, and F_{freq}, respectively) are also displayed. Expected genotype values were calculated using the Hardy-Weinberg equilibrium equation, and significant deviations were identified using the chi-square statistic (X^2) (p-value < 0.05). A positive F_{IS} value indicates an excess of homozygotes. A negative F_{IS} indicates an excess of heterozygotes. (-) indicates that the resistant allele has reached fixation.

	Site	Total	Observed			L_{freq}	95 % CI	V_{freq}	Expected			X^2	
			VV	VL	LL				VV	VL	LL	p-value	Fis
V410L	MC	48	12	20	16	0.54	(0.44-0.64)	0.46	10.08	23.83	14.08	0.27	0.16
	MLM	48	11	16	21	0.60	(0.50-0.70)	0.40	7.52	22.96	17.52	0.04	0.30
	VNC	48	13	26	9	0.46	(0.36-0.56)	0.54	14.08	23.83	10.08	0.53	0.09
	WCT-03	24	2	9	13	0.73	(0.59-0.83)	0.27	1.76	9.48	12.76	0.80	0.05
	WM5	24	0	7	17	0.85	(0.73-0.93)	0.15	0.51	5.98	17.51	0.40	0.17
	COMBINED	192	38	78	76	0.60	(0.55-0.65)	0.40	30.88	92.24	68.88	0.03	0.15
	Site	Total	Observed			I_{freq}	95 % CI	V_{freq}	Expected			X^2	
			VV	VI	II				VV	VI	II	p-value	Fis
V1,016I	MC	48	12	20	16	0.54	(0.44-0.64)	0.46	10.08	23.83	14.08	0.27	0.16
	MLM	48	10	17	21	0.61	(0.51-0.71)	0.39	7.13	22.74	18.13	0.08	0.25
	VNC	48	13	26	9	0.46	(0.36-0.56)	0.54	14.08	23.83	10.08	0.53	0.09
	WCT-03	24	2	7	15	0.77	(0.63-0.87)	0.23	1.26	8.48	14.26	0.39	0.17
	WM5	24	0	7	17	0.85	(0.73-0.93)	0.15	0.51	5.98	17.51	0.40	0.17
	COMBINED	192	37	77	78	0.61	(0.56-0.65)	0.39	29.69	91.62	70.69	0.03	0.16
	Site	Total	Observed			C_{freq}	95 % CI	F_{freq}	Expected				
			FF	FC	CC				FF	FC	CC		
F1,534C	MC	48	0	0	48	1	(0.96-1)	-	-	-	48		
	MLM	48	0	0	48	1	(0.96-1)	-	-	-	48		
	VNC	48	0	0	48	1	(0.96-1)	-	-	-	48		
	WCT-03	24	0	0	24	1	(0.93-1)	-	-	-	24		
	WM5	24	0	0	24	1	(0.93-1)	-	-	-	24		
	COMBINED	192	0	0	192	1	(0.99-1)	-	-	-	192		

Table S2. Chi-square analysis and inbreeding coefficient data for Mexico collections. Individuals for each collection, displayed in the total column, were screened via allele-specific polymerase chain reaction (AS-PCR) analysis. The genotype results for each of the three knockdown-resistant (kdr) sites (V410L, V1,016I, and F1,534C, respectively) are displayed in the observed columns. Resistant allele frequencies (L_{freq}, I_{freq}, and C_{freq}, respectively) are displayed with 95 % confidence intervals (CI). Susceptible allele frequencies (V_{freq}, V_{freq}, and F_{freq}, respectively) are also displayed. Expected genotype values were calculated using the Hardy-Weinberg equilibrium equation, and significant deviations were identified using the chi-square statistic (X^2) (p-value < 0.05). A positive F_{IS} value indicates an excess of homozygotes. A negative F_{IS} indicates an excess of heterozygotes. (-) indicates that the resistant allele has reached fixation.

	Site	Total	Observed			L_{freq}	95 % CI	V_{freq}	Expected			X^2	
V410L			VV	VL	LL				VV	VL	LL	p-value	Fis
	COL	76	27	35	14	0.41	(0.34-0.49)	0.59	26.06	36.89	13.06	0.66	0.05
	RAY	74	33	34	7	0.32	(0.25-0.40)	0.68	33.78	32.43	7.78	0.68	-0.05
	Site	Total	Observed			I_{freq}	95 % CI	V_{freq}	Expected			X^2	
V1,016I			VV	VI	II				VV	VI	II	p-value	Fis
	COL	76	27	35	14	0.41	(0.34-0.49)	0.59	26.06	36.89	13.06	0.66	0.05
	RAY	74	32	34	8	0.34	(0.27-0.42)	0.66	32.45	33.11	8.45	0.82	-0.03
	Site	Total	Observed			C_{freq}	95 % CI	F_{freq}	Expected			X^2	
F1,534C			FF	FC	CC				FF	FC	CC	p-value	Fis
	COL	76	2	0	74	0.97	(0.93-0.99)	0.03	0.05	3.89	72.05	2.8^{-18}	1.00
	RAY	74	0	0	74	1.00	(0.97-1.00)	0	0	0	74.00	-	-

Table S3A. Biological replicates for round one from Colinas del Rey. Displayed are the results for each biological replicate which are separated by collection site and round number. Each biological replicate has the wing length measurement (mm), the number of eggs laid, and the knockdown-resistant genotypes.

Colinas del Rey				
Round 1				
Wing Length (mm)	Number of eggs	Genotype		
		V410L	V1,016I	F1,534C
2.8	98	VV	VV	CC
2.67	111	VL	VI	CC
2.53	13	VL	VI	CC
2.62	111	VV	VV	CC
2.6	129	VL	VI	CC
2.6	64	VL	VI	CC
2.73	36	VL	VI	CC
2.65	75	VL	VI	FF
2.71	130	VL	VI	CC
2.76	83	VV	VV	CC
2.71	15	LL	II	CC
2.65	37	VV	VV	CC
2.62	123	LL	II	CC

2.64	42	LL	II	CC
2.53	42	LL	II	CC
2.76	128	VL	VI	CC
2.69	99	LL	II	CC
2.65	119	LL	II	CC
2.62	40	VV	VV	CC
2.65	20	VL	VI	CC
2.56	119	LL	II	CC
2.69	127	VV	VV	CC
2.67	118	VL	VI	CC
2.71	137	VV	VV	CC
2.69	126	VL	VI	CC
2.62	47	VV	VV	CC
2.59	126	VL	VI	FF
2.65	124	VV	VV	CC
2.69	125	VL	VI	CC
2.62	102	LL	II	CC
2.58	96	VL	VI	CC
2.73	106	VL	VI	CC
2.64	64	VL	VI	CC
2.65	133	VL	VI	CC
2.58	81	LL	II	CC
2.69	30	VV	VV	CC
2.53	92	VL	VI	CC
2.58	124	VV	VV	CC
2.75	89	VV	VV	CC
2.64	43	VL	VI	CC
2.73	106	VL	VI	CC
2.62	126	VV	VV	CC
2.68	99	VV	VV	CC
2.65	63	LL	II	CC

Table S3B. Biological replicates for round two from Colinas del Rey. Displayed are the results for each biological replicate which are separated by collection site and round number. Each biological replicate has the wing length measurement (mm), the number of eggs laid, and the knockdown-resistant genotypes.

Colinas del Rey				
Round 2				
Wing Length (mm)	Number of eggs	Genotype		
		V410L	V1,016I	F1,534C
2.65	57	VL	VI	CC
2.67	69	VL	VI	CC
2.73	158	VL	VI	CC
2.71	106	LL	II	CC
2.65	124	LL	II	CC
2.73	107	VL	VI	CC
2.8	92	VV	VV	CC
2.72	33	VV	VV	CC
2.69	82	VL	VI	CC
2.75	120	VV	VV	CC

2.76	133	VL	VI	CC
2.73	25	VV	VV	CC
2.58	54	LL	II	CC
2.73	114	VL	VI	CC
2.73	90	VL	VI	CC
2.8	129	VL	VI	CC
2.67	110	VV	VV	CC
2.69	94	VV	VV	CC
2.82	138	VL	VI	CC
2.68	37	VL	VI	CC
2.65	52	VV	VV	CC
2.76	106	VL	VI	CC
2.69	105	VV	VV	CC
2.65	21	VV	VV	CC
2.64	98	VL	VI	CC
2.59	47	VV	VV	CC
2.64	68	VL	VI	CC
2.58	87	VV	VV	CC
2.61	99	VV	VV	CC
2.74	90	VL	VI	CC
2.69	80	LL	II	CC
2.62	97	VV	VV	CC

Table S3C. Biological replicates for round one from Raymundo Enriquez.
Displayed are the results for each biological replicate which are separated by collection site and round number. Each biological replicate has the wing length measurement (mm), the number of eggs laid, and the knockdown-resistant genotypes.

Raymundo Enriquez				
Round 1				
Wing Length (mm)	Number of eggs	Genotype		
		V410L	V1,016I	F1,534C
2.71	39	VV	VV	CC
2.67	91	VV	VV	CC
2.73	111	VV	VV	CC
2.73	108	LL	II	CC
2.73	24	VL	VI	CC
2.65	86	VV	VV	CC
2.77	65	VV	VV	CC
2.67	40	VV	VV	CC
2.48	95	VV	VV	CC
2.75	125	VL	VI	CC
2.71	52	LL	II	CC
2.85	60	VV	VV	CC
2.73	48	VL	VI	CC
2.69	92	VL	VI	CC
2.51	122	VL	VI	CC
2.76	177	LL	II	CC
2.67	122	VL	VI	CC
2.58	34	VL	VI	CC
2.7	53	VL	VI	CC

2.65	66	LL	II	CC
2.75	111	VV	VV	CC
2.58	88	LL	II	CC
2.59	50	VV	VV	CC
2.63	45	VL	VI	CC
2.69	67	VV	VV	CC
2.69	41	VV	VV	CC
2.73	129	VL	VI	CC
2.8	112	VL	VI	CC
2.69	130	VV	VV	CC
2.69	33	VL	VI	CC
2.71	27	VL	VI	CC
2.84	41	VV	VV	CC
2.58	86	VL	VI	CC
2.8	124	VV	VV	CC
2.7	110	VV	VV	CC
2.67	119	LL	II	CC
2.7	127	LL	II	CC
2.73	144	VL	VI	CC
2.67	112	VL	VI	CC
2.76	123	VV	VV	CC
2.87	135	VV	VV	CC
2.61	36	VV	VV	CC
2.65	124	VL	VI	CC
2.58	102	VL	VI	CC
2.55	98	VL	VI	CC
2.75	1	VV	VV	CC

Table S3D. Biological replicates for round two from Raymundo Enriquez.
Displayed are the results for each biological replicate which are separated by collection site and round number. Each biological replicate has the wing length measurement (mm), the number of eggs laid, and the knockdown-resistant genotypes.

Raymundo Enriquez				
Round 2				
Wing Length (mm)	Number of eggs	Genotype		
		V410L	V1,016I	F1,534C
2.78	163	VL	VI	CC
2.79	139	VV	VV	CC
2.65	133	VV	VV	CC
2.76	28	VL	VI	CC
2.69	91	VL	VI	CC
2.8	157	VV	VV	CC
2.78	75	VV	VV	CC
2.77	73	VL	VI	CC
2.73	60	VV	VV	CC
2.73	73	VL	VI	CC
2.62	132	VL	VI	CC
2.71	47	VL	VI	CC
2.71	105	VL	VI	CC
2.64	105	VV	VV	CC

2.76	82	VL	VI	CC
2.73	41	VV	VV	CC
2.64	77	VV	VV	CC
2.67	121	VL	VI	CC
2.62	102	VL	VI	CC
2.73	69	VV	VV	CC
2.69	71	VV	VV	CC
2.8	111	VV	VV	CC
2.67	48	VL	VI	CC
2.73	106	VL	VI	CC
2.65	131	VL	VI	CC
2.62	139	VV	VV	CC
2.76	97	VL	VI	CC
2.66	56	VV	II	CC

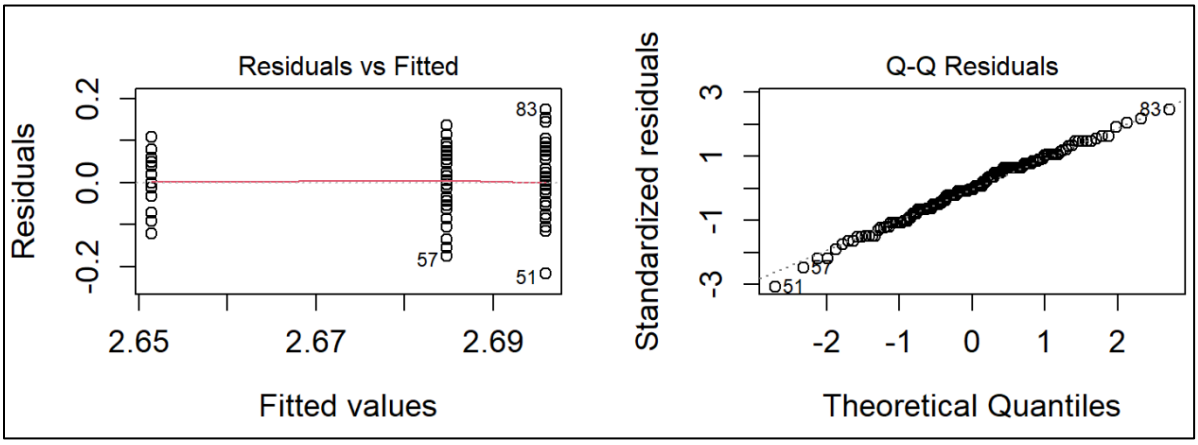

Figure S1. Homogeneity of variance and normality plots for the ANOVA model. Assumption of homogeneity of variance was visually checked by observing the residuals vs. fitted plot. Assumption of normality was visually checked by observing the Q-Q residuals plot.

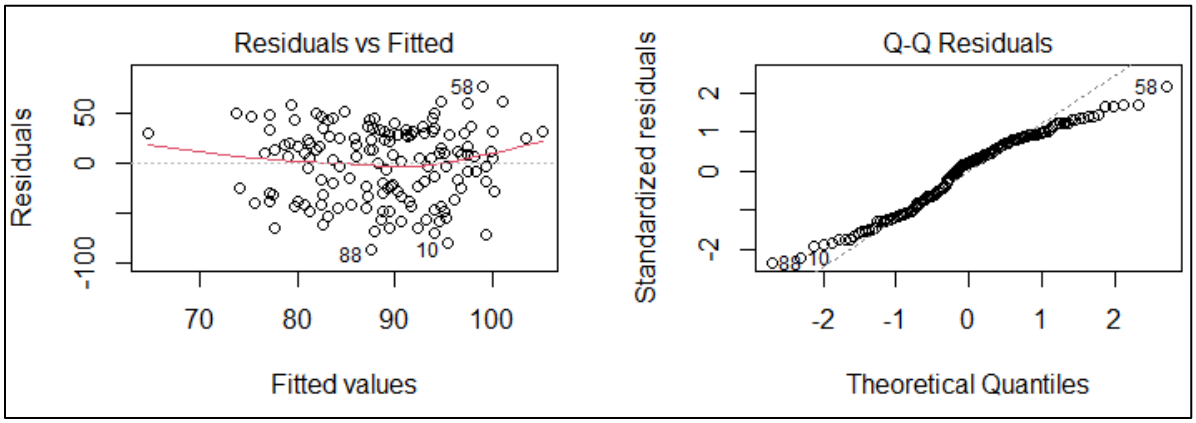

Figure S2. Homogeneity of variance and normality plots for the ANCOVA model. Assumption of homogeneity of variance was visually checked by observing the residuals vs. fitted plot. Assumption of normality was visually checked by observing the Q-Q residuals plot.

Table S4. ANOVA type III model. Summary of the effect of genotype on wing length (mm). Included in the table are the sum of squares, degrees of freedom (df), F-value, and p-value. A p-value < 0.05 indicates that the genotype had a statistically significant impact on wing length.

	Sum of Squares	df	F-value	p-value
Intercept	147.63	1	29036.22	$< 2^{-16}$
Genotype	0.03	2	3.02	0.05
Residuals	0.73	144		

Table S5A. Estimated marginal means of wing length. Tukey's (HSD) method was used to produce estimated marginal means (EMM). The standard error (SE), degrees of freedom (df), and confidence interval (95 % CI) are included for each genotype.

Genotype	Estimated Marginal Means	SE	df	95 % CI
RR	2.65	0.02	144	(2.62 - 2.68)
RS	2.68	0.01	144	(2.67 - 2.70)
SS	2.70	0.01	144	(2.68 - 2.71)

Table S5B. Estimates of means contrast between genotypes. Tukey pairwise comparison of adjusted means (estimate) between genotypes. The standard error (SE), degrees of freedom (df), t-ratio, and p-value are included for each contrast. A p-value < 0.05 indicates the statistical significance of each contrast.

Contrast	Estimate	SE	df	t.ratio	p-value
RR-RS	-0.03	0.02	144	-1.87	0.15
RR-SS	-0.04	0.02	144	-2.46	0.04
RS-SS	-0.01	0.01	144	-0.88	0.66

Table S6. ANOVA type III model without interaction. Summary of the effects of genotype, wing length, and round on egg production. Included in the table are the sum of squares, degrees of freedom (df), F-value, and p-value for each variable. A p-value < 0.05 indicates that the variable had a statistically significant impact on egg production.

	Sum of Squares	df	F-value	p-value
Intercept	1799	1	1.29	0.26
Genotype	2709	2	0.97	0.38
Wing Length	5062	1	3.64	0.06
Round	280	1	0.20	0.65
Residuals	197598	142		