Surveillance of Permethrin Resistance in Valdosta Populations of *Aedes albopictus* via the CDC Bottle Bioassay

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ABSTRACT

Mosquito populations are likely to develop genetic resistance to insecticides with prolonged and/or improper application. Regular assessments of insecticide resistance should be performed on exposed populations to optimize local integrated mosquito management strategies. Current strategies within Valdosta, Georgia, especially chemical insecticide application, may prove to be outdated and inefficient due to the absence of a designated resistance testing center. The current study aims to provide a baseline resistance report for the nuisance biter and potential disease vector, *Aedes albopictus* (Skuse) to the type I pyrethroid, permethrin. Although *Ae. albopictus* is primarily a nuisance biter in southern Georgia, this species has the physiological potential to be a vector of numerous arboviruses and other pathogens. *Ae. albopictus* colonies were established from eggs collected at 15 independent sites within and near Valdosta, GA between July and November of 2019. The majority of the sites were located in residential neighborhoods. Post-rearing, survivability upon permethrin exposure was tested using the Centers for Disease Control and Prevention (CDC) Bottle Bioassay Kit. This bioassay accounts for resistance by comparing a 'diagnostic time' (the maximum time required to kill 100% of susceptible mosquitoes of a particular species and insecticide dosage) to the actual time taken for experimental mosquitoes to die. The occurrence of permethrin resistance in Valdosta populations of *Ae. albopictus* was observed in this study. According to World Health Organization (WHO) standards, <90% mortality at the diagnostic time indicates resistance, and mosquitoes from all 15 collection sites exhibited mortality <82%. This level of resistance suggests that mosquito abatement methods currently used in Valdosta, Georgia should be modified to mitigate insecticide resistance. Population reduction of this potential vector species should then reduce the risk of rapid disease transmission should the respective pathogens be introduced into the area.

DEDICATION

I dedicate this body of work to my parents, Paula and Emile Evans.

CHAPTER I: INTRODUCTION

Globally, the list of insecticide resistant vector species has expanded since the mid-20th century (Georghiou & Mellon, 1983). Common insect vectors, particularly mosquitoes, have developed resistance to all synthetic insecticide classes currently available (Hemingway et al., 2002; Nauen, 2007; Rivero et al., 2010; Scott 1999; World Health Organization [WHO], 1957). With the extensive use of insecticides in urban environments, resistance in mosquito populations may arise from various sources. Sources such as unintentional exposure as a non-target species, lack of insecticidal class rotation, and improper product usage ultimately lower the intrinsic value of insecticides by rendering them unusable (Su et al., 2018). Moreover, protocols behind the development and distribution of new insecticidal compounds are now more meticulous than ever. The time and cost required to manufacture new insecticides have increased in order to prevent severe ecosystem disturbances such as bioaccumulation of DDT (Dunlap, 2008; Dunlap, 2014; Kinkela, 2011). Complicated resistance evolution and the limited availability of suitable insecticides has led to many difficulties in integrated mosquito management.

By definition, an insecticide is a chemical or biological agent that deters, incapacitates, or kills targeted insect species (Gupta & Milatovic, 2014). Larvicides, adulticides, or both may be utilized to manage different life stages of target species. Synthetic chemical insecticides are grouped into four classes: carbamates, organochlorines, organophosphates, and pyrethroids. Synergists, which are not directly toxic to insects, can be combined with chemical insecticides to boost the overall effectiveness of their delivery to the target site (Bernard & Philogène, 1993).

Pyrethrins are natural product compounds derived from flowers of *Tenacetum cinerariifolium* or *Chrysanthemum cinerariifolium*. Due to their low toxicity to mammals, pyrethrins have been favored for use in residential pesticide formulations (Sudakin, 2006). The chemical structure of these natural insecticides is universally defined by a cyclopropane-carboxylic acid moiety and cyclopentenolone alcohol moiety. One shortcoming to using pyrethrins is their tendency to rapidly degrade in the environment following to light exposure (Cycoń & Piotrowska-Seget, 2016). To increase photostability and maintain insecticidal properties, synthetic chemicals from processed pyrethrin mixtures were first manufactured in the mid 1970's (López & Fernández-Bolanos, 2011). Known as pyrethroids, these chemicals were modified to replace the cyclopentenolone ring with other heterocyclic structures like 3-phenozybenyl in the alcohol moiety (Sudakin, 2006). Pyrethroids are classified by the absence or presence of an alpha-cyano substituent to the 3-phenozybenyl alcohol group as type I and type II respectively. In addition, chlorine and bromine may substitute methyl groups in the acid moiety (Table 1).

The physiological pathway used by pyrethroids to incapacitate insects involves the functional disruption of voltage-gated sodium channels. Voltage-gated sodium channels serve a critical role in electrical signaling within cells, making them a primary target for neurotoxic insecticides like pyrethroids. Action potentials in the nervous system and other excitable cells are initiated and propagated by voltage-gated sodium channels (Du et al., 2016). These channels become activated (opened) upon membrane depolarization and allow an influx of sodium ions into the cell. The rising phase of an action potential commences as a result. After a millisecond of opening, the channel becomes physically occluded by an inactivation particle in a process called fast inactivation. Fast inactivation is responsible for the termination of the action potential and prevents excessive membrane depolarization. Meanwhile, slow inactivation is crucial to regulating membrane excitability, action potential firing patterns, and spike frequency adaptation. Pyrethroids prevent a sodium channel from closing by binding to it in place of the inactivation particle. Pyrethroids modify the channel gating transition and cause prolonged activation through binding. Since termination of the action potential is inhibited, pyrethroids cause repetitive firing and/or nerve conduction blocking at a cellular level. Prolonged activation ultimately leads to paralysis and death in insects.

Insecticide use has selected for genetic variants in mosquito populations with increased ability to tolerate doses that are lethal to the majority of unexposed natural populations of the same species (Hemingway et al., 2002; Scott, 1999; WHO, 1957). These responses entail behavioral avoidance and/or physiological resistance (Chareonviriyaphap et al., 2013). Grieco et al., (2007) states that behavioral avoidance occurs when a change of action prevents or mitigates exposure to an insecticide. This change of action can be stimulated dependently or independently from the insecticide itself (Chareonviriyaphap et al., 2013). Stimulus-dependent action involves repellency after chemical detection of the active ingredient though vapor-phase molecules in the air or direct contact. Alternatively, stimulus-independent action does not require chemical detection and has been considered genetically driven (WHO, 1986). This form of avoidance rather involves life history components of such as zoophilic feeding and exophilic resting preferences (Byford & Sparks, 1987).

Meanwhile, Mackenzie-Impoinvil et al. (2019) characterizes physiological resistance as more effective accommodation or biodegradation of the insecticide through compositional and/or functional changes in the body. Some examples of physiological resistance include (1) reduced penetration across the cuticle due to structural or compositional modifications; (2) increased excretion or sequestration of insecticide; (3) increased detoxification through the possession of higher levels or more efficient forms of enzymes such as cytochrome P450 monooxygenases, glutathione S-transferases, and esterases (metabolic resistance); and (4) increased target-site insensitivity due to mutations in insecticide target proteins such as gamma-aminobutyric acid receptors, acetylcholinesterase, and voltage-gated sodium channels Mutations in the voltage-gated sodium channels, specifically known as knockdown resistance (KDR), are most frequently implicated in response to pyrethroid resistance in mosquitoes (Gao et al., 2018).

Physiological resistance typically originates from some form of genetic duplication or mutation (e.g., single amino acid substitutions) according to Wood and Cook (1983). Findings from their research describe physiological resistance as heritable and therefore able to be circulated within a mosquito population through selective pressure. Prior to insecticide exposure, only a few individuals in the natural population are likely to have genotypes that promote resistance, or 'resistant genotypes.' Most mosquitoes have non-resistant (susceptible) genotypes. Individuals exhibiting resistant phenotypes then have increased fitness, leading to greater survivorship under the current selective pressure of insecticide exposure. Since surviving mosquitoes have a higher tendency to reproduce, there is an overall shift of the population's gene pool with offspring favoring resistant genotypes. Furthermore, each class of insecticide is categorized by a similar mode of action, and cross resistance may also occur. Cross resistance is defined as the resistance of one insecticide leading to the resistance of a different, unused insecticide with a similar mode of action.

Insecticide resistance can be initially monitored using susceptibility bioassays such as the Centers for Disease Control and Prevention (CDC) bottle bioassay created by research entomologists Dr. William G. Brogdon and Dr. Adeline Chan. This bioassay assesses the ability of an insecticide's active ingredient to control a snapshot sample of a mosquito population (CDC, 2016). The experimental population's mortality rate is compared to that of a completely susceptible population using a specified diagnostic dose and time. In this mortality rate, mosquitoes that are unable to stand upright are also included in count data. A diagnostic dose is the concentration of a particular insecticide at which all susceptible mosquitoes will die, whereas the diagnostic time is the maximum duration needed for all susceptible mosquitoes to die. The insecticide susceptibility status of a mosquito population can be interpreted by the percentage mortality at the diagnostic time. A mortality of ≥97% denotes a susceptibility, 90-96% denotes development of resistance, and ≤89% denotes resistance (CDC, 2016). Aspects of the CDC bottle bioassay that have led to its preference over other susceptibility bioassay alternatives are as follows: (1) capability for use on site and in the lab, (2) rapid results, (3) relatively economic cost, (4) re-usable apparatus, and (5) modifiable concentrations for insecticidal stock solutions. Biochemical and molecular assays can be used in conjunction with the CDC bottle bioassay to affirm observed resistances and determine underlying mechanisms of resistance.

Aedes albopictus (Skuse) is a diurnal tree hole mosquito with invasive breeding populations present in Valdosta, Lowndes, Co., GA. *Ae. albopictus* is a conspicuously dark-colored mosquito with a body length of approximately 2.0 to 10.0 mm. Adults are identified by a black scutum having a singular stripe of silvery white scales spanning the dorsal surface of the head to the thorax. These silvery white scales are also found on the palpi and the basal portion of each tarsal segment (Figure 1). The flight range typically does not exceed 200 m (Turell et al., 2005). *Ae. albopictus* adults are often found resting in shady areas with low-lying vegetation (Koehler & Castner, 1997). As aggressive, opportunistic feeders, female *Ae. albopictus* will seek blood meals from a variety of hosts during the peak periods of early morning and late afternoon (Hawley, 1988). Acquired blood meals from domestic and wild mammals, birds, reptiles, and amphibians have been observed (Eritja et al., 2005; Hawley, 1988; Turell et al., 2005). However, human hosts appear to be most favored within blood meal analysis studies (Paupy et al., 2009).

The short photoperiods and low temperatures of temperate climates can induce eggs to undergo a winter diapause (Eritja et al., 2005; Hanson & Craig, 1995; Lyon & Berry, 1991; Medlock et al., 2006), but *Ae. albopictus* is otherwise active throughout the year. *Ae. albopictus* eggs are dark brown to black and ~0.5 mm in length. Eggs are described as having a 'cigar-shape' with a blunt anterior and tapered posterior end. Females prefer to lay eggs above the surface of small, restricted, and shaded bodies of water such as naturally formed holes in vegetation (Hawley, 1988). However, this species may also utilize artificial containers namely tires, pet dishes, birdbaths, and flowerpots (Hawley, 1988). A gravid female lays the eggs via skip oviposition, meaning that eggs will be laid individually or in small clutches in multiple sites (Colton et al., 2003; Davis et al., 2015; Day, 2016). A female may have up to four gonotrophic cycles in her lifetime (Invasive Species Specialist Group [ISSG], 2021), with each clutch approximated to contain 23-46 eggs (Davis et al., 2016). Eggs are desiccation-resistant and rely on water submersion via rainfall to facilitate hatching (Hawley, 1988). Larval development is largely temperature and food dependent, and this life stage usually lasts for 5-10 d. Pupae will emerge as adults after 2 d (Hawley, 1988).

Aedes albopictus is native to the tropical forests of South-east Asia but has established breeding populations in at least 28 countries outside of its original range (Benedict et al., 2007). Within the continental United States, Texas documented the first observation of *Ae. albopictus* in 1985 after receiving shipments of rubber tires from Japan (Moore & Mitchell, 1997; Sprenger & Wuithiranyagool, 1986). Another isolated population was documented in Florida the following year (O'Meara, 1997). To date, *Ae. albopictus*' range has expanded to include more than 900 counties across 26 continental states and Hawaii (Center for Invasive Species Research [CISR], N.D.). *Aedes albopictus* is highly competitive in comparison to other container-breeding North American *Aedes* species and has been listed within the top 100 invasive species worldwide (Leisnham & Juliano, 2012). The rapid range expansion of *Ae. albopictus* (Figure 2) is a result of having the ability to: co-exist with humans in urban centers, breed in natural and artificial containers, develop and survive longer in a broader temperature range (15-35°C), and overwinter as eggs in more temperate climates (Reinhold et al., 2018).

Field and laboratory studies have shown *Ae. albopictus* as a competent vector of at least 22 arboviruses (Moore & Mitchell, 1997; Wong et al., 2013) including chikungunya (Leparc-Goffart et al., 2014), dengue (Simmons et al., 2012), yellow fever (Jentes et al., 2011), and Zika viruses (Likos et al., 2016; McKenzie et al., 2019). Within the continental United States, surveillance of human infection from these four pathogens indicates that cases are scarce and mostly limited to Florida. No reports of human infections by these viruses have been documented in Lowndes, Co., Georgia since the introduction of *Ae. albopictus*; it is unlikely that the respective pathogens of such infections are currently found in Valdosta *Ae. albopictus* populations. To date, *Ae. albopictus* has been deemed as only a biting nuisance within Valdosta due to having little ecological, economic, and social impact. Despite being a low priority species in the Valdosta region, *Ae. albopictus*' vector status may be subject to change in upcoming decades. Rising global temperatures and frequent interstate migration are likely to influence an increase in pathogen and vector distribution across the Southeastern United States.

Managing resistance is as complex and dynamic as the evolutionary pathways leading to it. Since population suppression methods frequently rely on chemical insecticides, resistance becomes a major threat to the success of integrated mosquito management programs (Rivero et al., 2010). The CDC bottle bioassay can be implemented as the initial step of resistance management by gauging susceptibility and identifying related insecticide modes of action (Parker, 2020). Resistance surveillance provides the information necessary for management programs to recommend and implement the best practices for population control. By reducing vector mosquito populations, the risk of contracting arboviral diseases can be mitigated. In addition, early detection of resistance through routine monitoring of natural populations is one of the most effective ways to maintain chemical insecticide integrity/sustainability (Aïzoun et al., 2013; Lopez-Monroy, 2018). Given the importance of insecticide resistance management, the objective of this study was to establish a baseline report of permethrin resistance among *Ae. albopictus* populations in Valdosta Lowndes Co., Georgia.

CHAPTER II: MATERIALS AND METHODS

Collection Sites

Collection sites were chosen by considering major population centers, distributing them almost equidistantly, and covering as much surface area within the city limits. Once the general location of the site was determined, specific addresses that had adequate vegetative cover within a 2-block area were listed as prospects. Prospects were then personally visited to acquire permission from residence/organization owners. Each collection site was considered an isolated *Ae. albopictus* population as the distance between any two sites surpassed 400 m.

Mosquito eggs were collected from July to November of 2019, while nighttime temperatures consistently exceeded 10°C. Collections were made on a weekly basis from 14 sites across Valdosta, GA and 1 site in Lake Park, GA (Figure 3). The Valdosta sites were denoted as follows: (1) Baytree, (2) Carter, (3) Charlton, (4) Conoley, (5) Gordon, (6) Kensington, (8) Myrtle, (9) Nan, (10) Oak, (11) Park, (12) Plantation, (13) Ravenwood, (14) Simpson, and (15) Wooding (Figure 4). With the exception of Gordon and Myrtle, all other sides were residential neighborhoods. Lake Park's only site, (7) Louise (officially named Lake Louise Field Station), was intended to serve as a control for resistance testing since it is a research preserve located in a rural area of the county.

Oviposition Cups

Black 266 mL plastic cups were used as oviposition cups or ovicups. At all collection sites, four ovicups were placed in shaded areas where tampering would be unlikely (Figure 5). Ovicups were fitted with a coarse-finished, germination paper cut to 5 cm x 23 cm. The ovicup's color and germination paper's texture were chosen to simulate the appearance of a tree-hole. A standard sized wooden clothespin was used to secure one piece of germination paper onto each ovicup. Lastly, hay-infused water was used to fill ovicups at approximately 1.25 cm from the rim to account for daily evaporation until the following recovery and replacement of germination paper. Hay-infused water helps to attract gravid mosquitoes (Santana et al., 2006) but is not necessary for collection as ovicups in the field should naturally accumulate organic debris overtime (Ponnusamy et al., 2010; Reiter et al., 1991). Any larvae present in the cups at the time of collection were also retained and reared for testing. Germination paper and hay-infused water were refreshed for ovicups during each collection instance regardless of any eggs or larvae being present. After the sampling period, ovicups were removed from each site prevent the creation of new breeding habitats. Additional tree-hole species such as *Toxorhynchites rutilus* (Coquillet) and *Aedes triseriatus* (Say) were occasionally detected as larvae within collection cups or early lab rearing; these specimens were immediately removed for specimen collections.

Specimen Storage and Rearing

Following retrieval, eggs and larvae were transported to a laboratory at Valdosta State University to be reared. Specimens from different collection sites were isolated in separate plastic containers for rearing. Containers had a clear bottom and opaque detachable lid with the following dimensions: 35.56 cm L x 20.32 cm W x 12.40 cm H. The center of each container's lid was cut out, and the remaining rim of the lid was used to secure a mesh screen underneath. The altered lid facilitated light entry and proper airflow for the enclosed larvae. Containers were prepared with 1 L distilled water, maintained at room temperature, and exposed to a natural day/night cycle (Figure 6). Oviposition papers were separated according to site and gently rinsed with distilled water to remove dirt and other debris. Optimal larval development is compromised by rearing over 250 larvae/L of water, so a maximum of four germination papers were placed into each container during a single rearing cycle (Asahina, 1964). Upon egg submersion (Figure 7), 2 mL of bovine liver solution (40 g/500 mL) were introduced to the water to induce hatching and provide food for developing larvae (Parker, 2020). Germination papers were removed from the rearing container after about 3 d, as they would have eventually deteriorated.

Upon pupation, mosquitoes were transferred to holding cages (also representative of each site) with a 1 mL pipette trimmed at the tip. Each holding cage housed one cotton ball dampened in 10% sucrose solution (Kauffman et al., 2017) and another with only distilled water. Cotton balls were replaced every 4 d. Finally, holding cages were placed into an incubator set to 25 °C with a 12-h day/night cycle. Optimal atmospheric conditions for survival assurance are 24-28 °C and 70-80% humidity, or as high of a humidity that can be attained (Kauffman et al., 2017). Once the first few adults of a holding age had emerged, mosquitoes were given 24 h to feed. Another 24-48 h then elapsed between removal of the sucrose solution and commencement of testing. Since active metabolic activity might skew results in favor of susceptibility, a 24-h minimum was necessary for complete digestion. Only the cotton ball dampened with distilled water remained during this digestive period.

CDC Bottle Bioassay Resistance Testing

The CDC bottle bioassay kit was used as the primary tool for testing the resistance of permethrin. It is important to note that at the time of resistance testing in this study, the CDC had not yet updated its bioassay protocol to include male mosquitoes. The CDC now recommends mixed sex testing as both male and female mosquitoes are equally responsible for relaying resistant genotypes to progeny (CDC, 2020). Species confirmation is crucial to determining which diagnostic dose and time should be applied to the current round of testing. Although color and scale patterns of *Ae. albopictus* are very distinct compared to other regional mosquitos, a standard dichotomous key was used to confirm species prior to testing (Darsie Jr. & Ward, 2005). Mortality due to susceptibility is accepted as either a mosquito's death or inability to right itself and fly. Materials necessary to carry out the CDC bottle bioassay kit included: insecticide stock solution, 90% acetone, 1 mL calibrated droppers, 250 mL Wheaton glass bottle with screw caps, mouth aspirator, timer, and stationary for marking (Figure 8). In order to simulate the diagnostic time of 10 min for a permethrin-susceptible *Ae. albopictus* mosquito, exposure to 43 µg of permethrin per bottle is required. (Table 2). This amount of permethrin in the defined space of the Wheaton bottle conforms to the legal allowance for local distribution. Due to the limited sample size, no synergists were tested in combination with permethrin.

After the stock solution of technical grade permethrin was prepared (CDC, 2016), 1 mL of the solution was dropped into four 250 mL Wheaton glass bottles. A fifth bottle, serving as a control for contamination, did not have any insecticide but instead contained 1 mL of 90% acetone. Mosquitoes placed in the control bottle, whether susceptible or resistant, should not die since there is no exposure to insecticide. All 5 bottles were rolled to ensure full coating and were then left to dry overnight with loose caps in a biological safety cabinet. An opaque covering was also placed upon the bottles to shield from exposure to light while drying. Once the Wheaton bottles were fully dried, a mouth aspirator was used to introduce up to 25 female mosquitoes to each bottle. Mouth aspiration was used because it is considered less damaging to adult mosquitoes during transfer than a vacuum aspirator. An individual timer was set for each bottle at mosquito introduction to increase the accuracy of mortality rate measurements. Mosquitoes were monitored at 5-min intervals for 2 h, and the 0-min mark represented introduction.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel (release 16.0.14026.20202) and Real Statistics Resource Pack software (release 7.6) (Microsoft Corporation, 2018; Zaiontz, 2020).

CHAPTER III: RESULTS

Over five months of field collections and rearing, a total of 1546 *Ae. albopictus* female adults were tested for permethrin resistance using the CDC bottle bioassay kit (Table 3). 1496 adults were tested between all the Valdosta trap sites, and 50 adults were tested from the control trap site of Louise. The number of tested mosquitoes from each trap site varied highly with a range of 17-195 and a mean of 103. All collection sites resulted in <82% mortality when exposed to permethrin at the diagnostic time of 10 minutes (Table 4). Collection sites with <60% mortality at the diagnostic time are as follows: Simpson (21.74%), Park (35.40%), Ravenwood (41.03%), Charlton (54.87%), and Baytree (55.42%). Louise, the control site, resulted in 62.00% mortality at the diagnostic time and ranked 7th most resistant out of all 15 sites (Figure 10). For all collection sites, only 59.06% of mosquitoes died at the diagnostic time (Figure 9). This value remains relatively constant even when Louise is excluded completely. Abbot's formula was not applied to the data to correct mortality rates because the percent mortality within control bottles remained <5% during testing (CDC, 2016).

For all statistical tests employed in this study, $p < 0.05$ was considered significant (Table 5). To determine if all collection sites yielded data with homogeneous variance, a Levene's Test was performed and indicated unequal variances ($df = 14$, $F = 6.08$, $p = 7.46^{-12}$). A Brown-Forsythe test was also carried out to reconfirm the presence of unequal variances ($df = 14$, $F = 4.80$, $p = 9.97^{-09}$). After variance homogeneity among collection sites was assessed, a Kruskal-Wallis test was performed in place of an ANOVA. The Kruskal-Wallis test indicated that medians of time of death (*df* = 14, *H* = 173.04, $p = 8.26^{-33}$) were significantly different between collection sites, but a *post hoc* test was needed to identify the exact comparisons where such significances lay.

A Games-Howell *post hoc* test was chosen for this task and returned 45 instances of significance ($p < 0.05$) across 105 paired collection site comparisons without replication (Table 7). With replication among sites (meaning each treatment level's comparison results were considered independently), Oak, Park, Ravenwood, and Simpson returned ≥8 instances of significance when compared to all other sites. Oak and Ravenwood individually returned 8 significant comparisons, while Park and Simpson individually returned 11. When the comparisons of these four sites were excluded from the total, all other sites returned ≤3 instances of significance. Upon further examination, Baytree and Conoley assumed most of the remaining instances of significance. Baytree and Conoley were therefore excluded in addition to the previous four sites. Only a single significant comparison persisted between Plantation and Wooding after this second exclusion.

CHAPTER IV: DISCUSSION

Increased urbanization, trade, and human mobility have led to frequent novel introductions of *Ae. albopictus* in the continental USA (Findlater & Bogoch, 2018; Kraemer et al., 2019; Li et al., 2014). A primary control strategy in vector mosquito management is the use of chemical insecticides such as pyrethroids. By lowering such mosquito populations, the risk of pathogen transmission from infective mosquitoes to humans and other wildlife can also be reduced. However, vector mosquito management does not stop at the application of chemical insecticides. Mosquito populations exposed to chemical insecticides can develop physiological resistances since these products serve as a selective pressure (David et al., 2018; Karunamoorthi & Sabesan, 2013; Machani et al., 2020). Insecticide resistance is local, temporary, dynamic, and situational (Lopez-Monroy et al., 2018). Although *Ae. albopictus* is currently regarded as a nuisance biter in Valdosta, this mosquito species does have the biological capacity to vector numerous pathogens lethal to humans. Moreover, vector-control strategies have not been optimized due to the absence of a locally designated susceptibility testing program or center. This study aimed to provide a baseline insecticide resistance report of permethrin for Valdosta *Ae. albopictus* populations.

Result integrity between the CDC bottle bioassay and WHO susceptibility test is reportedly conflicting. Some studies show no significant differences between results obtained from these two kits (Aïzoun et al., 2013; Vatandoost et al., 2019), but other studies show the opposite (Owusu et al., 2015). Field-collected adults can be directly tested for susceptibility via the CDC bottle bioassay; however, determining and accounting for the mosquitoes' physiological status before testing would have introduced additional complicating factors. The CDC instead recommends sampling from field-collected eggs and larvae for susceptibility testing. At least 50 viable eggs should be collected for a single cohort to represent adequate genetic diversity, but an ideal sample size would be near 250 (Parker, 2020). In this study, the number of screened mosquitoes differed considerably between trap sites with one site, site Myrtle falling below the threshold. If far larger sample sizes were able to be collected, susceptibility testing using both kits would be optimal.

Moreover, the type of resistance mechanism(s) present within a population tends to influence the rate at which it increases in frequency for subsequent generations. Knockdown resistance, a mechanism typically associated with permethrin (Table 2), can appear and rapidly proliferate in a population as a single nucleotide polymorphism (Saavedra-Rodriguez et al., 2007; Yanola et al., 2011). Valdosta populations of *Ae. albopictus* were sampled within as narrow of a time frame as possible to limit sample variation that can occur within a single mosquito season (Parker, 2020). Since *Ae. albopictus* exhibits skip oviposition (Colton et al., 2003; Davis et al., 2015), efficiently sampling for eggs and larvae proved challenging. Several weeks of field collections were necessary to obtain adequate sample sizes for analysis with eggs being laid individually over multiple sites. So far, ovicups have been the preferred solution to counteract low sampling efficiency. Ovicup materials are highly accessible, cheap, and reusable; these attributes allow for many ovicups to be produced, maintained, and replaced. The CDC bioassays performed in this study were optimized for larger sample sizes and shorter collection periods of field-collected eggs and larvae.

Based on WHO's guidelines for determining resistance in a mosquito population, less than 90% mortality at the diagnostic time suggests that resistance is present. All 15 collection sites exhibited <82% mortality at the diagnostic time. The designated control site of Louise displayed a surprising level of resistance that ranked greater than half of Valdosta's sites. Based on this result, properties surrounding Louise are suspected to have some degree of insecticidal contamination. If Louise was a truly a pristine site, it should have had at least 90% mortality at diagnostic time. A secondary control site or record of a historical population would assist in better modelling the mortality rate of an unexposed mosquito population within the area. No published records of mosquito insecticide resistance in Valdosta are available. As a result, comparisons to historical data within the region could not be established. This lack of historical data highlights the need for baseline resistance reports of commonly used insecticides such as permethrin.

The categorical independent variable of collection site consisted of 15 treatment levels. The quantitative predictor variable, dependent on collection site, represented the time of mortality based upon the CDC bottle bioassay. Based on the experimental variables defined in this study, a one-way Analysis of Variance (ANOVA) or nonparametric counterpart would be necessary to interpret the data. The ANOVA allows for simultaneous comparison between three or more treatment levels (groups) by calculating whether the means of the treatment levels are statistically different from the overall mean of the dependent variable. If a significant *p*-value is returned from the ANOVA, the null hypothesis of no significant differences being present between any of the 15 sites is rejected. To confidently perform an ANOVA, certain assumptions of sampling methods and acquired data must be confirmed. Sampled populations must be normally distributed with homogeneous variance, samples and related observations must be drawn randomly and independently of each other, and factor effects must be additive. By violating any one of these assumptions, only nonparametric approaches would be left available for use. Therefore, a Levene's test was used to assess homogeneity or equality of variances. Levene's test is quite similar to a one-way ANOVA, but the dependent variable is instead calculated as the absolute value of the difference between a score and the mean of that score's treatment level. The null hypothesis for a Levene's test states that variances among treatment levels are the same. This test yielded a *p*-value of 7.46^{-12}, meaning that the null hypothesis was rejected and the difference between treatment level variances was statistically significant. A Brown-Forsythe test, sometimes referred to as Modified Levene's test, was also performed. Both the Levene's and Brown-Forsythe tests are functionally identical except that the dependent variable calculation of the Brown-Forsythe test inputs the median of a score's treatment level. The Brown-Forsythe test (*p*-value was 9.97^{-09}) re-confirmed nonhomogeneous variance of the data.

With the knowledge that at least one of ANOVA's assumptions had been violated, only a nonparametric test could be utilized. The Kruskal Wallis test was substituted in place of an ANOVA. Since nonparametric tests do not assume that the data comes from a particular distribution, the data is then ranked or ordered. Simultaneous comparisons can still be made between three or more treatment levels, however the Kruskal Wallis test uses the ranks of the data scores instead of the actual data scores. If the distribution shape of the dependent variable for all treatment levels are similar, the median for these levels would be compared. For an otherwise dissimilar-shaped distribution, the mean would be compared. The null hypothesis for the Kruskal Wallis test used in this study states that the median ranks of all treatment levels are the same. The *p*-value returned was 8.26^{-33}, meaning that the null hypothesis was rejected and the difference between treatment level medians was statistically significant.

Results from the Kruskal Wallis test only confirm that there is a statistically significant difference between medians within the data, but they do not indicate which median comparisons are responsible for this difference. A *post hoc* test would be required to further interpret the data in this way. The Games-Howell *post hoc* test is designed similarly to Tukey's studentized range distribution with Welch's degrees of freedom correction. Since the data variance is nonhomogeneous, this nonparametric approach was chosen to compare all possible combinations of treatment levels. The Games-Howell test controls for type I error and maintains the established alpha value by not assuming samples have equal variances or sizes. For each comparison, the null hypothesis states that the means of the two treatment levels being compared are the same. 41 out of 105 comparisons rendered significant differences between means without replication. The Oak, Park, Ravenwood, and Simpson sites were involved in the majority of the significant comparisons, while Baytree and Conoley sites are additional sites of concern. The Park, Ravenwood, and Simpson sites exhibited the three lowest percentages of mosquito mortality at the diagnostic time. Baytree exhibited the fifth lowest percentage. Conoley and Oak exhibited the highest and second highest percentages. The significant paired comparisons involving these collection sites directly relate to their percentages of mosquito mortality residing on the far lower and upper boundaries.

Valdosta populations of *Ae. albopictus* do show various levels of permethrin resistance based on their medians of time of mortality. The underlying causes for such results are currently unknown and unable to be effectively hypothesized without further research on each collection site and respective population. Further research on collection sites could include a questionnaire or interview for residents on mosquito activity and insecticide use, testing soil and plant concentrations of insecticidal contamination, and accounting for biotic and abiotic factors that are likely to promote or hinder resistance. In addition, potential mechanisms responsible for these physiological resistances can be investigated by implementing a 24 h holding period after insecticidal exposure, testing with synergists, and genotyping. Since a mosquito population's resistance status does tend to fluctuate, egg collections over multiple periods in a single season (e.g., early, middle, and late season) would also be necessary to detect frequency changes.

Without follow-up studies involving a broader range of mosquito species and chemical insecticides, alternatives to permethrin cannot be recommended outright. Yet, it is possible to speculate that these populations may exhibit cross resistance to other pyrethroids, such as deltamethrin, as they share the same mode of action (Moyes et al., 2021). These findings not only highlight the importance of routine resistance surveillance, but also the importance of consideration for mosquito-control alternatives to chemical insecticides. Due to associated biological and environmental hazards, insecticide concentrations recommended for distribution cannot be simply increased to maintain efficacy. If pesticide resistance is not monitored and managed within a mosquito population, decreased efficacy of an insecticide may require complete withdrawal of that product from use (Aïzoun et al., 2013). Clearly, increased awareness for insecticide sustainability and routine monitoring for resistance are vital to the success of current and future mosquito management programs. This study bridged a gap between vector control, public health, and scientific communities of Valdosta by providing a baseline report of *Ae. albopictus* permethrin resistance.

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APPENDIX A: TABLES

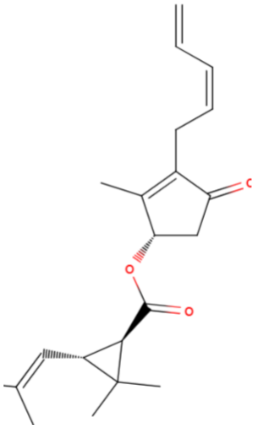
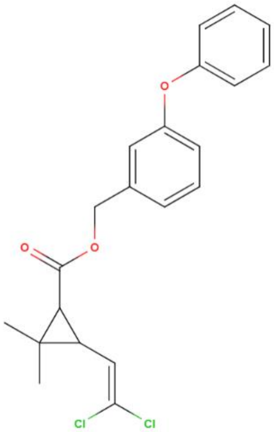
Pyrethrin	**Permethrin**
	

Table 1. Molecular structures of insecticides pyrethrin and permethrin (Bergwerf, n.d.).

Insecticide					**Insecticide concentration (µg/bottle)**	**Diagnostic time (min)**
Common name	Trade name	Chemical name	IRAC Classification	Type	*Aedes albopictus*	
Permethrin	DeLice	3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate	3A	Pyrethroid	43	10

Table 2. Description of permethrin.

Elapsed Time (min)	Site														
	Baytree	*Carter*	*Charlton*	*Conoley*	*Gordon*	*Kensington*	*Louise*	*Myrtle*	*Nan*	*Oak*	*Park*	*Plantation*	*Ravenwood*	*Simpson*	*Wooding*
5	4	32	12	10	58	6	16	0	17	38	11	24	18	5	24
10	42	73	50	43	55	81	15	11	23	42	29	87	62	10	15
15	16	26	37	10	36	8	12	5	10	16	16	34	56	20	13
20	5	25	10	2	10	5	2	1	6	2	41	18	28	17	6
25	13	3	2		6	4	3			1	7	11	20	1	
30	0	5	1		3	2	1				4	5	5	12	
35	2	2	0		1	1	1				3	3	2	3	
40	0		1		1	0					0		1	0	
45	0				1	1					2		0	1	
50	0					0							1		
55	1					0							0		
60						1							0		
65													1		
70													0		
75													0		
80													0		
85													1		
Site Count	83	166	113	65	171	109	50	17	56	99	113	182	195	69	58
Total Count	1546														

Table 3. *Aedes albopictus* site counts and distribution of mortality over 5-min intervals.

Site	**% Mortality at Diagnostic Time**	**Range**
Simpson	21.74%	< 60%
Park	35.40%	
Ravenwood	41.03%	
Charlton	54.87%	
Baytree	55.42%	
Plantation	60.99%	60–69.9%
Louise	62.00%	
Carter	63.25%	
Myrtle	64.71%	
Gordon	66.08%	
Wooding	67.24%	
Nan	71.43%	70–79.9%
Kensington	79.82%	
Oak	80.81%	≥ 80%
Conoley	81.54%	

Table 4. Percentage mortality of *Aedes albopictus* by site at the 10-min diagnostic time.

Site	Mean	Standard Error	Median	Mode	Standard Deviation	Sample Variance	Kurtosis	Skewness	Range	Min	Max	Sum	Count
Baytree	14.82	0.88	10	10	8.02	64.30	6.86	2.11	50	5	55	1230	83
Carter	12.50	0.50	10	10	6.44	41.44	1.48	1.19	30	5	35	2075	166
Charlton	12.70	0.50	10	10	5.30	28.14	6.02	1.65	35	5	40	1435	113
Conoley	10.31	0.41	10	10	3.29	10.84	1.30	0.61	15	5	20	670	65
Gordon	11.35	0.54	10	5	7.00	49.06	4.54	1.78	40	5	45	1940	171
Kensington	12.48	0.73	10	10	7.60	57.70	16.91	3.67	55	5	60	1360	109
Louise	11.80	1.00	10	5	7.05	49.76	1.79	1.32	30	5	35	590	50
Myrtle	12.06	0.75	10	10	3.09	9.56	0.88	1.28	10	10	20	205	17
Nan	10.45	0.64	10	10	4.79	22.98	-0.51	0.59	15	5	20	585	56
Oak	9.24	0.42	10	10	4.19	17.53	1.08	0.93	20	5	25	915	99
Park	16.77	0.75	20	20	7.96	63.36	1.69	0.91	40	5	45	1895	113
Plantation	13.13	0.49	10	10	6.57	43.18	1.43	1.26	30	5	35	2390	182
Ravenwood	15.74	0.67	15	10	9.39	88.23	18.51	3.26	80	5	85	3070	195
Simpson	18.84	1.04	15	15	8.62	74.37	0.08	0.62	40	5	45	1300	69
Wooding	10.09	0.68	10	5	5.17	26.75	-0.94	0.56	15	5	20	585	58

Table 5. Summary statistics across all collection sites for time at mosquito mortality recorded in 5-min intervals.

Site 1 (I)	Site 2 (J)	Mean Difference (I-J)	Std. Error	Sig.	Confidence Interval	
					Lower Bound	Upper Bound
Baytree	Carter	2.319	0.716	0.598	-1.177	5.816
Baytree	Charlton	2.120	0.715	0.735	-1.377	5.617
Baytree	**Conoley**	4.512	0.686	**0.001**	1.148	7.876
Baytree	Gordon	3.474	0.729	0.063	-0.082	7.030
Baytree	Kensington	2.342	0.807	0.765	-1.588	6.272
Baytree	Louise	3.019	0.941	0.615	-1.593	7.632
Baytree	Myrtle	2.760	0.818	0.533	-1.313	6.834
Baytree	**Nan**	4.373	0.770	**0.008**	0.612	8.134
Baytree	**Oak**	5.577	0.690	**0.000**	2.197	8.957
Baytree	Park	1.951	0.817	0.934	-2.025	5.926
Baytree	Plantation	1.687	0.711	0.936	-1.789	5.164
Baytree	Ravenwood	0.924	0.783	1.000	-2.886	4.734
Baytree	Simpson	4.021	0.962	0.185	-0.678	8.721
Baytree	**Wooding**	4.733	0.786	**0.003**	0.893	8.573
Carter	Charlton	0.199	0.499	1.000	-2.219	2.617
Carter	Conoley	2.192	0.456	0.055	-0.022	4.407
Carter	Gordon	1.155	0.518	0.963	-1.348	3.658
Carter	Kensington	0.023	0.624	1.000	-3.008	3.053
Carter	Louise	0.700	0.789	1.000	-3.212	4.612
Carter	Myrtle	0.441	0.637	1.000	-2.857	3.739
Carter	Nan	2.054	0.574	0.429	-0.757	4.864
Carter	**Oak**	3.258	0.462	**0.000**	1.021	5.495
Carter	**Park**	4.270	0.637	**0.000**	1.179	7.360
Carter	Plantation	0.632	0.493	1.000	-1.752	3.016
Carter	**Ravenwood**	3.244	0.592	**0.011**	0.381	6.106
Carter	**Simpson**	6.341	0.815	**0.000**	2.334	10.347
Carter	Wooding	2.414	0.596	0.229	-0.505	5.332
Charlton	**Conoley**	2.391	0.456	**0.022**	0.173	4.610
Charlton	Gordon	1.354	0.518	0.875	-1.152	3.860
Charlton	Kensington	0.222	0.624	1.000	-2.810	3.254
Charlton	Louise	0.899	0.789	1.000	-3.013	4.811
Charlton	Myrtle	0.640	0.637	1.000	-2.659	3.940
Charlton	Nan	2.253	0.574	0.275	-0.560	5.065
Charlton	**Oak**	3.457	0.462	**0.000**	1.216	5.698
Charlton	**Park**	4.071	0.636	**0.001**	0.979	7.162
Charlton	Plantation	0.433	0.493	1.000	-1.954	2.820

Charlton	**Ravenwood**	3.044	0.592	**0.025**	0.180	5.909
Charlton	**Simpson**	6.141	0.815	**0.000**	2.135	10.148
Charlton	Wooding	2.613	0.596	0.133	-0.307	5.533
Conoley	Gordon	1.037	0.476	0.969	-1.273	3.348
Conoley	Kensington	2.169	0.590	0.378	-0.705	5.044
Conoley	Louise	1.492	0.762	0.986	-2.306	5.291
Conoley	Myrtle	1.751	0.604	0.753	-1.431	4.934
Conoley	Nan	0.139	0.537	1.000	-2.507	2.784
Conoley	Oak	1.065	0.415	0.888	-0.956	3.086
Conoley	**Park**	6.462	0.603	**0.000**	3.525	9.400
Conoley	**Plantation**	2.824	0.449	**0.001**	0.643	5.005
Conoley	**Ravenwood**	5.436	0.556	**0.000**	2.741	8.131
Conoley	**Simpson**	8.533	0.789	**0.000**	4.640	12.426
Conoley	Wooding	0.221	0.560	1.000	-2.538	2.981
Gordon	Kensington	1.132	0.639	0.995	-1.968	4.232
Gordon	Louise	0.455	0.801	1.000	-3.508	4.418
Gordon	Myrtle	0.714	0.652	1.000	-2.639	4.066
Gordon	Nan	0.899	0.590	0.999	-1.986	3.783
Gordon	Oak	2.103	0.482	0.129	-0.230	4.435
Gordon	**Park**	5.425	0.651	**0.000**	2.266	8.584
Gordon	Plantation	1.787	0.512	0.466	-0.687	4.260
Gordon	**Ravenwood**	4.399	0.608	**0.000**	1.462	7.336
Gordon	**Simpson**	7.496	0.826	**0.000**	3.438	11.553
Gordon	Wooding	1.259	0.612	0.980	-1.731	4.248
Kensington	Louise	0.677	0.873	1.000	-3.615	4.969
Kensington	Myrtle	0.418	0.739	1.000	-3.296	4.132
Kensington	Nan	2.031	0.685	0.736	-1.310	5.372
Kensington	**Oak**	3.235	0.594	**0.013**	0.342	6.127
Kensington	**Park**	4.293	0.738	**0.005**	0.711	7.875
Kensington	Plantation	0.655	0.619	1.000	-2.352	3.661
Kensington	Ravenwood	3.267	0.701	0.073	-0.126	6.659
Kensington	**Simpson**	6.364	0.896	**0.000**	1.981	10.746
Kensington	Wooding	2.391	0.704	0.517	-1.040	5.821
Louise	Myrtle	0.259	0.882	1.000	-4.152	4.670
Louise	Nan	1.354	0.838	0.998	-2.788	5.495
Louise	Oak	2.558	0.766	0.550	-1.255	6.370
Louise	**Park**	4.970	0.882	**0.010**	0.637	9.303
Louise	Plantation	1.332	0.785	0.997	-2.563	5.227
Louise	Ravenwood	3.944	0.851	0.087	-0.242	8.129
Louise	**Simpson**	7.041	1.018	**0.000**	2.050	12.031

Louise	Wooding	1.714	0.853	0.983	-2.497	5.924
Myrtle	Nan	1.612	0.697	0.940	-1.941	5.165
Myrtle	Oak	2.816	0.608	0.128	-0.379	6.012
Myrtle	**Park**	4.711	0.749	**0.003**	0.952	8.470
Myrtle	Plantation	1.073	0.632	0.995	-2.206	4.353
Myrtle	**Ravenwood**	3.685	0.712	**0.039**	0.091	7.279
Myrtle	**Simpson**	6.782	0.906	**0.000**	2.287	11.277
Myrtle	Wooding	1.973	0.715	0.815	-1.657	5.602
Nan	Oak	1.204	0.542	0.961	-1.460	3.868
Nan	**Park**	6.323	0.697	**0.000**	2.929	9.718
Nan	Plantation	2.685	0.569	0.071	-0.100	5.470
Nan	**Ravenwood**	5.297	0.657	**0.000**	2.103	8.492
Nan	**Simpson**	8.394	0.863	**0.000**	4.161	12.627
Nan	Wooding	0.360	0.660	1.000	-2.878	3.598
Oak	**Park**	7.527	0.607	**0.000**	4.572	10.483
Oak	**Plantation**	3.889	0.455	**0.000**	1.686	6.093
Oak	**Ravenwood**	6.501	0.561	**0.000**	3.787	9.216
Oak	**Simpson**	9.598	0.792	**0.000**	5.692	13.504
Oak	Wooding	0.844	0.565	0.999	-1.934	3.622
Park	**Plantation**	3.638	0.632	**0.006**	0.571	6.705
Park	Ravenwood	1.026	0.712	1.000	-2.420	4.473
Park	Simpson	2.071	0.905	0.952	-2.352	6.494
Park	**Wooding**	6.684	0.715	**0.000**	3.201	10.167
Plantation	Ravenwood	2.612	0.587	0.109	-0.225	5.449
Plantation	**Simpson**	5.709	0.811	**0.000**	1.720	9.698
Plantation	**Wooding**	3.046	0.591	**0.029**	0.152	5.940
Ravenwood	Simpson	3.097	0.875	0.446	-1.181	7.375
Ravenwood	**Wooding**	5.657	0.676	**0.000**	2.368	8.946
Simpson	**Wooding**	8.754	0.877	**0.000**	4.453	13.056

Table 6. Games-Howell test results inclusive of all collection site comparisons without replication; site comparisons that returned a significant *p*-value are bolded.

APPENDIX B: FIGURES

Figure 1. Adult female *Aedes albopictus* (Evans et al., 2019).

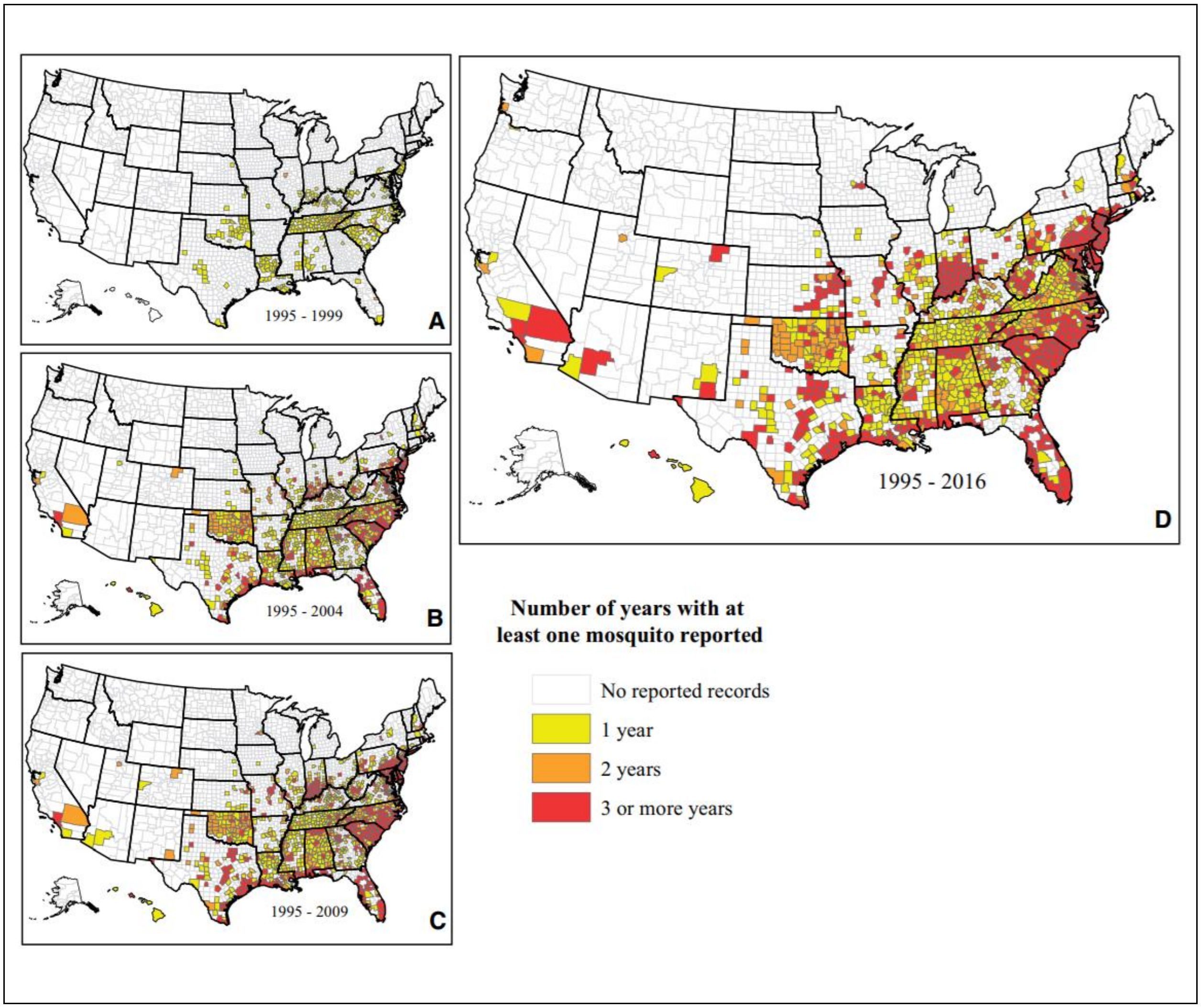


Figure 2. *Aedes albopictus* estimated range in continental United States between years 1995 and 2016 (Hahn et al., 2016).

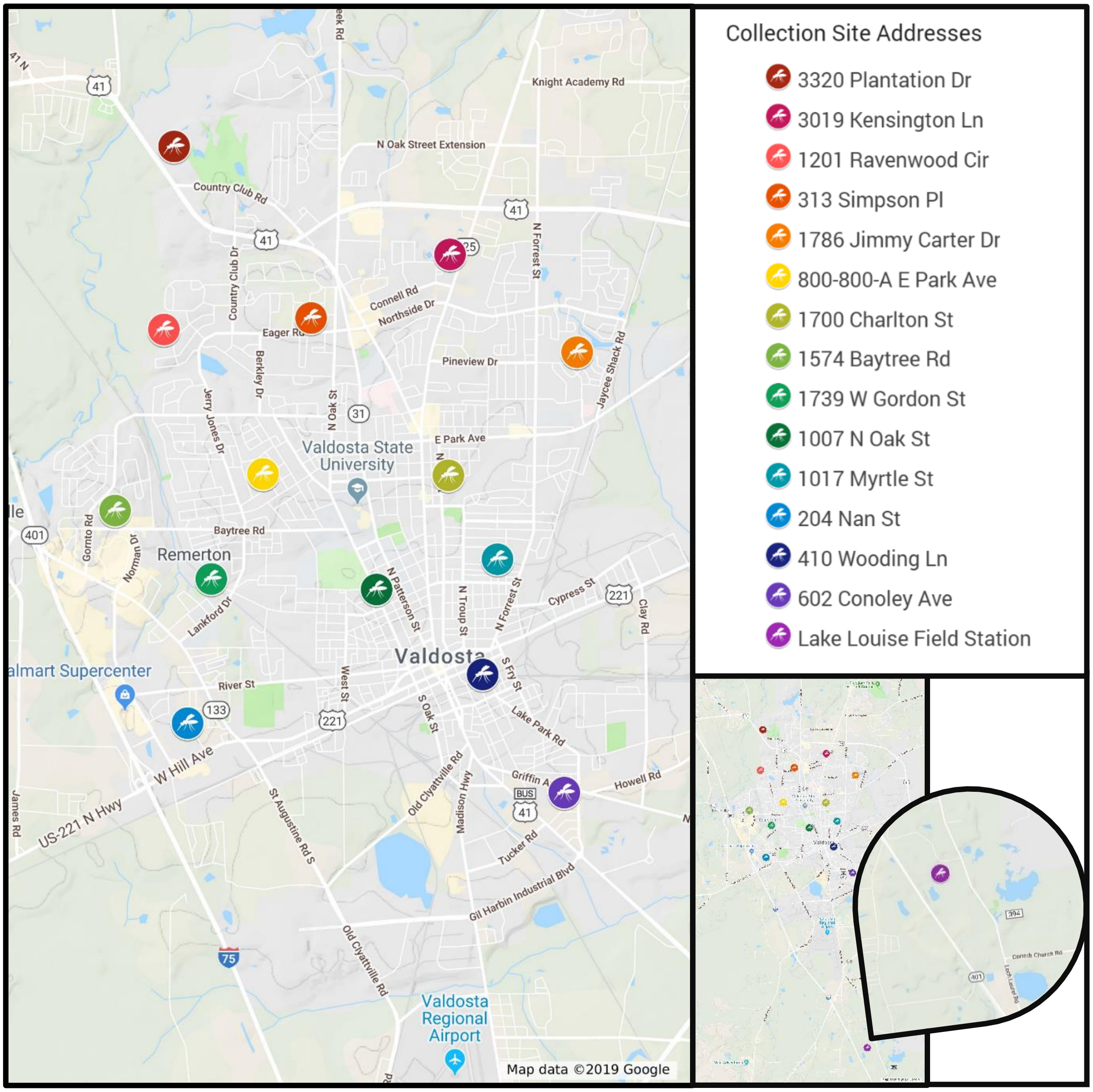


Figure 3. *Aedes albopictus* egg collection site locations within Valdosta and Lake Park, Georgia. Map constructed using Google Maps (2019).

Figure 4. Collection sites within Valdosta and Lake Park, Georgia (from top left to right): Baytree, Carter, Charlton, Conoley, Gordon, Kensington, Louise, Myrtle, Nan, Oak, Park, Plantation, Ravenwood, Simpson, and Wooding.

Figure 5. Examples of oviposition cup placement in the field.

Figure 6. Egg and larval rearing containers.

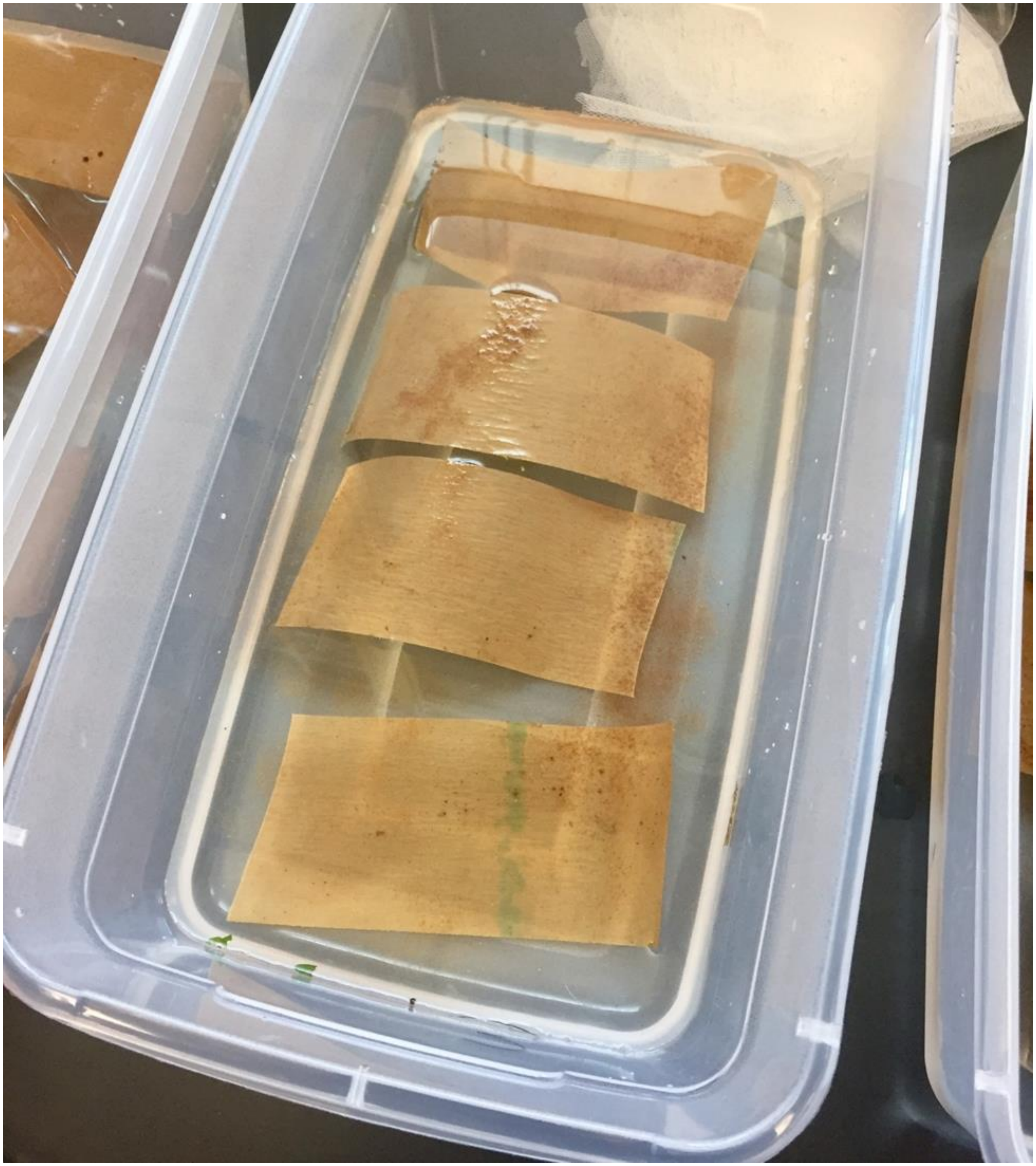


Figure 7. Germination papers with *Aedes albopictus* eggs submerged in distilled water and bovine liver solution.

Figure 8. CDC bottle bioassay apparatus (CDC, 2016).

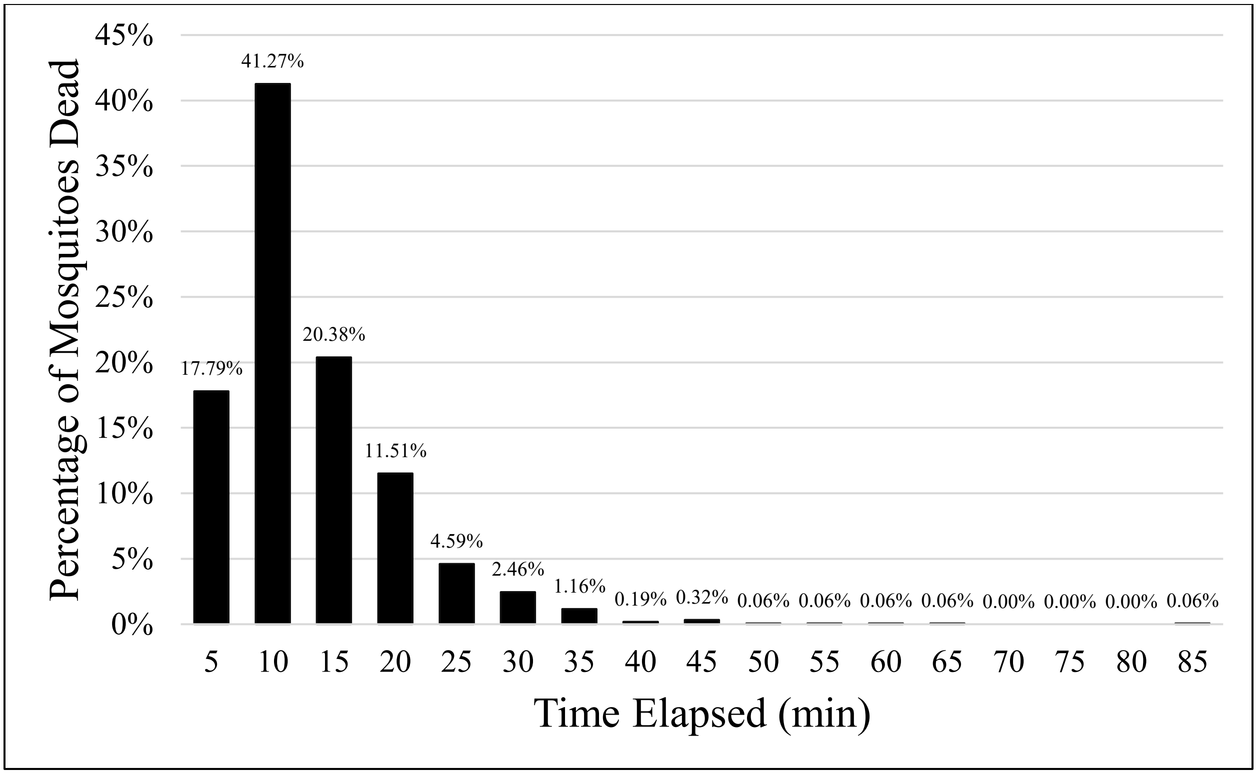


Figure 9. *Aedes albopictus* percentage of mortality comprising all collection sites for each 5-min interval.

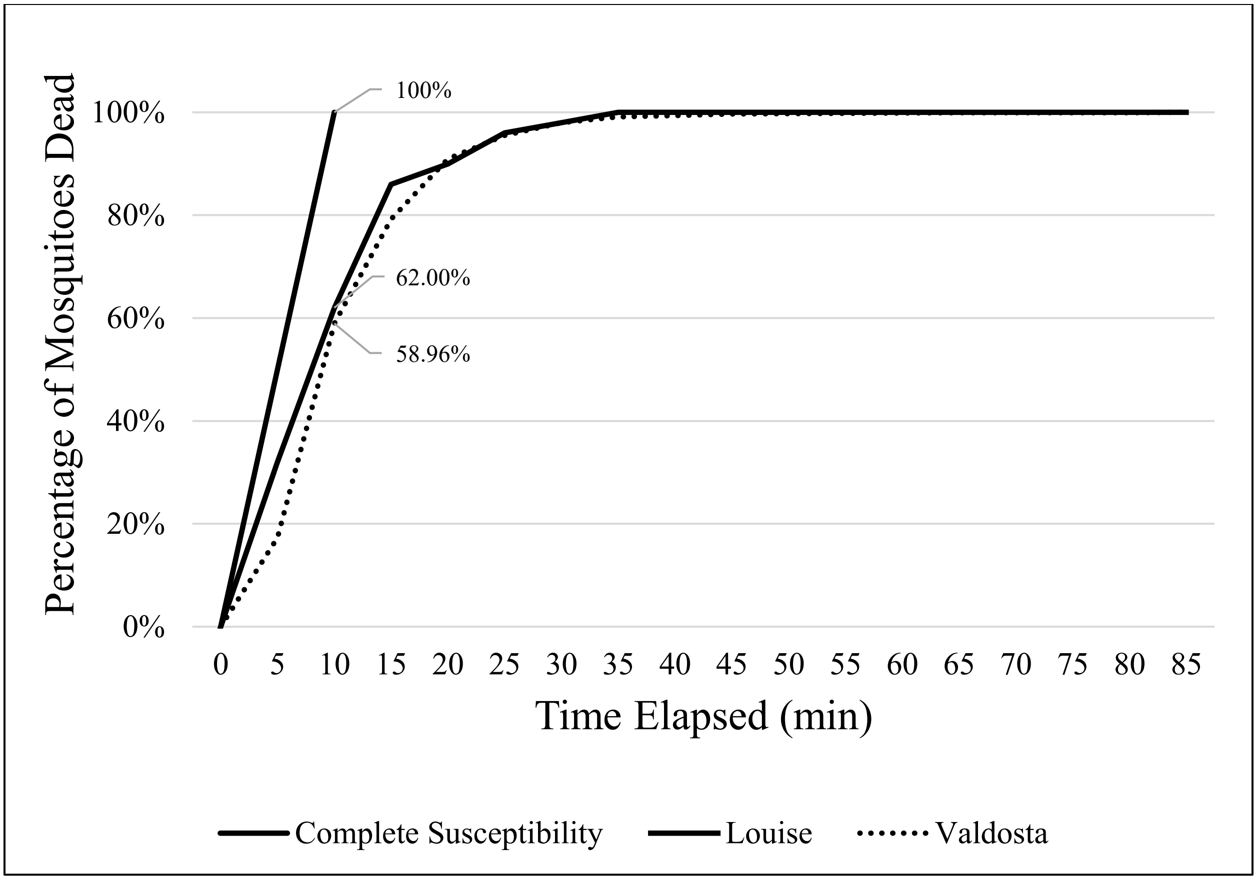


Figure 10. Mortality rates compared across *Aedes albopictus* populations that represent complete susceptibility (theoretical), Louise, and all Valdosta sites.