Assessment of Insecticide Resistance to Organophosphates and Pyrethroids in *Aedes aegypti* (Diptera: Culicidae)

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Aedes aegypti is the primary vector of pathogens such as Zika, dengue, yellow fever and chikungunya viruses, making mosquito control for this species a vital part of protecting public health. Pesticides used to control the infestation of adult mosquitoes that transmit disease, also known as adulticides, prevent the onset and spread of vector-borne disease outbreaks. It is essential to conduct mosquito surveillance and to determine the insecticide resistance status for populations before adulticiding. Only the most effective insecticides should be used to avoid financial loss and ineffective control of mosquitoes. Pyrethroids and organophosphates are the most commonly used insecticides for mosquito control. Permethrin (a pyrethroid) accounts for 25% of all insecticides used throughout the world, and thus pyrethroid resistant mosquito populations are of public health concern. Here, the efficacy of active ingredients (AIs) (permethrin [pyrethroid], chlorpyrifos [organophosphate]), formulated products (Mosquitomist™ [contains chlorpyrifos], Biomist© [contains permethrin]), and synergists (piperonyl butoxide, diethyl maleate, s-s-s-tributyl phosphorotrithioate) were evaluated for controlling two populations (pyrethroid resistant and susceptible) of Ae. aegypti in a laboratory setting. We show that Mosquitomist™ performed best against the pyrethroid

resistant population with a mortality rate of 100% at the diagnostic time. The addition of synergists to AIs did not increase the efficacy against the pyrethroid resistant mosquito population. This resistance to synergists may due to the mechanism of action working to enable this population of mosquitoes to be pyrethroid resistant. Further research is needed to discover how mechanisms of resistance may impact synergist effectiveness.

Assessment of Insecticide Resistance to Organophosphates and Pyrethroids in *Aedes aegypti*

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CHAPTER I – INTRODUCTION

Aedes (Ae.) aegypti L. is the primary vector of Zika, dengue, chikungunya, and yellow fever viruses, making mosquito control for this species a vital part of protecting public health (Centers for Disease Control and Prevention [CDC] 2016). The most effective method currently available for reducing vector-borne disease is through mosquito control (Sun et al. 2014). Integrated mosquito management programs often use insecticides to control adult populations of *Ae. aegypti* (CDC 2016). However, with frequent use by mosquito control programs and other sources (e.g., agricultural, homeowner), insecticide resistance may develop, especially for active ingredients (AIs) used in adulticides. Hence, routine monitoring of insecticide resistance should be included in mosquito control programs (Sun et al. 2014, CDC 2016). Most insecticides used in mosquito control include AIs such as pyrethroids (e.g., permethrin, bifenthrin, deltamethrin) or organophosphates (e.g., malathion, chlorpyrifos). Pyrethroids, used in bed nets and other indoor uses to control pests, comprise 25% of the global insecticide market due to their low toxicity in humans and high toxicity in insects (Hemingway et al. 2004). Organophosphates, while not as widely used as pyrethroids, contain AIs that are of human health and environmental concerns (Environmental Protection Agency [EPA] 2018a).

The objectives of the current study on *Ae. aegypti* are to:

1) Determine insecticide resistance status to pyrethroid and organophosphate formulated products and their AIs; and

2) Evaluate the extent to which synergists impact insecticide resistance to pyrethroid and organophosphate AIs.

CHAPTER II – LITERATURE REVIEW

Aedes Aegypti

Ae. aegypti are most commonly found in tropical and subtropical areas of the world and prefer to blood feed on humans (Centers for Disease Control and Prevention [CDC] 2012). *Ae. aegypti* is considered the primary vector for dengue, yellow fever, chikungunya, and Zika (Smith et al. 2016). Dengue is a risk to an estimated 40% of the world's population and yellow fever is responsible for 30,000 deaths worldwide (Smith et al. 2016). It is important to understand the status of mosquito resistance to best treat a geographic area. If resistance is present in a class of insecticides, ways to efficiently identify resistance and knowledge about alternative treatments is vital to prevent a public health emergency. In areas with high occurrences of vector-borne disease, bed nets treated with permethrin are a cheap, effective solution to combat the incidence of disease, especially malaria (Wilson et al. 2014). A meta-analysis concluded that even a 30% reduction in mosquitoes mortality due to pyrethroids resistance could result in 245 additional cases of malaria per 1000 people, with an even larger impact on areas that depend on bed nets treated with pyrethroids (Churcher et al. 2016). Because pyrethroids are currently the only approved insecticides for use on bed nets, it is especially important to understand how to combat pyrethroid resistant populations. Understanding a mosquito populations current resistance status allows mosquito control programs to be more efficient with their budgets, reduce the amount of pesticides being sprayed in the environment and prevent vector-borne outbreaks.

Insecticide Resistance

Globally, mosquito-borne diseases are on the rise due, in part, to increased global travel, insecticide resistance, and other factors, leading researchers worldwide to develop more effective strategies to control mosquitoes (Liu 2015, Hemingway et al. 2004, Brogdon & McAllister 1998). In both developing and developed countries, public health agencies have implemented mosquito control programs that use adulticides as one method for controlling mosquito populations and surveillance-based targeted control in the form of either formulated products (FPs) and active ingredients (AIs) (Sun et al. 2014). The two most common classes of chemicals used as adulticides are organophosphates and pyrethroids (CDC 2016). Organophosphate and pyrethroid insecticides target the nervous system of mosquitoes (Hemingway & Ranson 2000). With long-term and repeated use of insecticides, mosquitoes may develop insecticide resistance. Hence, routine testing for insecticide resistance should be conducted to ensure that effective products are being used in mosquito control programs (CDC 2016). Mosquitoes may be exposed to insecticides from private/government mosquito control programs, agricultural, and/or home-owner applications (Richards et al. 2018, Brogdon et al. 1998, EPA 2018a, EPA 2018b).

There are multiple types of insecticide resistance including 1) altered target-site resistance, 2) metabolic resistance, 3) behavioral resistance, 4) and penetration resistance. Behavioral resistance is the ability for an insect to avoid the insecticide and has been reported in organophosphate, pyrethroids, and carbamates (Southern Region Integrated Pest Management Center [SRIPMC] 2013). Penetration resistance occurs when an insect develops a thickened outer cuticle that slows the rate of insecticide

absorption and is often present alongside other types of resistance (SRIPMC 2013). Metabolic resistance is defined as genetic change, such as mutations in protein-coding genes that increase metabolic detoxification, of an insect in response to the exposure to a specific toxicant (CDC 2016, SRIPMC 2013). Altered target-site resistance occurs when the target-site for a toxin is modified due to a genetic mutation in protein-coding genes that decrease sensitivity of target proteins in an insect to reduce the effects of the toxin (SRIPMC 2013). Individual mosquitoes that carry resistance alleles may survive exposure to the stressor (e.g. AI), and potentially pass this resistance characteristic to their offspring, thereby increasing the proportion of resistant insects in a population (Liu 2015, SRIPMC 2013).

Of the four types of insecticide resistance, the two most common types are: 1) target-site resistance: insecticide no longer binds to the target-site, and 2) metabolic detoxification enzyme-based resistance where levels of esterases, glutathione s-transferases (GST), or oxidases are modified to prevent the insecticide from reaching the action site (Brogdon & McAllister 1998). Esterases, GST, and oxidases are members of multigene families responsible for detoxification of xenobiotics, substances foreign to the body such as insecticides, in living organisms (Brogdon & McAllister 1998). The development of resistance varies by geographical area, mosquito species, and other environmental factors to which mosquitoes are exposed. Due to the fact that several factors are responsible for mosquito resistance, the timeline for the development for mosquito resistance is not well characterized in mosquitoes.

CDC Bottle Bioassay

The most direct, efficient, and cost-effective method for determining insecticide resistance is through the CDC bottle bioassay (Brogdon & McAllister 1998). Bottle bioassays allow researchers to determine insecticide resistance in a potential vector population. The CDC bottle bioassay uses timed mortality data to provide initial evidence of insecticide resistance in a given mosquito population. The timed mortality data measures the time it takes for the insecticide to penetrate the mosquito, traverse the intervening tissues, move to the action site and act on it. The diagnostic dose (DD) and diagnostic time (DT) are determined for the tested insecticide in a susceptible mosquito population and this serves as reference points for comparison to tested field population. Once a DD and DT is determined, it is important to consistently use these parameters to assess potential changes in resistance over time for that population (CDC 2013).

Using a single AI in a bottle bioassay provides data on insecticide resistance to that AI in adult mosquitoes. If resistance is detected in a bottle bioassay, one can test for resistance mechanisms using a bottle bioassay with synergists. Synergists act by stopping a certain detoxification enzyme, oxidase, esterase, or glutathione s-transferases, from causing resistance to an insecticide and indicates to researchers which resistance mechanism or mechanisms may be causing resistance. When a synergist is used on a resistant population, one of three things may occur: 1) resistance to the insecticide is abolished which dictates that the mechanism of resistance is related to the synergist mechanism of action; 2) resistance to the insecticide is partially abolished suggesting that the mechanism for that synergist is related to the resistance but it is not the only mechanism occurring in the resistance; or 3) resistance to the

insecticide is unaffected suggesting that the mechanism of the synergist is not related to the mechanism of resistance for that insecticide. If resistance in present that cannot be attributed to the detoxification enzymes, then the cause is likely to be a target site mechanism such as sodium channel mutation or insensitivity to acetylcholinesterase (CDC 2013).

Insecticides

a. Pyrethroids

Pyrethroids are a class of insecticides that are a synthetic formulation of pyrethrins, which are derived from chrysanthemum flowers (EPA 2018b). Pyrethroid AIs bind to the sodium channels in mosquitoes, thereby altering the gate properties (keeps the gate open) (Liu 2015). At the cellular level, pyrethroids cause the cell membrane to be persistently depolarized and disrupt nerve function, causing synaptic disturbances, paralysis, and eventually death (Dong et al. 2014). The consistent opening of sodium channels allows the excessive release of acetylcholine, overstimulating nerve and muscle fibers, causing death in invertebrates (Leake 1982). Resistance to pyrethroids may occur when genetic mutations modify the sodium channel structure, also known as knock down resistance, so that the binding affinity of the insecticide AI to the protein is altered, potentially diminishing the effects of the insecticide (Liu 2015). Resistance resulting from a reduced sensitivity of sodium cannels is called knockdown resistance (Liu 2015).

Permethrin has been registered as an insecticide with the EPA since 1967 to be used in bed nets, flea products for dogs, treating clothing, outdoor insect sprays,

agricultural products, and mosquito repellants. Permethrin is the most commonly used mosquito adulticide in the United States and is used in 9-10 million acres out of 32-39 million acres treated with mosquito adulticide annually. Pyrethroids may be applied as ultra-low volume spray (ULV) aerosolized droplets that are suspended in the air to kill flying mosquitoes on contact and/or as a barrier spray to keep mosquitoes from entering an area for a certain period of time. Formulated pyrethroid products can only be applied by trained personnel (i.e., public health pest control license) and public health officials. When applied in accordance to label instructions, pyrethroids do not pose unreasonable risks to humans. Pyrethroids have a low toxicity in mammals and birds but are toxic to fish and bees. Label use requires buffer zones to protect water bodies for uses other than mosquito control and specific instructions to reduce risk to pollinators. The two major mechanisms of pyrethroid resistance in mosquitoes are increased detoxification through cytochrome 450 monooxygenases, an oxidase enzyme which is responsible for broad detoxification, and mutations in Vssc, the gene that codes for the sodium channel proteins and is responsible for knockdown resistance (Smith et al. 2016).

b. Organophosphates

The target-site for organophosphates is acetylcholinesterase, an enzyme that breaks down the neurotransmitter acetylcholine in nerve synapses (Brogdon & McAllister 1998, Fukuto 1990, Essandoh et al. 2013). Acetylcholine is involved in the transmission of nerve impulses to effector cells in the neuromuscular, cholinergic, and synaptic junctions (Fukuto 1990). When a nerve impulse is sent down the parasympathetic neuron, the acetylcholine that is stored in the vesicles is released into the synaptic or neuromuscular junction (Fukuto 1990). The acetylcholine binds to the

acetylcholine receptor on the postsynaptic membrane which then stimulates the nerve or muscle fiber (Fukuto 1990). Acetylcholinesterase reduces and regulates the concentration of acetylcholine in the junction (Fukuto 1990). Organophosphates inhibit acetylcholinesterase, which results in a high concentration of acetylcholine in the junction, causing the continuous stimulation of the muscle or nerve fiber and the eventual exhaustion and tetany, intermittent muscle spasms, for the invertebrate (Fukuto 1990). In the case of resistance to organophosphates, esterases commonly contribute to resistance (Hemingway & Ranson 2000). Esterase-based resistance occurs when mosquito populations select for individuals producing an elevated amount of esterases (Hemingway & Ranson 2000). Esterases bind to insecticide AIs, and sequester the AIs, hence rendering them ineffective (Hemingway & Ranson 2000).

Chlorpyrifos, an insecticide, acaricide, and miticide, is used in agricultural markets since 1965 to control pests on foliage and soil and on crops such as soybeans, fruit, broccoli, and corn. In non-agricultural settings, chlorpyrifos can be used to prevent termites in non-structural wood products, such as fencing, and to kill mosquitoes and ants on golf courses and turf. Chlorpyrifos is also registered for use as a mosquito adulticide and for roach/ant bait stations. Chlorpyrifos products are either applied using ULV, liquid, granules, water dispersible granules, water soluble packets, or wettable powders and can be applied to the ground or through aerial equipment. Applying chlorpyrifos requires extra precautions such as wearing chemical resistant gloves, coveralls, and respirators while also restricting entry to treated areas for a period ranging from 24 h to 5 d. In humans, chlorpyrifos acts as a cholinesterase inhibitor and can overstimulate the nervous system, causing nausea, dizziness, confusion, and at

high doses, respiratory paralysis and death. Chlorpyrifos has been linked to incidents of wildlife mortality related to residential, termite, and golf course applications. The EPA has since eliminated chlorpyrifos use in residential settings, restricted its use as a termiticide, and reduced its use rate on golf courses. During the 2016 EPA assessment of chlorpyrifos, it was indicated that with current labeled use, expected residue of chlorpyrifos on food crops exceeded the safety standard put in place by the Federal Food, Drug, and Cosmetic Act of 1938. The EPA analysis also found that estimated drinking water exposure exceeded safe levels, including those from current registered food and non-food uses. Even with the assessment's findings, in March 2017, the EPA denied petitions to revoke pesticide tolerances for chlorpyrifos and cancel chlorpyrifos registrations. The EPA is currently reviewing the potential effects of chlorpyrifos on endangered species and neurodevelopmental issues in humans (EPA 2018a).

c. Formulated Products and Active Ingredients

The FPs tested in the current analysis are Biomist® and Mosquitomist™. Biomist®, a pyrethroid based insecticide, contains the AI permethrin and is distributed by Clarke Mosquito Control (Clarke 2015). Biomist® components include 3% permethrin (AI), 15% piperonyl butoxide (PBO) (synergist), and no more than 82% petroleum distillate mixture (Clarke 2015). Biomist® produces toxic effects in fish, aquatic invertebrates, and aquatic plants, hence is not allowed to be applied near water bodies (Clarke 2015). Mosquitomist™ is an organophosphate which contains the AI chlorpyrifos and is also distributed by Clarke Mosquito Control (Clarke 2017). Mosquitomist™ contains 19.36% chlorpyrifos (AI), 8.27-11.02% light aromatic solvent naphtha, 2.76-1.13% 1,2,4 Trimethylbenzene, 0.028-0.11% xylene, and no more than 65% white mineral oil (Clarke

2017). However, Mosquitomist™ does not contain any synergists (unlike Biomist®). Mosquitomist™ has toxic effects on fish, aquatic invertebrates, and aquatic plants so is not used near water bodies (Clarke 2017).

Synergists

Some FPs have a combination of synergists and AIs to enhance the effectiveness of the AI. Some synergists are enzyme inhibitors for specific detoxification enzymes present in insects (CDC 2013). Synergists that inhibit metabolic detoxification enzymes are esterases, oxidases, and glutathione s-transferases (CDC 2013). If resistance to a FP is observed/suspected in a mosquito population, it is important to understand what is causing this resistance (CDC 2013). For instance, synergists used in a FP may mask underlying resistance to an AI (CDC 2018). Using synergists in CDC bottle bioassays that test AIs may help uncover the mechanism of resistance (e.g. detoxification enzyme or other cause of resistance) (CDC 2013). Commonly used synergists in CDC bottle bioassays are: 1) S-S-S-tributyl phosphorotrithioate (esterase inhibitor), 2) diethyl maleate (glutathione transferase inhibitor), and 3) PBO (oxidase inhibitor) (CDC 2013).

Using S-S-S-tributyl phosphorotrithioate as a synergist will test for esterase-based resistance (CDC 2013). If there is suspected resistance to an organophosphate, then one should use S-S-S-tributyl phosphorotrithioate to test for detoxification-based resistance (Hemingway & Ranson 2000). There is a correlation between decreased esterase activity and increased resistance to organophosphates where esterase activity

was significantly lower in resistant *Ae. aegypti* when compared to susceptible *Ae. aegypti* (Mazzarri & Georghiou 1995).

The synergist diethyl maleate inhibits glutathione transferase, an enzyme that detoxifies a broad range of xenobiotics including insecticides (CDC 2013, Hemingway & Ranson 2000). Hence, glutathione transferase may affect both pyrethroid and organophosphate susceptibility (Hemingway & Ranson 2000). Numerous studies have shown that insects resistant to insecticides may have elevated levels of glutathione transferase (Hemingway & Ranson 2000, Sani et al. 2014, Che-Mendoza et al. 2009).

Piperonyl butoxide inhibits oxidase activity and reduces pyrethroid resistance in multiple mosquito species, including *Culex* and *Aedes* genera, as it acts as a P450 inhibitor (CDC 2013, Smith et al. 2016). P450 monooxygenases are an integral part of insects' adaptation to toxic chemicals and are generally the rate-limiting enzyme (Hemingway & Ranson 2000). Monooxygenases are important in the metabolism of xenobiotics and are a part of the endogenous metabolism system in insects (Hemingway & Ranson 2000). Elevated levels of monooxygenase activity are associated with pyrethroid resistance in various mosquito species (Hemingway & Ranson 2000). Because a major mechanism for resistance in pyrethroids is the increased detoxification of xenobiotics through elevated P450 enzymes, using PBO as a synergist will help test whether or not resistance to permethrin is due to an increase in P450 monooxygenase enzymes (Smith et al. 2016). A previous study on pyrethroid resistance in *Ae. aegypti* found that genes for P-450 and glutathione S-transferases were overexpressed in resistant mosquito populations (McAllister et al. 2012).

Previous Studies on Resistance

Previous studies have shown pyrethroid resistance in various *Ae. aegypti* populations (Richards et al. 2018, Mazzarri & Georghiou 1995, and Lopez et al. 2014). Richards et al. (2018) found that, in an *Ae. aegypti* population from Dallas, Texas, 54% of the mosquitoes died in the appropriate diagnostic time, the time required for 97% of mosquitoes to die, for permethrin (indicating resistance) (Richards et al. 2018). Mazzari & Georghiou (1995) found moderate resistance to multiple pyrethroids, including permethrin, in field populations of *Ae. aegypti* in Venezuela. Mazzarri & Georghiou (1995) show that four of six tested *Ae. aegypti* field populations that were resistant to 8 pyrethroid insecticides were also resistant to chlorpyrifos. Bisset et al. (2013) found 100% susceptibility to chlorpyrifos in *Ae. aegypti* in a Costa Rica population, Mazzari & Georghiou (1995) found moderate resistance to chlorpyrifos in an *Ae. aegypti* populations from Venezuela. Researchers and mosquito control programs should be wary of drawing conclusions from past studies to implement in their area as multiple factors such as species, geographical location, temporality, and on-going mutations can affect the resistance of a mosquito population at any given time (Richards et al. 2018, McAllister et al. 2012). Even if there has been previous data finding susceptibility to certain AIs, resistance should be evaluated at least annually to improve control measures.

CHAPTER III – MATERIALS AND METHODS

Mosquito Colonies: Susceptible and Resistant

Two populations of *Ae. aegypti* were used in this study (BEI Resources, Manassas, VA): 1) Susceptible colony: insecticide susceptible population (generation F-48) from Costa Rica (catalog # MRA-726), 2) Resistant colony: pyrethroid resistant population (generation F-18) from Puerto Rico (catalog # NR-48830). Both mosquito colonies were reared using standard procedures (Richards et al. 2009). Two to three mosquito egg strips were placed in plastic pans (24 cm x 36 cm x 5 cm) (BioQuip, Rancho Dominguez, CA) containing approximately 700mL of tap water from a faucet located in a university lab. The mosquitoes were reared in an incubator at 28ºC with a 14h:10h (light:dark) cycle and 80% humidity. Larvae were fed a 2:1 mixture of liver powder and Brewer's yeast *ad libitum* until pupation. Pupae were placed in 500 mL plastic cups which were then transferred into metal cages (12in x 12in x 12in) (BioQuip, Rancho Dominguez, CA) so that mosquito adults could emerge in containment. Adults were provided a 20% sucrose solution *ad libitum* (Richards et al. 2009).

Insecticides

Technical grade permethrin (Chem Service, Inc., Coon Rapids, MN; lot # 7281900) and chlorpyrifos (Chem Service, Inc., Coon Rapids, MN; lot # 7515500) were used. Synergists are as follows: S-S-S-tributyl phosphorotrithioate (Chem Service, Inc., Coon Rapids, MN; lot # 7157700), piperonyl butoxide (Chem Service, Inc., Coon Rapids, MN; lot # 7361000), and diethyl maleate (Frontier Scientific, Logan, UT; lot # L270046).

Biomist© was obtained through Clarke Mosquito Control (St. Charles, IL) (lot #1704183091). Mosquitomist™ was obtained through Clarke Mosquito Control (St. Charles, IL) (lot # 1704170002). The next step was to create stock solutions of the AIs, FPs, and synergists at the diagnostic dose, the dose required to produce susceptibility or enhance the effects of insecticides (CDC 2013). AIs, FPs, and synergists were added to acetone to create a concentration recommended by the CDC (Table 1) (CDC 2013). Stock solution were kept in a refrigerator, at 4ºC, and in light proof bottles (CDC 2013). Stock solutions were taken out of the refrigerator at least an hour before use and gently swirled (CDC 2013).

Bioassays

The CDC bottle bioassay procedure for synergist testing was used for this study (CDC 2013). Several 12-ounce white paper cardboard food containers (Instawares, LLC, Kennesaw, GA) and lids were modified to be resting containers for mosquitoes between steps. The tops of the lids were cut out and replaced with mesh screen to allow air flow into the cartons when mosquitoes were present. A “x” was cut into the side of the carton to allow a space for the aspirator to enter and place the mosquitoes into the carton. This “x” was then covered with a cotton ball and taped to close the portal into the carton and keep the mosquitoes from escaping. The cardboard cartons were carefully labeled by study group. Around 12 hours before the study was conducted, 250mL Wheaton® bottles were coated with 1 mL of stock solution or 1 ml of acetone for the control following the CDC bottle bioassay procedure (CDC 2013). The bottles were then placed on a bottle roller, with the caps removed, at 20 revolutions/min for 2-3 minutes until dry. Next, we removed the cap and rolled the bottles until all visible signs of the

liquid were gone and the bottles are completely dry (CDC 2013). These bottles were stored in a dark drawer overnight before use in the bottle bioassay procedure. Each bottle and cap were labeled with the name of the chemical coated in the bottle, what step in the bioassay it was to be used, and to which group it belonged.

The study had six different study groups: 1) Control (Figure 1), 2) FP (Figure 2), 3) AI alone (Figure 3), 4) S-S-S-tributyl phosphorotrithioate (DEF) and chlorpyrifos (Figure 4), 5) Diethyl maleate (DM) and both permethrin and chlorpyrifos (Figure 5), and 6) Piperonyl butoxide (PBO) and permethrin (Figure 6). PBO is paired with pyrethroid AIs, as they inhibit the oxidase enzymes that break down pyrethroids in insects (Smith et al. 2014). DEF is combined with organophosphate AIs, as they inhibit esterase, the enzyme that breaks down organophosphates (Hemingway & Ranson 2000). DM can be combined with either class of insecticide AIs, as it inhibits glutathione transferase, a general detoxifying enzyme (Hemingway & Ranson 2000).

Each group experienced three steps with approximately 15 adult female mosquitoes per bottle. Step 1) mosquitoes were exposed to clean bottle or synergist-treated bottles for 60 minutes, 2) mosquitoes were transferred to cardboard cartons and held for 30 minutes, 3) mosquitoes were exposed to clean bottle, AI, or FP for 120 minutes. Each bottle was coated, following the CDC bottle bioassay protocol, with 1 mL of the appropriate stock solution the night before the experiment and kept in a cool, dark drawer until the experiment was ready to be conducted (CDC 2013).

The bioassay procedure was split into two groups. The first bioassay was conducted using the resistant group of mosquitoes and the second bioassay was conducted using the susceptible group of mosquitoes. The second bioassay was

conducted immediately after the first using the same bottles as the first bioassay. Five researchers assisted in the bottle bioassay procedure, so each was assigned one of the six treatment groups, with one researcher having the control and another group. Mosquitoes were aspirated out of the colony cage and put into the step one bottles in groups of approximately 15 female mosquitoes per/bottle. Once the step one bottles for each group were filled, the timer for that group began. This process was repeated for each group. When the timer for the group reached one hour, the mosquitoes were aspirated out of the bottles and placed into labeled cardboard cartons for 30 minutes so that each bottle of mosquitoes was transferred to separate cartons. The mosquitoes were then placed into step three bottles. Step three included an assessment of insecticide resistance using the CDC bottle bioassay procedure where the dead and alive female mosquitoes are counted at time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min), based on the time sheet provided by the CDC (CDC 2013).

Data Analyses

The mortality rate was calculated by finding the average mortality rate of all 4, step 3 bottles, at each time interval on the bottle bioassay. According to CDC guidelines, resistance or susceptibility at the DT can be judged by the following criteria: 97%-100% mortality is susceptible, 90%-96% mortality is possible resistance, and ≤ 90% mortality is resistance (CDC 2013). The diagnostic time for each insecticide and synergist/insecticide was determined by finding what time interval in which 97% mortality or greater was achieved in the insecticide susceptible mosquito colony, because this colony was used as the reference group.

The predetermined DT is the most critical time point as it represents susceptibility and resistance in the population (CDC 2013). This average was assessed with a 0 or 1 code for each time interval's average mortality rate. The binary outcome was 0-96% mortality, resistance/developing resistance, (coded as 0) or 97%-100% mortality, susceptibility, (coded as 1). Binary logistic regression applying a significance level of $P < 0.05$ in SPSS (SPSS Inc., Chicago, IL) was used to determine if there were differences in susceptibility or resistance, using the mortality rate at the DT determined by the susceptible population.

This analysis was conducted for two objectives: 1) To compare each bottle group in the resistant population to the same bottle group in the susceptible population (Tables 2-9) and 2) To compare AI only groups in the resistant population to the AI combined with synergist groups in the resistant population (Tables 10-13).

CHAPTER IV – RESULTS

Insecticide resistance status to pyrethroid and organophosphate formulated products and AIs

At the DT, the pyrethroid resistant mosquito colony only showed susceptibility to Mosquitomist™ (100%). This population showed resistance to Biomist® (46%), permethrin (71%), chlorpyrifos (87%), DM and chlorpyrifos (84%), DEF and chlorpyrifos (71%), DM and permethrin (0%), and PBO and permethrin (5%) (Figure 7). Statistically significant differences were found between the insecticide susceptible population and the pyrethroid resistant population in the Biomist®, DM and permethrin, and PBO and permethrin groups (Table 14). For the Biomist® group, there was an odds ratio of 26 which means Biomist®, in the susceptible mosquito colony, is 26 times more likely to result in susceptibility than the pyrethroid resistant population (Table 14). In the susceptible colony the synergist DM in combination with permethrin was 36 times more likely to result in susceptibility than in the pyrethroid resistant population (Table 14). In the susceptible colony the synergist PBO in combination with permethrin was 45 times more likely to result in susceptibility than in the insecticide resistant population (Table 14).

Synergists impact on insecticide resistance to pyrethroid and organophosphate AIs

Neither DM nor PBO, in combination with permethrin, showed a statistically significant impact on mean mortality rates compared to permethrin alone (Table 15). The mortality rate of permethrin alone was 71% at the DT. In comparison, DM in combination with permethrin resulted in a mortality rate of 6% and PBO in combination

with permethrin resulted in a 4% mortality rate (Figure 8). Neither DM nor DEF, in combination with chlorpyrifos, had a statistically significant impact on mortality rates compared to chlorpyrifos alone (Table 16). The mortality rate of chlorpyrifos alone was 87% at the DT. In comparison, DEF in combination with chlorpyrifos had a mortality rate of 71% and DM in combination with chlorpyrifos resulted in a 6% mortality rate at the DT (Figure 8).

CHAPTER V – DISCUSSION

Susceptibility and resistance to AIs and FPs were analyzed in both insecticide resistant and susceptible colonies. We show a statistically significant difference in three groups' mortality rates (Biomist® [p= .009], DM in combination with permethrin [p= .019], and PBO in combination with permethrin [p= .004]). PBO/permethrin (46% mortality) and DM/permethrin (0% mortality) both resulted in the 2 of the lowest mortality rates at the DT in the pyrethroid resistant colony (Figure 7). This finding was expected in the pyrethroid resistant population, as permethrin is classified as a pyrethroid. This was also found in a studies examining synergists in combination with permethrin in a permethrin resistant *Culex pipiens quinquefasciatus* and an *Ae. aegypti* population (Priester and Georghiou 1978 & Astari and Ahmad 2005). Our results demonstrate that this combination of synergists and AIs are the least likely to control a pyrethroid resistant population. Here, mosquitoes exposed to Mosquitomist™ showed the only susceptible mortality rates (100%) at the DT. The pyrethroid resistant colony showed resistance to chlorpyrifos (AI in Mosquitomist™), with a mortality rate of 87% at the DT. This may suggest that other compounds in Mosquitomist™ are masking resistance to chlorpyrifos. This finding is consistent with reported resistance to chlorpyrifos in pyrethroid resistant *Ae. aegypti* populations from Mexico (Lopez et al. 2014; Flores et al. 2009). Our findings may be useful for determining which insecticides to use on a known pyrethroid resistant population.

None of the synergists tested resulted in a statistically significant difference in the mortality rate in pyrethroid resistant mosquitoes. Previous findings suggest some types of synergists may increase mortality in mosquitoes with metabolic resistance (Dadzie et

al. 2017). Hence, some synergists may be ineffective in combating resistance due to target site mutations, such as knock-down resistance, the mutation found in pyrethroid resistant mosquitoes (Kumar et al. 2002 and Sumarnrote et al. 2017). Mazzari & Georghiou (1995) found that synergists DEF and PBO did not affect the mortality of a pyrethroid resistant *Ae.aegypti* population. The same study also found that this lack of effect on mortality rates was because the resistance was not caused by metabolic enzymes. Our findings that synergists did not increase mortality rates in a pyrethroid resistant population, in combination with the findings in previous studies (Mazzari & Georghiou 1995 and Sumarnrote et al. 2017), suggests that the pyrethroid resistance status of our population is due to a target-site mutation. This is an important deciding factor when choosing FPs, which may contain synergists, for adulticiding and may cause potential issues as many programs lack the capability to differentiate between different types of resistance (e.g., metabolic resistance and target-site resistance).

Future studies should compare the difference in mortality rates, when using synergists, in pyrethroid resistant mosquitoes with a metabolic resistance against those with a target site resistance. In addition, understanding the methods to combat the effects of other classes of insecticides on pyrethroid resistant mosquitoes could be advantageous to discover the best method to combat a pyrethroid resistant mosquito population.

Strengths and Limitations of this Study

Few studies have examined resistance versus susceptible status to insecticides in a pyrethroid resistant population and even fewer have investigated the extent to which synergists may affect mortality rates in resistant mosquitoes. This study was

conducted using lab colonized mosquito populations, but future studies may focus on field collected mosquitoes. Lab colonized mosquitoes provide assurance that one type of resistance mechanism is present in that population, knock-down resistance, in this case. This study examined a single AI and FP from two different insecticide classes. Numerous types of organophosphates and pyrethroids that can be studied to determine differences in susceptibility and resistance. The current study investigated one synergist dose for three different synergists, but future studies may want to expand this work to include a dose-response assessment.

CHAPTER VI – CONCLUSION

Insecticide resistance is an increasing concern for mosquito control programs globally (Liu 2015). The goal of mosquito control programs is to reduce the population of mosquitoes and most importantly, to reduce the risk of vector-borne diseases in a geographic area. Because pyrethroids are the most commonly used class of insecticide internationally, understanding how to best combat this issue is important when making choices on what insecticide to spray to control a mosquito population. It is important to understand the status of resistance to best treat an area and if there is a current resistance to a class of insecticides, to know how to treat that population and prevent a public health emergency. We found that exposure to Mosquitomist™ in the mosquito resistant colony resulted in the highest mortality rates but mosquitoes exposed to the AI (chlorpyrifos alone) in this product showed resistance. Here, the addition of synergists to AIs did not increase mortality rates in the pyrethroid resistant mosquito colony; however, factors such as mosquito species, geographic area, insecticides, synergists, insecticide/synergist doses and other factors may have affected the outcome. More research should be conducted to understand the complex relationships between each factor and mortality rates. Results of this research shows that synergists may not be effective when combating target site resistance in pyrethroid resistant mosquitoes.

TABLES

TABLE 1: Stock Solution Preparation and Diagnostic Times

Type of Insecticide	*Amount of Product*	*Amount of Acetone*	*Concentration of AI per Bottle*	*Diagnostic Time*
Permethrin	8 mg	1000 mL	8 µg/mL	60 minutes
Chlorpyrifos	30 mg	1000 mL	30 µg/mL	35 minutes
Biomist©	150 mg	1000 mL	8 µg/mL	15 minutes
Mosquitomist™	258 mg	1000 mL	30 µg/mL	75 minutes
S-S-S-tributyl phosphorotrithioate	125 mg	1000 mL	125 µg/mL	N/A
Piperonyl Butoxide	400 mg	1000 mL	400 µg/mL	N/A
Diethyl Maleate	80 mg	1000 mL	80 µg/mL	N/A

TABLE 2: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with Mosquitomist™

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Group(1)	-.847	1.345	0.397	1	0.529	0.429	0.031	5.985
	Constant	-1.099	0.816	1.810	1	0.178	0.333		

a. Variable(s) entered on step 1: Group.

TABLE 3: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with Biomist©

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	3.283	1.248	6.918	1	**0.009**	**26.667**	2.309	30.8000
	Constant	-.981	0.677	2.099	1	0.147	0.375		

a. Variable(s) entered on step 1: Group.

TABLE 4: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with Permethrin

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	2.120	1.211	3.065	1	0.080	8.333	0.776	8.9470
	Constant	-2.303	1.049	4.820	1	0.028	0.100		

a. Variable(s) entered on step 1: Group.

TABLE 5: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with Chlorpyrifos

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	.421	0.923	0.208	1	0.648	1.524	0.250	9.295
	Constant	.560	0.627	0.797	1	0.372	1.750		

a. Variable(s) entered on step 1: Group.

TABLE 6: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with DEF and Chlorpyrifos

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	-.875	1.366	0.411	1	0.522	0.417	0.029	6.064
	Constant	-.916	0.837	1.199	1	0.273	0.400		

a. Variable(s) entered on step 1: Group.

TABLE 7: Susceptible Colony Compared to Resistant Colony Mortality Rates with DM and Permethrin

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	3.584	1.528	5.504	1	**0.019**	**36.000**	1.803	71.867
	Constant	-1.792	1.080	2.752	1	0.097	0.167		

a. Variable(s) entered on step 1: Group.

TABLE 8: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with DM and Chlorpyrifos

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	.421	0.923	0.208	1	0.648	1.524	0.250	9.295
	Constant	-.981	0.677	2.099	1	0.147	0.375		

a. Variable(s) entered on step 1: Group.

TABLE 9: Susceptible *Aedes aegypti* Colony Compared to Resistant Colony Mortality Rates with PBO and Permethrin

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	3.807	1.308	8.469	1	**0.004**	**45.000**	3.465	58.4339
	Constant	-1.504	0.782	3.702	1	0.054	0.222		

a. Variable(s) entered on step 1: Group.

TABLE 10: Chlorpyrifos without Synergist Compared to Chlorpyrifos with DEF in the Resistant *Aedes aegypti* Colony

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	0.377	0.872	0.187	1	0.665	1.458	0.264	8.048
	Constant	0.182	0.606	0.091	1	0.763	1.200		

a. Variable(s) entered on step 1: Group.

TABLE 11: Chlorpyrifos without Synergist Compared to Chlorpyrifos with DM in the Resistant *Aedes aegypti* Colony

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Group(1)	1.540	0.923	2.788	1	0.095	4.667	0.765	28.466
	Constant	-.981	0.677	2.099	1	0.147	0.375		

a. Variable(s) entered on step 1: Group.

TABLE 12: Permethrin without Synergist Compared to Permethrin with DM in the Resistant *Aedes aegypti* Colony

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Group(1)	-18.900	12118.63	0.000	1	0.999	0.000	0.000	.
	Constant	-2.303	1.049	4.820	1	0.028	0.100		

a. Variable(s) entered on step 1: Group.

TABLE 13: Permethrin without Synergist Compared to Permethrin with PBO in the Resistant *Aedes aegypti* Colony

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Group(1)	-.799	1.308	0.373	1	0.542	0.450	0.035	5.843
	Constant	-1.504	0.782	3.702	1	0.054	0.222		

a. Variable(s) entered on step 1: Group.

TABLE 14: Mortality Rates of Pyrethroid Resistant and Susceptible *Aedes aegypti* Colonies. The synergist acronym for S-S-S-tributyl phosphorotrithioate is DEF, piperonyl butoxide is PBO, and diethyl maleate is DM. *Susceptible colony used as reference group.

	Diagnostic Times	*Mortality Rate (%) Susceptible Colony**	*Mortality Rate (%) Resistant Colony*	*OR*	*P-value*	*95% CI*
			Formulated Products			
Mosquitomist™	75 min	99 N= number dead/ number tested N=75/76	100 N=72/72	0.429	0.53	(0.031-5.98)
Biomist©	**15 min**	**99** **N=90/91**	**46%** **N=41/82**	**26.66**	**0.009**	**(2.31-30.8)**
Permethrin	60 min	100% N=116/116	71% N=62/87	8.333	0.080	(0.776-8.94)

Chlorpyrifos	35 min	100% N=97/97	87% N=95/110	1.52	0.648	(0.250-9.29)
			Synergists and Active Ingredients			
DEF and Chlorpyrifos	35 min	100 N=79/79	71 N=62/87	0.417	0.522	(0.029-6.06)
DM and Permethrin	**60 min**	**97 N=73/76**	**6 N=4/72**	**36**	**0.019**	**(1.80-71.8)**
DM and Chlorpyrifos	35 min	98 N=75/77	6 N=4/78	1.524	0.648	(0.250-9.29)
PBO and Permethrin	**60 min**	**100 N=88/88**	**46 N=46/102**	**45**	**0.004**	**(3.46-58.4)**

TABLE 15: Difference in Permethrin Alone and Permethrin/Synergist Mortality Rates in Resistant *Aedes aegypti* Colony

	AI alone Mortality Rate (%)	*Synergist and AI Mortality Rate (%)*	*OR*	*P-value*	*95% CI*
DM	71	06	0.00	0.99	(0.00-0.00)
PBO	71	04	0.45	0.54	(0.03-5.84)

Diagnostic time is based on diagnostic time for permethrin without a synergist in the susceptible population

TABLE 16: Difference in Chlorpyrifos Alone and Chlorpyrifos/Synergist Mortality Rates in Resistant Colony

	AI alone Mortality Rate (%)	*Synergist and AI Mortality Rate (%)*	*OR*	*P-value*	*95% CI*
DEF	87	71	1.458	0.66	(0.26-8.04)
DM	87	06	4.667	0.09	(0.76-28.46)

Diagnostic time is based on diagnostic time for chlorpyrifos without a synergist in the susceptible population.

FIGURES

FIGURE 1: Control Flow Chart

FIGURE 2: Formulated Product without Synergist Trial

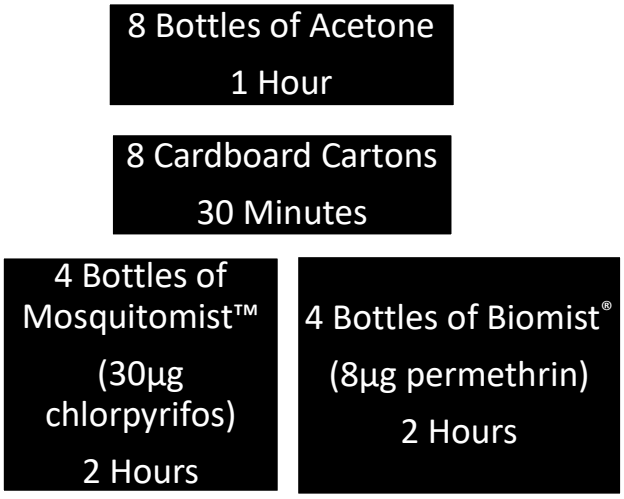


FIGURE 3: Active Ingredient without Synergist Trial

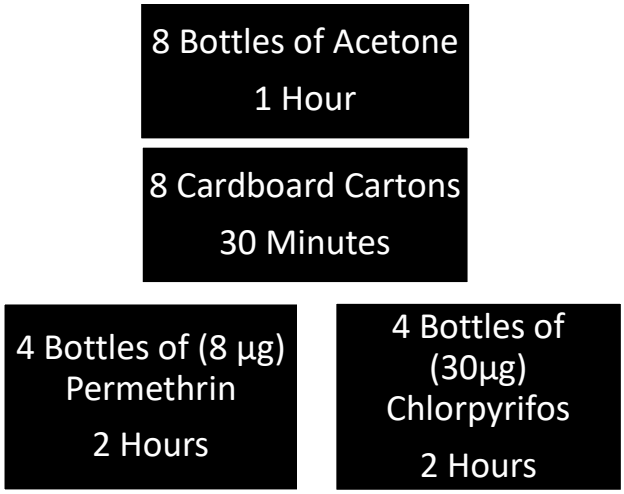


FIGURE 4: Synergist Trial S-S-S-tributyl Phosphorotrithioate

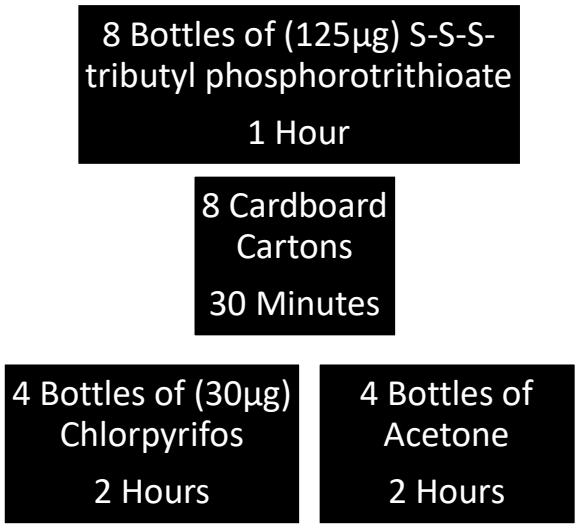


FIGURE 5: Synergist Trial Diethyl Maleate

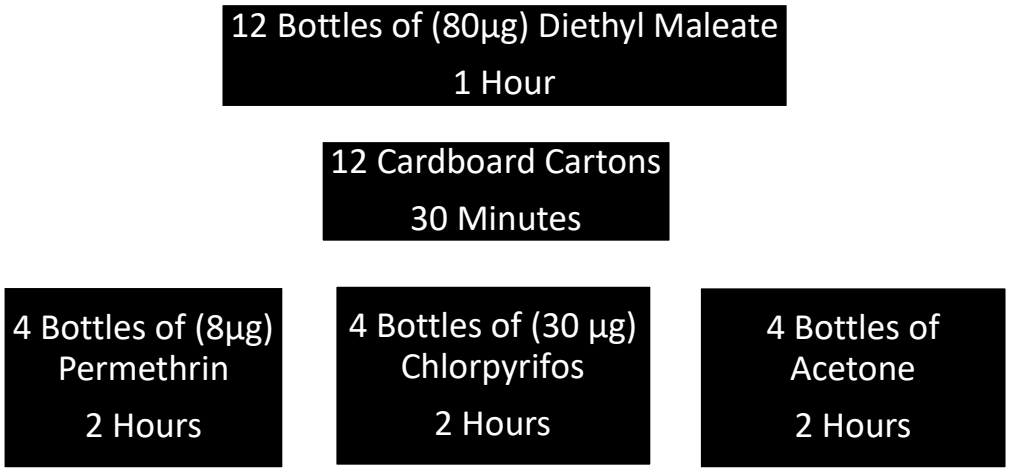


FIGURE 6: Synergist Trial Piperonyl Butoxide

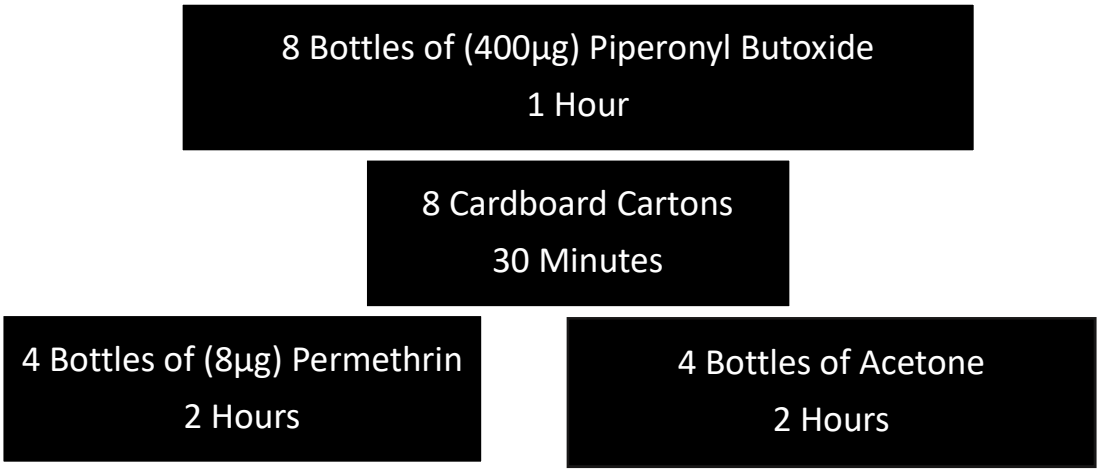
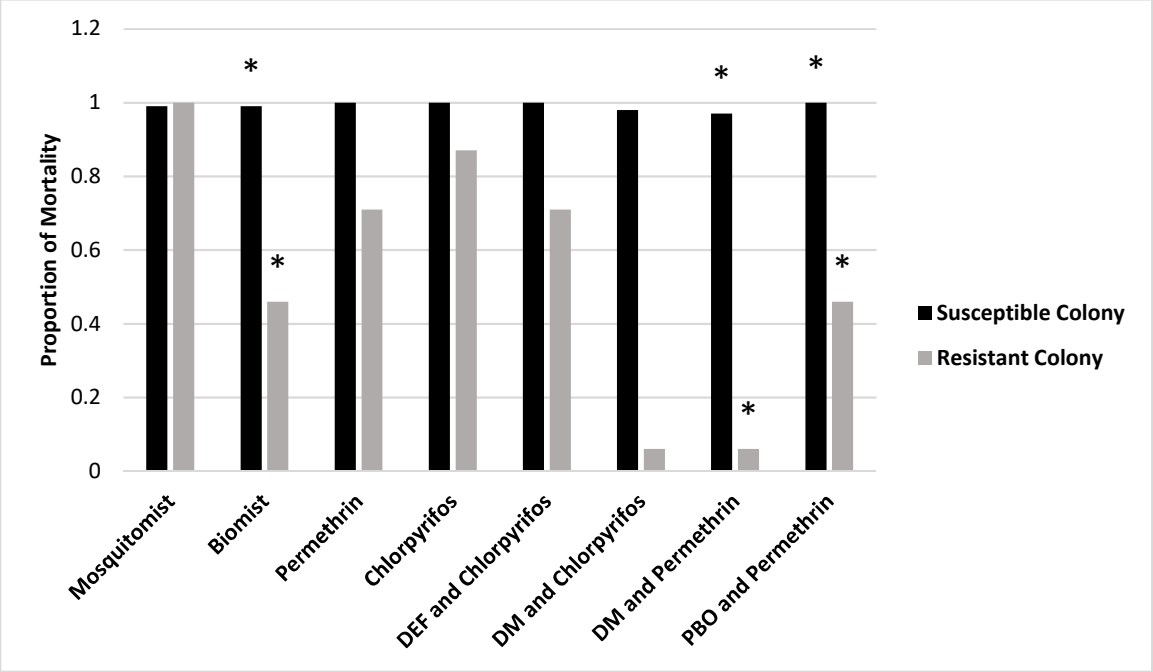
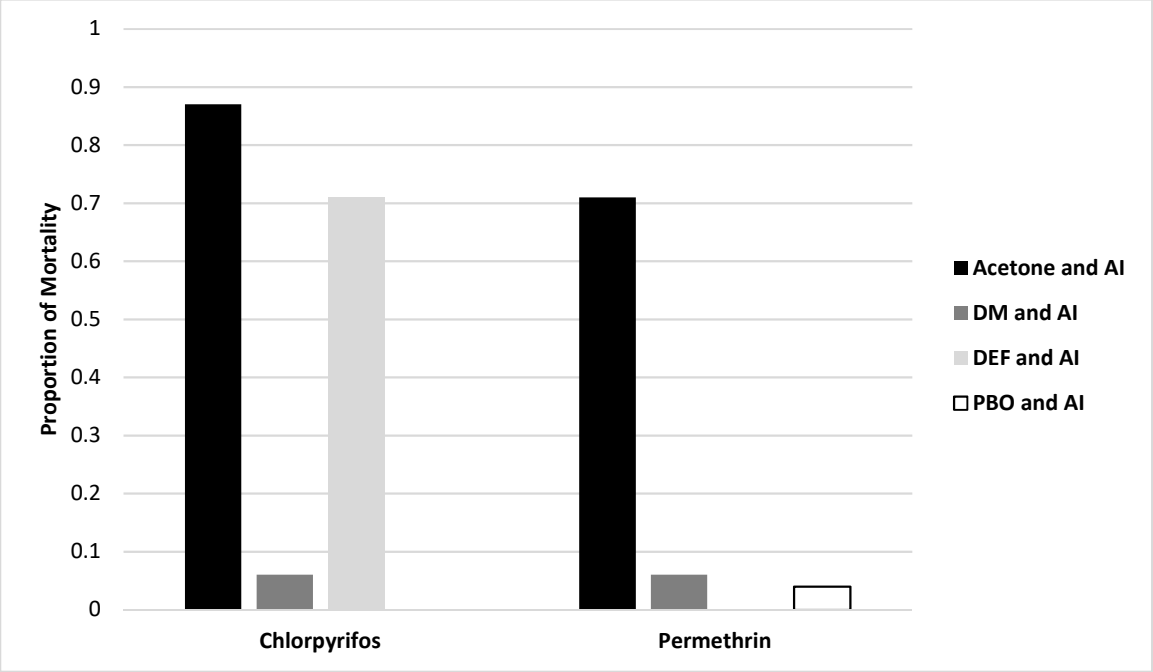


FIGURE 7: Proportion of Mortality at Diagnostic Time in Susceptible and Resistant *Aedes aegypti* Colonies



"*" indicates a statistically significant difference between groups. Diagnostic time for Mosquitomist™ is 75 minutes, Biomist© is 15 minutes, permethrin is 60 minutes, and chlorpyrifos is 35 minutes. The synergist acronym for S-S-S-tributyl phosphorotrithioate is DEF, piperonyl butoxide is PBO, and diethyl maleate is DM. Diagnostic times for synergists and AI was based on the AI diagnostic time.

FIGURE 8: Proportion of Mortality at Diagnostic Time using Synergists in Pyrethroid Resistant *Aedes aegypti* Colonies



Diagnostic time for permethrin is 60 minutes and chlorpyrifos is 35 minutes. The synergist acronyms for S-S-S-tributyl phosphorotrithioate is DEF, piperonyl butoxide is PBO, and diethyl maleate is DM. Diagnostic times for synergists and AI were based on the AI diagnostic time.

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