Increasing Our Understanding of Insecticide Resistance Evolution by Expanding and Comparing Insecticide Susceptibility Bioassays

by

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ABSTRACT

Insecticide resistance is a continuing issue that negatively affects both public health and agriculture and allows vector-borne diseases to spread throughout the globe. To improve resistance management strategies (RMS), robust susceptibility bioassays need to be performed in order to fill the gap of the relationship between resistant and susceptible genotype and phenotype, and a deeper knowledge of how bioassay data relates to vector control success or failure is imperative. A bioassay method that is infrequently used but yields robust results is the topical application bioassay, where the insect is directly treated with a constant volume and concentration of an insecticide via a syringe. To bring more attention to this method, my colleagues and I published a paper in the Journal of Visualized Experiments where the optimized protocol of the topical application bioassay for mosquitoes and fruit flies is described, and the strengths and limitations to the method are explained. To further investigate insecticide susceptibility tests, I set up my individual project where I used *Aedes aegypti* mosquitoes to compare the topical application bioassay to the commonly used Centers for Disease Control and Prevention (CDC) bottle bioassay and World Health Organization (WHO) tube test. The objective of this study was to test which method exhibited the most variability in mortality results, which would guide the choice of assay to determine the link between resistant and susceptible genotype and phenotype. The results showed that the topical application method did indeed exhibit the least amount of variation, followed by the CDC bottle bioassay (WHO data is currently being collected). This suggests that the topical application bioassay could be a useful tool in insecticide resistance surveillance studies, and, depending on the goal, may be better than the CDC and WHO tube tests for assessing resistance levels at a given site. This study challenges

the value of the widely used CDC and WHO assays and provides a discussion on the importance of technical and practical resistance assays. This will help vector control specialists to collect accurate surveillance data that will inform effective RMS.

This thesis is dedicated to my parents, David and Melissa.
I cannot thank them enough for their support, love and encouragement.

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PREFACE

Originally, my MSc thesis project was focused on determining how competition between different genotypes affects the insecticide susceptibility phenotype of resistant and susceptible *Drosophila* strains. Data on this topic is lacking in the literature, and it is crucial to address this gap because 1) insects undoubtedly experience direct competition between different genotypes in the field, 2) knowledge about the fitness of heterozygotes is especially lacking, and 3) answering this question will help break down the complexity of the Window of Selection (WoS), which is a framework that can inform the intensity of selection for resistance at various insecticide concentrations. This research would better inform resistance management strategies (RMS) by increasing our understanding of how certain resistance profiles may behave based on the existing direct competition between genotypes. To do this, I originally proposed to determine the different fitness of resistant phenotypes at various deltamethrin concentrations using *Drosophila melanogaster*. I was interested in performing this research with *D. melanogaster* because they are a widely used model system, are inexpensive to rear, and have a short generation time. I hypothesized that selection for resistance is weakest at low insecticide concentrations, and that fitness is negatively associated with inter-genotypic competition.

This project began by using the topical application bioassay to create dose-response curves for the resistant *para*^{ts1} and susceptible Canton-S *Drosophila* strains. Unfortunately, *Drosophila* did not appear to be the best model organism for answering my research questions in the time I had available. The fruit flies were more fragile than expected, and it would have required a large amount of investment to perfect the system. Additionally, the *para*^{ts1} strain I was working with possessed a resistance mutation on the X chromosome, making it difficult to execute competition experiments with males and females heterozygous for the resistance allele.

The strain also experienced a heat-sensitive fitness cost because of its resistance mutation, where it would become paralyzed at high temperatures (Suzuki *et al*., 1971). In the end, it did not make sense to continue troubleshooting when I only had 8 months left of my Masters program. Therefore, I decided to wrap up this project by fine-tuning the topical application bioassay for fruit flies, and published a methodology paper in the Journal of Visualized Experiments (JoVE) on this method with Brook Jensen and colleagues (Jensen, Althoff *et al*., 2022), which is presented in Chapter 1.

For our JoVE publication, my main role was to optimize the method for fruit flies and collect the mortality data by creating dose-response curves for the Canton-S (susceptible) strain using the topical application bioassay. I also assisted with making the deltamethrin insecticide solutions (and calculating the actual concentrations), completing the data analysis and writing, as well as addressing the reviewers' comments. I also participated in the filming production of our protocol, where we show how we execute the topical application bioassay in our laboratory and explain the significance of it. Brook Jensen (first author) collected the mosquito topical bioassay data using two different strains, completed data analysis, and participated in the filming. Brook and I co-wrote the first manuscript draft together. Sarah Rydberg (third author) helped rear the mosquitoes and fruit flies, and also provided rearing details for our manuscript. Emma Royster (fourth author) assisted in the mosquito rearing, topical bioassay setup, and data collection. Alden Estep (fifth author) provided mosquito eggs, visited ASU to train Brook and me on the topical bioassay, and provided troubleshooting advice. Dr. Silvie Huijben (corresponding author), provided supervision, methodology conceptualization, and major writing reviews and editing. Dr. Silvie Huijben also participated in the JoVE filming.

During the production of our JoVE manuscript, I became increasingly interested in the accuracy of insecticide susceptibility tests in assessing insecticide resistance. I was curious as to how much variation occurred in the CDC bottle bioassay, WHO tube test, and topical application bioassay and how all three methods compared using an identical, inbred laboratory mosquito strain. I decided to focus my new project on comparing the above three methods using the inbred, insecticide resistant MC1 *Aedes aegypti* strain. I designed, developed, and implemented this individual project in the fall of 2021, which is presented in Chapter 2.

Finally, I am also in the process of writing a review paper on the Window of Selection (WoS) (to which I have a shared first authorship) with two fellow graduate students (Brook Jensen and Theodore Ransome) as well as my PI, Dr. Silvie Huijben. Note: This review does not appear in this thesis. In this theoretical review, we discuss previously published work addressing the WoS as it relates to antibiotic, cancer and insecticide resistance research, and address the gaps within the literature. We argue for a greater focus on the heterozygote WoS for diploid organisms as it plays an important role in the evolution of resistance, as well as a greater focus on the window of negative selection. This review will stretch and challenge the current use of the WoS in resistance research and will give insight into what insecticide/drug concentrations will result in a certain selection for resistance. We aim to submit our manuscript to the journal 'Evolutionary Applications' by November 2022.

Finally, Chapters 1 and 2 are each written as a standalone paper, and there may be some overlap in the background information and discussion. Chapter 1 reads exactly as our publication is found in JoVE, with minor changes to figure numbers to assist with the thesis outline. I aim to submit my manuscript of Chapter 2 to the Malaria Journal by September 2022.

INTRODUCTION

Insecticide resistance is a continuing evolutionary issue that negatively affects both public health and agriculture (Auteri *et al*., 2018; Matowo *et al*., 2020). Insecticides are widely used in various treatment regimens to combat the spread of vector-borne diseases and crop pests, and insects are acquiring resistance faster than new insecticides are being developed (Paaijmans & Huijben, 2020). There are two major processes that are well understood to cause this resistance: 1) the random appearance of a *de novo* mutant, and 2) selection acting upon a mutant in the presence of an insecticide (Stepniewska & White, 2008). Vector control specialists have worked to combat the spread of vector-borne diseases with various interventions, including insecticide fogging, indoor residual spraying (IRS), and the use of long-lasting insecticidal nets (LLINs), to name a few. However, due to the observed plateau in reduction of malaria infections over the past five years despite continued eradication efforts (possibly due to resistance) (World Malaria Report, 2021), improved insecticide resistance surveillance and resistance management strategies (RMS) are crucial. Surveillance data is used to identify resistance profiles in a given area, which can help identify when and where resistance is emerging or spreading. Accurate data collection in these settings is imperative, as it is important to not under or over-estimate resistance. Underestimating resistance could lead to further spread of the resistance, while over-estimating resistance could result in an effective treatment being removed too soon or in over-treating an area which could further select for *de novo* resistance mutations.

There are four resistance mechanisms: target-site resistance, metabolic resistance, cuticular resistance, and behavioral resistance (Namias *et al*., 2021). Metabolic resistance allows an organism to detoxify insecticides over time through amplification or overexpression of detoxification genes, such as cytochrome P450s, esterases, and glutathione S-transferases (Liu,

2015). Cuticular resistance decreases the insecticide's ability to penetrate the cuticle, and is associated with either a thickening cuticle or change in the cuticle's composition (Yahouédo *et al*., 2017). Finally, behavioral resistance causes the organism to avoid insecticides, but more research needs to be done on this type of resistance (Carrasco *et al*., 2019; Corbel & N'Guessan, 2013).

Knockdown resistance (*kdr*) mutations in mosquitoes are an example of a target-site resistance mechanism, and they are widespread due to the continuous use of pyrethroids in the field (and other insecticides with similar modes of action, such as DDT), which results in high selective pressure for these mutations (Brengues *et al*., 2003). *kdr* mutations are nonsynonymous mutations caused by single nucleotide polymorphisms (SNPs), which causes a change in the amino acid sequence in the voltage gated sodium channel (VGSC). VGSCs are transcellular membrane proteins that are important for the proper function of a cell because they control the flux of ions responsible for action potential, and *kdr* mutations occur in the VGSC because that is the target site of pyrethroids and DDT (Soderlund & Knipple, 2003). These insecticides prevent the cell's ability to control polarity when they bind to the VGSC, which results in rapid paralysis (known as knockdown) and eventually death of the organism by preventing normal transmission of nerve impulses (Bowman *et al*., 2018; Silver *et al*., 2014). (Note: Throughout this thesis, "knockdown" means the mosquito is not dead and could possibly recover, while the use of "mortality" means the mosquito is dead).

The most commonly found *kdr* mutation is L1014F, which is found in *Anopheles* and Culex mosquitoes, but not *Aedes*. However, one of the *kdr* mutations in *Aedes* is located at codon 1016, which is very close to the 1014 mutation location in *Anopheles* (Antonio-Nkondjio *et al*.,

2017; Chen *et al*., 2019). This may be seen as a limitation in my study since I used the MC1 *Ae. aegypti* strain, and this is further discussed in Chapter 2.

One way resistance (with the exception of behavioral resistance) is detected in vector populations is through insecticide susceptibility bioassays, with the CDC bottle bioassay and WHO tube tests widely used to test mosquito populations. The CDC bottle bioassay involves coating glass bottles with insecticide (and one acetone or ethanol only control bottle) and aspirating 20 – 25 mosquitoes into each bottle. Knockdown is assessed at 30 min and total mosquito number is counted per bottle. For the WHO tube test, filter paper impregnated with insecticide (two with acetone and olive oil only for control) is inserted into plastic tubes, and 20 – 25 mosquitoes are aspirated into each tube. Knockdown is assessed at 1 hour, and mortality is scored 24 hours after a 1-hour exposure. These bioassays mimic the way mosquitoes may pick up insecticides in the field (e.g., through tarsal contact), but can result in highly variable results due to the untreated ends of the tubes and other factors further discussed later. The topical application bioassay, an infrequently used assay, can provide more reliable data as the mosquitoes are exposed to a consistent volume and concentration of an insecticide. Colleagues and I published an in-depth protocol on this method in the Journal of Visualized Experiments (Chapter 1). The CDC and WHO assays are used interchangeably but since both assays (as well as the topical assay) measure resistance slightly differently, it is important to know how they compare to each other. The advantages and drawbacks to these bioassays are discussed in detail in Chapters 1 and 2.

Once resistance has been detected in a population (using the above discussed methods, for example), different theoretical frameworks can be used to decide the best course of action to prevent the development of more *de novo* resistance mutations, as well as the spread of the

existing resistance. One of these theoretical frameworks that has been used by evolutionary biologists includes the Window of Selection (WoS) (Figure 1), also called the Mutant Selection Window (MSW). In short, the WoS gives insight into the impact a certain insecticide concentration has on genotype, which can help identify the intensity of selection for resistance at various insecticide concentrations (South *et al*., 2020). The WoS opens when the mortality rate of RR (homozygous resistant) is less than 100%, and the mortality rate of RR slowly decreases as insecticide concentrations decrease. As insecticide concentrations further decrease, RS (heterozygous resistant) will begin to experience a mortality rate less than 100% and will subsequently open the window of dominance within the WoS until RS no longer experiences a mortality rate lower than SS (homozygous susceptible). SS will experience mortality less than 100% at the lowest dosages, and as insecticide concentrations continue to decrease and SS experiences 0% mortality, the WoS will close and negative selection for the resistance allele will occur. This will result in SS experiencing higher fitness than RS and RR due to the fitness costs associated with the resistance allele (Note: this description is for diploid organisms). This framework has been used for many years in antibiotic resistance research (Drlica & Zhao, 2007), but the WoS was only recently introduced into insecticide resistance studies (South *et al*., 2020). If studied more widely in diploid organisms, this framework could help vector control specialists to pick insecticide concentrations that would be more effective based on the vector population's genotype and fitness costs. More research needs to be done on the heterozygote WoS, as well as how selective pressures could influence the amount of time that the "window" is open, in order to know how to best incorporate this framework into future studies.

To help address the gaps in research addressed above, I have collected the first data showing the comparison of three insecticide susceptibility bioassays (CDC, WHO, and topical) (Figure 2),

where I used an inbred laboratory mosquito strain to compare variation in mortality (and knockdown) for each method (Chapter 2). This data addresses the gaps in the WoS research by allowing a more accurate evaluation of the relationship between genotype and phenotype for each bioassay, which is currently lacking in the literature. This will further help establish the WoS for different organisms and genotypes and will inform RMS on how to best combat resistance at a given site. This research also highlights the need for bioassays to be standardized at the local level to determine if control tools are remaining effective in order to prevent detrimental policy decisions (such as under or overtreating an area) (Namias *et al.*, 2021). This is significant because if resistance is to be understood, then it must be known whether the mosquito population of interest is evolving (technical resistance, which measures resistance under standardized, controlled environments) and whether or not the vector control tools are still working (practical resistance, which determines the response of mosquitoes to actual interventions under natural conditions). Both topics are important, but both need a different approach. There are undoubtedly gaps in both areas of research, including how genotype influences resistance phenotype (in technical resistance assays) and how vector populations evolve over time under the impact of a vector control tool (practical resistance assays). My research helps address the gaps within the understanding of technical resistance and is further discussed in Chapter 2. Overall, the goal of my research is to expand the framework of evolutionary biology with new and improved theories and methods that will help win the fight against the development and spread of resistance.

CHAPTER 1

TOPICAL APPLICATION BIOASSAY TO QUANTIFY INSECTICIDE TOXICITY FOR MOSQUITOES AND FRUIT FLIES

This chapter appears as found with some minor alterations in Jensen, B. M., Althoff, R. A., Rydberg, S. E., Royster, E. N., Estep, A., & Huijben, S. (2022). Topical Application Bioassay to Quantify Insecticide Toxicity for Mosquitoes and Fruit Flies. *Journal of Visualized Experiments*, *179*. https://doi.org/10.3791/63391
To download supplemental files, use the above link and scroll down to the supplemental files section.

Abstract

The continued use of insecticides for public health and agriculture has led to widespread insecticide resistance and hampering of control methods. Insecticide resistance surveillance of mosquito populations is typically done through Centers for Disease Control and Prevention (CDC) bottle bioassays or World Health Organization (WHO) tube tests. However, these methods can result in a high degree of variability in mortality data due to variable insecticide contact of the insect, the relatively small numbers of organisms tested, extensive variation in mass between populations, and constantly changing environmental conditions, leading to variable outcomes. This paper presents the topical application bioassay, adapted as a high-throughput phenotypic bioassay for both mosquitoes and fruit flies, to test large numbers of insects at a range of insecticide concentrations.

This assay 1) ensures consistent treatment and insecticide contact with every organism, 2) produces highly specific dose–response curves that account for differences in average mass between strains and sexes (which is particularly important for field-

collected organisms), and 3) allows for the calculation of statistically rigorous median lethal doses (LD_{50}), which are necessary for resistance ratio comparisons—an alternative surveillance approach from diagnostic dose mortality, which is also used for larvicide resistance surveillance. This assay will be a complementary tool for accurately phenotyping mosquito populations for resistance and, as illustrated using fruit flies, is easily adaptable for use with other insects. We argue that this assay will help fill the gap between genotypic and phenotypic insecticide resistance in multiple insect species.

Introduction

Mosquitoes are responsible for over 700,000 deaths each year due to the diseases they transmit to humans, with over half of those deaths due to malaria alone (World Health Organization, 2020). The main preventive method against transmission of malaria and other vector-borne diseases is the use of insecticides, often in the form of long-lasting insecticide nets or indoor-residual spraying (World Health Organization, 2012). However, insecticide resistance is widespread among mosquitoes and other insect vectors, as well as agricultural pests (Hemingway & Ranson, 2000; Liu, 2015). To effectively manage resistance, surveillance is of key importance (World Health Organization, 2016a). For this, highly accurate and high-throughput resistance detection methods are needed. Currently, the most widespread insecticide resistance surveillance tools for mosquitoes are the WHO tube test (World Health Organization, 2016b) and the CDC bottle bioassay (McAllister & Scott, 2020). For fruit flies, the residual contact application method (similar to the CDC bottle bioassay) is a commonly used insecticide bioassay (Duneau et al., 2018; Pittendrigh et al., 1997; Rinkevich et al., 2013). However, variability in data from these methods is typically high, with measurements of the same

laboratory mosquito strain ranging from ~20 to 70% mortality in CDC bottle assays and 0–50% in WHO tube tests when exposed to sublethal dosages (Lissenden et al., 2021). Such variation is surprising because the limited genetic variation in most laboratory strains is expected to lead to limited insecticide susceptibility variation in the population. Yet, there is still a high level of variation being observed in the bioassay results.

Other potential sources of this variation could be a result of heterogeneous insecticide exposure between specimens within the bioassay due to indirect insecticide exposure via the surface, heterogeneous environmental effects, normal biological variation between individuals of the same genotype, and variation in mass of specimens of the same population (Owusu et al., 2017). An infrequently used method with higher replicability is the topical application bioassay. In this assay, the insecticide is directly applied to each insect (Brito-Sierra et al., 2019; Burgess et al., 2020), removing the factor of heterogeneous exposure of different specimens within the same assay. However, due to the slow-throughput nature of this method, it is not routinely used as an insecticide susceptibility surveillance tool for mosquito populations. This paper presents a modified protocol for the topical application bioassay that allows for higher-throughput exposures while also correcting for variation in insect mass, a parameter that correlates to changes in insecticide susceptibility (Owusu et al., 2017). A reduction in noise and mass-associated variation in mortality data from variable insecticide exposure would allow for more accurate technical resistance surveillance (Lissenden et al., 2021; Namias et al., 2021). Such data could be used to more accurately associate phenotypic resistance with genetic markers, fitness parameters, and/or vector competence. Additionally, we

demonstrate how this assay could easily be adapted to other insect species by using the topical application bioassay on fruit flies, a smaller-bodied insect species.

The main limitation of the aforementioned residual contact applications is that insecticide exposure may vary from specimen to specimen within the same assay and, particularly in the case of CDC bottle bioassays and the contact method, insecticide exposure may vary between replicates of the same assay. The insects are exposed to insecticide that is either distributed on the inside of a glass bottle (CDC bottle bioassay and contact method) or on impregnated papers (WHO tube test). The concentration of insecticide on both surfaces (glass and paper) is known and predetermined by testing different species of insects with known genotypes. However, the amount available to potentially be absorbed by the insect can greatly vary depending on the surface used, the insecticide mixture components, and how homogeneously the insecticide is distributed across the surface material (Dang et al., 2017; Zhu et al., 2019). In the CDC bottle bioassay, the insecticide coating on the inside of the bottle is dependent on procedures employed by each laboratory and user. In the WHO tube test, the insecticide-treated papers are centrally produced and thus most likely quite homogeneous across labs. However, in the WHO tube test, the exposure tube allows specimens to land and rest on non-insecticide-exposed metal mesh, also leading to potential heterogeneous insecticide exposure among the specimens within each test. The actual amount of insecticide picked up and absorbed by specimens via each method still needs to be explored further (Spielmeyer et al., 2019).

Additionally, the CDC bottle bioassay, WHO tube test, and contact method are most commonly used as threshold assays testing only one predetermined insecticide

concentration but can also be used as intensity assays using multiple insecticide concentrations. This approach can accurately detect the presence of resistance and is valuable for resistance surveillance (especially when resistance is spreading). However, threshold assays cannot quantify the strength of the resistance, which might be more predictive of the efficacy of intervention tools. Intensity assays for both the CDC bottle bioassay and the WHO tube test have been introduced by testing 5x and 10x the predetermined discriminating dosages to address this gap in surveillance (Bagi et al., 2015; World Health Organization, 2016b). While providing greater ability to differentiate between resistant populations, 3–5 (predetermined) dosages provide limited resolution to calculate lethal concentrations. Additionally, mosquitoes of various sizes are used in such assays. Yet, the mass is important to measure as larger specimens might need a higher dose to be killed as the effective dose per unit of mass will be much lower than that of a smaller organism (and different groups of mosquitoes can have substantial differences in weight) (Owusu et al., 2017). Calculating a mass-relativized lethal dose (amount of insecticide per insect mass) would be a more useful metric than the more common lethal concentration (e.g., amount of insecticide per surface area) as it considers the variation of insect mass between sexes, populations, and genotypes. Such data would help fill the gap between genotypic (genetics) and phenotypic (observable characteristics) resistance within the laboratory and the field and could also provide an easy way to calculate the needed application concentration to treat a population of insects of a known average mass.

The use of mass-relativized lethal dosages that kill 50% of the specimens (LD_{50}) also incorporates several other benefits. Assessment of the toxicity of a specific

compound in mg/kg (= ng/mg) is standard in human and veterinary toxicology (Burgess et al., 2020), and LD_{50} values are found on material safety data sheets. Lethal dosages also allow direct comparison of toxicity between different chemicals toward a particular species or the same chemical toward different species (Pridgeon et al., 2009), as well as high-quality evaluation of novel insecticides and chemicals (Brito-Sierra et al., 2019). Additionally, the LD_{50} can provide more meaningful and accurate resistance ratios than those derived from diagnostic dose mortality results, which can result in an overestimation of the resistance level present in a population. Therefore, this assay would be suitable for routine surveillance programs by providing more rigorous resistance monitoring based on mass-relativized lethal doses derived from more specimens than what is recommended for other bioassays (World Health Organization, 2009).

The topical application method has been used in insecticide susceptibility surveillance for mosquitoes and flies as an alternative for the standard insecticide susceptibility bioassays when resistance is already known or suspected (Estep et al., 2018; Waits et al., 2017), as well as for surveillance in some pest insects (Kostromytska et al., 2018) to more accurately assess resistance profiles and insecticide intrinsic toxicity (World Health Organization, 2009). In topical application bioassays, the insecticide is applied to each individual organism, resulting in minimal variation in insecticide exposure. This paper presents a slightly adapted and improved method that allows for insecticide exposure to be applied to a large number of insects in a short period while also controlling for insect mass (Estep et al., 2018). This higher-throughput method with good levels of replicability could be a useful additional tool for routine insecticide susceptibility surveillance.

Protocol

NOTE: Insecticides can cause human, animal, and environmental hazards[25]. Caution, training, and personal protective equipment are highly advised. Be sure to follow the material safety data sheets for all insecticides and solvents used.

1. Rear specimens

1. Rear 3-5-day-old adult mosquitoes.

   NOTE: The protocol below reflects conditions for *Aedes aegypti* rearing, closely following Food and Agriculture Organization of the United Nations guidelines[26].

   1. Rear mosquitoes of all life stages at 27 ± 1 °C and 75 ± 5% relative humidity with 12:12 h light and dark cycling.
   2. Hatch the mosquito eggs by submerging them in deionized water and adding yeast[26], or place the submerged eggs inside a vacuum chamber for 30 min.

      NOTE: Both methods decrease the oxygen content within the water and increase hatching[27].
   3. Feed the newly hatched larvae fish food (or an equivalent diet such as ground cat kibble) within trays and keep the larval density as similar as possible between trays as larval density impacts development[12] (e.g., 200-250 larvae per tray containing a total of 1.5 L of water).
   4. Feed the larvae every other day until they reach the pupal stage (approximately 7-10 days), increasing the amount of food as needed.

      NOTE: When fed too little, larval growth will be stunted, and the larvae

may eat one another. When fed too much, the larvae may die, causing the water to go foul.

5. Once pupae develop, transfer them daily to a water bowl in adult mosquito cages and provide 10% sucrose solution *ad libitum*.
6. Record the first day of adult emergence. Remove the remaining pupae from the cage 2 days after emergence starts.

   NOTE: Male mosquitoes emerge faster. Note the emergence of males and females separately and ensure sufficient males and females are available for each test.
7. Wait for 3 days after removing the pupae to achieve 3-5-day-old mosquitoes for testing.

2. Rear fruit flies (loosely following protocols of the University of Zurich[28]).
   1. Rear *Drosophila* strains in stock bottles at 23 ± 1 °C and 60 ± 5% relative humidity with 12:12 h light and dark cycling.

      NOTE: *Drosophila* stock bottles should contain 75 mL of a standard fly medium (Jazz-Mix™, Fisher Scientific, Waltham, MA), which is first poured as a liquid into the bottom of the bottles and then allowed to solidify overnight. (Note: The ingredients in the medium are premixed by Fisher Scientific, and boiling water is added to the medium for stock bottle preparation).
   2. Transfer colonies to new stock bottles with fresh food every two weeks to prevent overpopulation and mold growth. To do this, knock down flies using a hand-held carbon dioxide (CO_2) dispenser, transfer the

anesthetized flies to a weighing paper on an ice pack or chill table, and brush the flies into a fresh stock bottle using a fine-tipped paintbrush. Be sure to keep the bottles on their sides during this process to prevent flies from falling into the food and drowning.

2. Prepare insecticide formulations using the gravimetric approach

1. Make the first stock solution following the gravimetric approach using an analytical scale with 0.1 mg accuracy inside a fume hood.

   NOTE: The gravimetric approach uses mass to measure the amounts of insecticide and solvent added. The standard practice (volumetric approach) will require an analytical scale to measure the amount of (solid) insecticide added when the first stock solution is prepared; however, the amount of solvent added and all following dilutions are measured by volume only. The gravimetric approach has a higher level of accuracy and is therefore preferred.

   1. Determine the target insecticide concentration and target volume (maximum 10 mL is recommended if using 15 mL conical tubes to prevent spillage when storing in a freezer) for the first stock solution and calculate how much insecticide active ingredient (AI) to add using Eq (**1**):

$$AI\ to\ add(mg) = Target\ concentration\ \left(\frac{mg}{mL}\right) \times Target\ volume\ (mL) \times \left[1 + \frac{(100\% - (purity\ of\ AI\%)}{100}\right] \quad (\mathbf{1})$$

2. Prepare a storage tube (15 mL conical tube recommended for larger volumes, 1.5 mL microcentrifuge screw cap tubes recommended for volumes of 1 mL or less) and label with insecticide and solvent name, target concentration, and preparation date. Place the tube and lid on the scale within a rack or holder and tare the scale.
3. Weigh the desired amount of solid or liquid insecticide AI determined from step 2.1.1. (e.g., deltamethrin used for the representative data) into the tube and record the mass.
4. Tare the scale and add the desired volume of solvent (equivalent to the target volume) to the tube, close the lid immediately, and record the mass. Close the tube's lid immediately after adding the solvent (acetone used here) to avoid evaporation and mix the solution.
5. Record the room temperature. Some solvents, such as acetone, can have significant changes in volume (and consequently density) depending on temperature.
6. If storing immediately, wrap the tube's lid in parafilm (to reduce evaporation), place it in a tube rack/holder (to keep upright and prevent leaking), cover in foil (to prevent UV exposure), place it in a resealable plastic bag (to reduce evaporation), and place the bag in a -20 °C freezer. If not stored immediately, make sure the lid is secured and cover in foil or a light-protected container.
7. Calculate the stock solution's actual concentration (mg/mL) by dividing the mass of insecticide AI added by the volume of solvent added (and the

volume of insecticide added if in liquid form). To calculate the volume of added solvent (or liquid insecticide), divide the mass added by the known density that is appropriate for the recorded temperature.

8. Calculate the density (g/mL) of the stock solution by dividing the total mass added (insecticide and solvent) by the total volume added (solvent and insecticide, if in liquid form). See step 2.1.7 for converting liquid mass to volume.

2. Serially dilute the initial stock solution via 10% dilutions. If needed, use these serial dilutions to create an initial dose-response curve to identify the target range of insecticide concentrations for the bioassay.
    1. Calculate the volume of insecticide stock solution and the solvent to add to each tube (e.g., 1 mL of insecticide stock solution diluted in 9 mL of solvent for a 10 mL dilution of 10% of the previous concentration).
    2. Vortex the stock solution for 10 s. Tare a prelabeled first dilution tube on the scale. Add the required volume of stock solution to the first dilution tube using a pipette. Immediately close the lid of both tubes and record the mass in the first dilution tube.
    3. Tare the first dilution tube again and add the required volume of solvent. Close the lid immediately, record the mass of the added solvent, and vortex the first dilution for 10 s.
    4. Repeat steps 2.2.2 and 2.2.3 for the remaining dilutions.
    5. Store all dilutions as described above in step 2.1.6.

6. Calculate the actual concentrations of the dilutions by following step 2.1.7.
7. Calculate the density of each insecticide dilution by dividing the total mass added (insecticide solution and solvent) by the total volume added (insecticide solution and solvent). For each serial dilution, use the previous insecticide stock dilution's density to calculate the new dilution's density following Eq (**2**):

$$Dilution\ Density\ \left(\frac{g}{mL}\right) = \frac{Insecticide\ stock\ mass\ (g) + Solvent\ mass\ (g)}{\left(\frac{Insecticide\ stock\ mass\ (g)}{Insecticide\ stock\ density\ \left(\frac{g}{mL}\right)}\right) + \left(\frac{Solvent\ mass\ (g)}{Solvent\ density\ \left(\frac{g}{mL}\right)}\right)} \quad \ldots$$

(**2**)

3. Optional: Create insecticide dilutions with smaller increments by serial dilution.
    1. Select the concentrations and volumes of each new solution to make with the aid of a dose-response curve of the initial serial dilutions, previous trials, or published literature.

       NOTE: Chosen concentrations should result in a mortality range of 0-100%, with a minimum of three concentrations from this range to allow for Probit analysis.
    2. Use the serial dilutions as stock solutions to make each new dilution and follow step 2.2 to create the new dilutions between the 10-fold dilutions.
4. Optional: Aliquot the insecticide solution. If larger volumes of the insecticide solutions are made, aliquot the solutions into 1.5 mL screw-cap tubes to avoid contamination, evaporation, and degradation of the stock solutions from frequent handling and exposure to light.

1. Aliquot the solutions, starting from the lowest concentration and work towards the highest concentration to reduce potential contamination. Mix each stock solution by vortexing for 10 s before opening and pipetting the desired volume (e.g., 0.5 mL) into a prelabeled screw cap tube.
2. Store the aliquots in a light-resistant container in a -20 °C freezer.

   NOTE: It is recommended to regularly (monthly) replace aliquots with small new aliquots taken directly from the stock pesticide dilutions. This will limit the potential for contamination to be carried over into other experiments or changes due to evaporation or UV degradation while the samples are used on the bench. The protocol can be paused here and restarted even years later, as long as the insecticide solutions are stored properly (see step 2.1.6) and kept in the -20 °C freezer.

5. Use a permanent marker pen to mark the meniscus before storing to monitor solvent evaporation. When removing insecticide solution to make aliquots, mark the meniscus every time the solution is removed.

3. Prepare topical application bioassay workspace

NOTE: It is recommended to work in a benchtop insect handling tent for easier capture of escaping mosquitoes or flies. See **Supplemental Figure S1** for images of an insect handling tent.

1. Remove the needed insecticide solutions from the freezer, vortex immediately, and place them in a light-resistant container at room temperature to let the insecticides warm to room temperature before using.

   NOTE: Insecticide AIs can separate from the solvent at cooler temperatures.

Additionally, acetone volume changes with temperature, which can alter the applied insecticide dose. Mixing the solutions and allowing them to warm to room temperature helps ensure consistency when using the insecticide solutions.

2. Set out all needed tools and materials for the topical application assay in the insect handling tent as referenced in the **Table of Materials**.
3. Clean the syringe barrel and needle with analytical grade acetone by completing 5 washes per acetone aliquot. Complete this with 5 separate aliquots for a total of 25 washes. See **Supplemental Figure S2** for syringe and repeater pipettor parts.
    1. Set out 5 microcentrifuge tubes with 0.5 mL of acetone each.
    2. Fill the syringe barrel with 0.025 mL of acetone from the first tube and then expel the acetone into a waste container by swiftly pushing down on the plunger. Repeat four more times to complete a total of five acetone washes from the same acetone aliquot. Then, fill the syringe barrel completely with air and expel the air and potential acetone remnants into the waste container. Repeat two more times to complete three "washes" with air.
    3. Repeat step 3.3.2 for the remaining 4 tubes of acetone.
    4. Create an air pocket within the barrel between the syringe plunger and the top of the needle by pulling up the plunger slightly into the barrel (~5 mm).

        NOTE: This air pocket protects the plunger from contacting the insecticide solutions and reduces insecticide carryover.
    5. Set the syringe aside until ready to use for topical application.

4. Create a key containing the doses to be applied and assign random IDs following random number or letter generators (see **Supplemental File 1**).
5. Label the plastic holding cups with the random ID for blind mortality assessment.

   NOTE: If needed, the protocol can be paused here and restarted at a later day and time. If more than a few hours pass while pausing, it is encouraged to repeat step 3.3 to ensure the syringe is clean and to place the insecticide solutions back in the freezer until about an hour before dosing the insects and then repeat step 3.1.

4. Prepare specimens for the topical bioassay. See Figure 3 for a procedural overview

1. Sort and weigh the mosquitoes
    1. Using an aspirator powered by suction from inhalation, aspirate the desired number of 3-5-day-old adult mosquitoes needed for the assay, including an excess to account for damaged individuals. Transfer the mosquitoes into a conical tube (up to 100 mosquitoes per tube) by placing the tip of the aspirator into the tube with cotton wrapped around the tip and gently exhale and tap the aspirator. Use the cotton to plug the tube when the aspirator tip is removed and then cap with the lid. Avoid filling the aspirator and tubes with too many mosquitoes at once, as this adds additional stress on the mosquitoes and can cause death.
    2. Briefly knock down the mosquitoes in the tubes by placing them for a minimum of 10 min at 4 °C or burying them under ice in an ice tray.

       NOTE: Mosquitoes can be held at 2 °C for several hours with minimal

mortality[29]; however, it is best to minimize the duration for which the mosquitoes are on ice to reduce potential negative effects.

3. Transfer the knocked down mosquitoes to the insect handling tent and carefully tip the mosquitoes out onto a plastic tray (e.g., Petri dish) placed on the ice. Pour only about 50 mosquitoes at a time to ensure each touches the cool tray beneath it and stays knocked down.
4. Sort the mosquitoes by sex by gently picking them up by the leg(s) (or wings) with forceps and place each sex into a separate holding cup. Count the number of mosquitoes of each sex while sorting and stop when the desired number is reached. While sorting, remove any mosquitoes that are injured (e.g., missing legs) or are extra-large (e.g., abnormally enlarged abdomen) or small (easily distinguished with the naked eye as smaller than the average mosquito size of that population).

   NOTE: Handling the mosquitoes by the appendages reduces structural damage to their soft primary bodies (e.g., abdomen).
5. Record the weight of each cup of mosquitos using an analytical scale with 0.1 mg precision.
    1. Place an empty cup with a Petri dish as a lid on the scale and tare the scale. Pour the mosquitoes into the container, place the lid on top, and place the container on the scale.
    2. Record the combined weight and number of specimens on the score sheet (see **Supplemental File 2**). Immediately place the cup of specimens back on ice to keep them immobilized.

3. Repeat steps 4.1.5.1-4.1.5.2 until all cups of specimens are weighed.

6. Divide the prepared mosquitoes into groups of 20-25 in separate cups placed on ice labeled with the random IDs. When transferring mosquitoes, aim to reduce stress and physical damage caused by the forceps. Ideally, pick the mosquitoes up using forceps 1-2 times only: once for sorting/weighing and a potential second time for transfer to the experimental cups.

   NOTE: An ideal number of mosquitoes per cup is 20-25, which is enough for a replicate, is reasonable to assess the mortality, and should not result in density-induced stress/death in the cup.

2. Sort and weigh the fruit flies

   1. Anesthetize the flies using CO_2 for 7 s.

      NOTE: If flies are exposed to CO_2 for more than 7 s, they may have trouble crawling and flying when they awaken[30].

   2. Pour the flies onto an ice pack wrapped in bench paper and use a fine-tipped paintbrush to separate and count the males and females.

   3. Use the paintbrush to gently pick up the chosen flies and place them into a clean, empty stock bottle. Choose equal numbers of male and female fruit flies (e.g., 15 males and 15 females) and label the stock bottles with the strain name and fruit fly total (e.g., Canton-S, 30 flies).

      NOTE: It is important to have equal numbers of female and male fruit flies because male fruit flies can experience heightened aggression

towards each other after being removed from the presence of females[31]. Therefore, to avoid non-insecticide mortality or injuries, it is best to have equal numbers of males and females (or omit male fruit flies completely).

4. Record the weight of each bottle of fruit flies using an analytical scale.
    1. Place an empty vial (labeled with a random ID, refer to step 3.4) with a Petri dish as a lid on the scale and tare the scale.

       NOTE: Glass vials are recommended for use with fruit flies as they significantly reduce the static.
    2. Anesthetize the bottle of fruit flies corresponding to the vial's random ID using CO_2 for 7 s.
    3. Pour the fruit flies onto weighing paper and use the paper as a funnel to introduce the flies into the vial. Place the Petri dish lid on top of the vial of fruit flies and place it on the scale.
    4. Record the combined weight and number of specimens on the score sheet and then immediately place the vial of fruit flies in a tray of ice, with the lid still on top to prevent the flies from escaping.
    5. Repeat steps 4.2.4.1-4.2.4.4 for each bottle of fruit flies.
3. When the above steps are complete, immediately move on to the next section.

5. Dose specimens

1. Load the syringe with the proper insecticide concentration. Start with the least concentrated dose and work towards the most concentrated dose with each group

of organisms. To prevent waste, only load the syringe with the needed volume of insecticide plus a recommended extra 2 µL.

2. Tip the specimens onto weighing paper(s) placed atop a tray on the ice. Separate the specimens that are close together using a clean, insecticide-free paintbrush or cotton swab to allow easy access to each specimen for dosing. For mosquitoes, use the paintbrush also to ensure that each specimen is laying on their dorsum and their ventral surface is facing up.
3. Using the syringe, apply one droplet of insecticide solution (or acetone for the control) to the ventral thorax and abdomen area for mosquitoes and the notum for fruit flies. Apply a 0.2 µL droplet (which requires a 10 µL syringe) for smaller sized insects such as fruit flies and a 0.5 µL droplet (which requires a 25 µL syringe) for mosquitoes.

   NOTE: Insecticide sensitivity does not significantly differ between primary body parts (such as the head, thorax, and abdomen) compared to appendages (such as wings, legs, or proboscis)[32]. Therefore, the application site does not have to be exact as long as the dose droplet is consistently applied to the primary body. The ventral thorax and abdomen area are chosen for mosquitoes because they often lay on their dorsal side when knocked down, whereas the notum is chosen for fruit flies because they often lay on their ventral side when knocked down. This decreased specificity of the application site helps increase the throughput of this method.
4. Immediately pour the specimens back into the labeled plastic cup and cover the cup with netting and a rubber band. Place the cup into a holding tray and note on

the cup any specimens that were killed, damaged, or escaped in this process (to exclude them in the final count of specimens in that cup). For the first cup, record the time when dosing is completed.

5. Replace the weighing paper(s) on which the specimens are placed to avoid insecticide contamination between doses.
6. Repeat dosing for each cup until all specimens have been dosed with the proper insecticide concentrations and record the ending time when all specimens have been dosed.
7. Provide 10% sucrose solution to each cup via a soaked cotton ball and set the cups aside until mortality is assessed the following day. Store the mosquitoes at 27 ± 1 °C with 75 ± 5% relative humidity[5] and the fruit flies at 23 ± 1 °C with 60 ± 5% relative humidity.

   NOTE: Be careful while squeezing the cotton balls to avoid oversaturation or undersaturation. The cotton balls should be moist but not dripping. Dripping sugar water in the cup can lead to mortality of the specimens and thus impact the mortality assessment of the insecticide.

6. Assess mortality

1. Record specimen mortality at 24 h after the start of insecticide exposure. Classify mosquitoes as alive if they can fly and hold themselves upright; as dead if they are immobile or ataxic (unable to stand or take off for flight), as described by the WHO[6]. Follow the same mortality assessment for fruit flies[8,33].

   NOTE: To assess delayed mortality, mortality can additionally be assessed after 48 and 72 h with daily sugar water changes.

2. After mortality is recorded, place all the cups of specimens in a contained bag in a freezer for at least 1 h to ensure all specimens are dead before disposal or subsequent use (e.g., molecular or chemical analysis).

7. Perform replicates

1. Repeat steps 3-6 on a new set of specimens, taking care to perform replicates at the same time each day, as insecticide susceptibility can change depending on the time of day due to changes in specimen metabolism[34].
2. Ensure a minimum of 3 replicates for each concentration for accurate estimation of the lethal dose that kills 50% of the specimens (LD_{50}). Include more replicates if a high level of variability is observed.
3. Complete the analysis after all data are collected.

8. Analyze the results

1. Record data in a spreadsheet program and use the random ID key to unmask the data (reference step 3.4). Save the data as a text file (see example data in **Supplemental File 3**) for analysis in the statistical program R[35] (see example R code in **Supplemental File 4**) or other software of choice[36].
2. Within the software program, complete the following analysis. See **Supplemental File 4** for an example R code.
    1. Calculate the dose of insecticide (ng) per specimen mass (mg) following Eq (**3**) below:

$$Dose\ \left(\frac{ng\ AI}{mg\ specimen\ mass}\right) = \frac{\left(\text{Insecticide Conc. } \frac{\text{Ng}}{\text{mL}}\right) * (\text{Dose Volume mL})}{\frac{(Specimen\ mass\ mg)}{(Total\ specimen)}}$$

(3)

2. Calculate mortality and apply Abbott's formula[37] to correct mortality relative to the mortality observed in each control[37]. Alternatively, use the Schneider-Orelli (1947) formula to correct mortality[38]. With either formula, apply the correction to all data regardless of mortality in each control, as previously described[37] and implemented[39], unless the control data are unusually high (see discussion below).

   NOTE: Abbott's formula and equivalent alternatives, such as the Schneider-Orelli formula, adjust mortality values proportionately to the extent of mortality not observed in the controls and will not cause a decrease in mortality for cups that had 100% mortality. For more information, see the cited references for these formulas.

3. Transform corrected mortality data into probit (probability unit) values[40] and perform linear regression between the insecticide dose and transformed mortality data. Use a chi-square test to assess the fit of the linear model(s).

   NOTE: Mortality values of 0 (0% mortality) or 1 (100% mortality) are removed from the data before completing the probit transformation. This is necessary due to the nature of the probit transformation. As such, the graphed data will not include positive or negative controls or any other

data that resulted in 0% or 100% mortality (after Abbott's correction has been applied).

4. Calculate the LD_{50} and 95% confidence intervals (CIs) per specimen strain, population, and/or sex following previously published methods[39,41,42].
5. NOTE: If the 95% CIs of two strains do not overlap, the strains have significantly different dose responses.
6. If applicable, calculate resistance ratios (RRs) by dividing the LD_{50} of the strain of interest by the LD_{50} of the reference/control strain.

Representative Results

These representative results feature two different strains of *Ae. aegypti*, Rockefeller (ROCK) and an isolated field strain from Florida with known knockdown resistance mutations F1534C and V1016I (IICC genotype). Additionally, *Drosophila melanogaster* (Canton-S strain) is featured.

Figure 4 and **Figure 5** illustrate the dose response of each organism by strain and sex tested following the above protocol. As no differences were observed between the dose–response curve of male and female mosquitoes within each strain ($t = 1.70$, $p = 0.098$ for ROCK and $t = 0.64$, $p = 0.527$ for IICC), data from both sexes within each mosquito strain were pooled. The mass-relativized LD_{50} for ROCK and IICC are 0.008 ng/mg (95% CI: 0–0.104) and 0.336 ng/mg (95% CI: 0.235–0.438), respectively. The 95% Cis of these values do not overlap, indicating that the strains' dose responses are significantly different. The RR of the IICC strain (relative to the ROCK strain) is 41.7, which according to the WHO is considered highly resistant (World Health Organization,

2016a). For the Canton-S fruit flies, the mass-relativized LD_{50} is 0.213 ng/mg (95% CI: 0–0.490).

Supplemental Figure S1: Benchtop insect handling tent. Benchtop insect handling tent is used for easier capture of escaping mosquitoes or flies during the topical application assay. Structure is closed in **A** and open in **B**. This structure was built with PVC pipe and fine-mesh fabric.

Supplemental Figure S2: Syringe and repeater applicator unit. Syringe and repeater applicator unit used for dosing insects. Main parts include 1) needle, 2) syringe barrel, 3) plunger, 4) repeater, and 5) repeater button.

Supplemental Document 1: Randomization script: Randomization script to create non-biased labels for all cups of each experiment.

Supplemental Document 2: Mortality score sheet: Mortality score sheet to assist morality assessment. Sheet also includes places to record all other important information to record, as referenced in the protocol, such as the insecticide application start and end times.

Supplemental Document 3: Example mortality data: Example data file used to create **Figure 4**. The column heading descriptions are as follows: "id" = identification code of each data point; "species" = species name (e.g., *Aedes aegypti*); "insecticide" = name of insecticide topically applied (e.g., Deltamethrin); "strain" = name of mosquito strain (e.g., ROCK); "date" = start date topical application; "sex" = sex of mosquitoes; "age" = age of mosquitoes (young = 3–5-day-old; old = 4 weeks old); "total.mosq" = total number of mosquitoes weighed in batch; "weight" = weight (mg) of all mosquitoes within batch; "concentration" = concentration of insecticide (µg/mL); "syringe" = droplet volume (mL) of syringe; "dose" = amount of insecticide active ingredient applied to each mosquito (ng); "total" = number of mosquitoes in each cup; "dead" = number of dead mosquitoes in each cup.

Supplemental Document 4: R analysis code: Example R code that can be used to complete the Probit analysis (as described in step 8 of the protocol). The representative results (accessible via the supplemental example data file) can be used with this R code.

Discussion

This paper presents an adapted protocol for the topical application assay for mosquitoes and fruit flies. This procedure could be easily adapted to be used in the field and with other organisms as it requires minimal specialized equipment. Addressed below

this protocol's critical steps, potential modifications, troubleshooting advice, limitations of the method, and significance of this method.

Critical steps in the protocol: There are three critical steps in the protocol that, if completed incorrectly, can drastically impact the results of the bioassay: insecticide concentration accuracy, specimen knockdown, and mortality assessment.

Insecticide concentration accuracy:

It is extremely important to have accurate insecticide solutions to obtain replicable dose–response curves and meaningful results. The volumetric approach to insecticide solution preparation is more common within the literature for both CDC bottle bioassay (McAllister & Scott, 2020) and topical applications (Brito-Sierra et al., 2019; Burgess et al., 2020; Miller et al., 2010). However, the gravimetric approach described here is inherently more accurate due to the consideration of temperature through the inclusion of (temperature-specific) density and therefore leads to more accurate formulation preparation.

Specimen knockdown:

Knocking down the specimens is a critical component of this method and allows for the accurate administration of the insecticide and weight measurements. However, knocking down organisms inevitably contains risk of physical stress and damage, as previously demonstrated (Bartholomew et al., 2015). Therefore, be cautious and mindful when knocking down the specimens to ensure i) each specimen is knocked down for a similar duration, ii) the length of knockdown is kept to a minimum, and iii) the method of

knockdown is kept consistent across all specimens. Additionally, it is advised to test the knockdown method separately, prior to insecticide application, to ensure the method is successful and does not induce control mortality greater than 10%. The initial test may take longer for an inexperienced user, leading to longer knockdown times. Therefore, be cautious when interpreting results from the first assays.

Mortality assessment:

Assessing mortality can be challenging, especially when the insecticide does not completely kill but only knocks down or maims the mosquito or fly. Therefore, it is important to be aware of how the insecticide impacts the target organism and have a clear definition for "dead" (or knocked down) organisms before starting. Additionally, it is recommended to have the same person assess mortality between doses and replicates to reduce variation.

Protocol modifications: There are several modifications described below that can be applied to this protocol to improve its versatility and accessibility.

Adapting the assay to smaller or larger sized insects:

When using smaller or larger specimens, it is advised to apply a smaller or larger dose volume of insecticide, respectively. As an example, we adapted the mosquito protocol to fruit flies by reducing the 0.5 µL dose to a 0.2 µL dose. Ensure the correct syringe size is chosen for the chosen dose volume.

Adapting the assay to field insects:

When using field insects, there may be more variation in insect size and therefore weighing the insects in smaller groups (e.g., per cup) would be recommended as opposed to as a large group (e.g., all insects used for one experiment). This can help capture the potential variation in insecticide susceptibility associated with the differences in field insect mass.

Equipment modifications:

Insect handling tent: Dosing of the specimen can be completed under an insect handling tent that is simply constructed with PVC pipe and mosquito netting. This can be an alternative to an enclosed room (e.g., insectary) and help eliminate potential insecticide contamination in areas where insect rearing might occur. This insect handling tent is easy to construct and low-cost (~$70US). Alternatively, an insect handling cage could be purchased (~$425US).

Chill table: Ice packs or trays of ice can be used for knocking down the specimen and/or keeping the specimen knocked down.

Incubator: Incubators are recommended for rearing the specimen and for holding the specimen for 24 h after insecticide treatment. If an incubator is not available, it can be constructed. Equipment needed to build the incubator include an insulated container, humidifier, heat cables, humidity and temperature controller, and a light, which should add up to a total cost of ~$170US, following and expanding upon previous methods (Glunt et al., 2015).

Holding cups: Although plastic cups are used to sort and hold the treated specimen, wax-lined paper cups or glass containers would be suitable alternatives.

Organism and life stage modification:

This method is very adaptable for use with other vectors, insects, and/or arthropods such as *Culex quinquefasciatus* mosquitoes (Aldridge et al., 2016), house flies (Aldridge et al., 2016), and cockroaches (ffrench-Constant & Roush, 1990), as well as non-adult life stages, such as mosquito larvae (Akdag et al., 2014).

Topical application location modification:

This method describes applying the insecticide to the ventral thorax and abdomen region for mosquitoes (and the dorsum for fruit flies). However, other application locations can be used as long as the exposure site is consistent. Consistency is important because insecticide sensitivity can vary based on application location (Aldridge et al., 2016).

Troubleshooting advice: This method has several steps that are initially challenging. Described below are some of the most common issues one might encounter.

Leaking/evaporating insecticide solutions:

Insecticides are commonly dissolved in acetone, a highly volatile compound. This means acetone evaporates easily at room temperature, leading to increasing insecticide concentrations over time. If the insecticide solutions appear to be leaking or evaporating, remake the solutions, ensure the tube's lid is on tightly, and double-check that the storage protocols are being properly followed (e.g., parafilm is being used and the tubes are stored upright). If leaking persists, try filling the tubes with a lower volume to allow more

room for the change in volume the acetone experiences at different temperatures. Additionally, if using acetone as the solvent, ensure the tubes are rated for acetone storage (e.g., FEP, TFE, and PFA plastics), and if using hydrophobic insecticides, store the solutions in glass vials (as hydrophobic insecticides adhere to plastic more than glass). It is also good practice to mark the meniscus of the solution prior to storing to monitor evaporation.

Weight drifting on microbalance when weighing organisms:

If the weight reading on the scale is drifting (slowly going up or down), this could be due to static. Drift most often occurs when weighing organisms in plastic items, as plastic can easily hold a static charge. To avoid this, a weighing paper can be placed underneath the plastic container being weighed, or a non-plastic container such as glass can be used instead.

Abnormal mortality results:

There are many ways by which the mortality results may seem abnormal, such as observing high mortality in the controls or high/low mortality throughout all insecticide doses. Review the following cases for troubleshooting each scenario.

High control mortality

If there is high mortality in the control group (10% or greater), evaluate the knockdown method and length of time for which the specimens are knocked down. If possible, shorten the length of time for which the specimens are knocked down. Other potential

factors to consider for high mortality in the controls include i) checking if the incubator settings are correct—abnormal temperatures and/or humidity could lead to increased mortality. Temperature and humidity should be checked with an independent data logger. ii) Assess insect handling. Handling insects too much or too roughly could lead to high mortality. iii) Check if there is no contamination of insecticide in the 100% acetone used to treat the control group or on the instrumentation. Replace acetone and clean all instruments with acetone or ethanol. Avoid contamination by frequently replacing gloves, preventing spillage, and cleaning instruments. Note that in the **Supplemental Document 3**, a maximum of two mosquitoes died within the control (acetone-only) cups. This level of morality is not considered high (it is less than 10%), and therefore, there was no cause for concern.

High mortality in all exposed groups (but not in control groups)

Use lower insecticide concentrations or smaller dose volumes for testing. The dosages used might all be above the minimum dose that will not induce mortality. Use several 10-fold dilutions to identify the correct dose range, and rule out contamination. To avoid contamination, start dosing with the lowest concentration and work towards the highest concentration. Additionally, make sure all equipment used is regularly cleaned with acetone and/or ethanol—the doses applied to the specimen are very small and even the slightest cross-contamination could impact the results.

Low mortality in all exposed groups

Use higher insecticide concentrations. The dosages used might all be too low to cause mortality in the population. Expose specimens to several more 10-fold concentrated dosages to identify the correct dose range. Ensure the insecticide solutions have not expired or degraded (potentially due to high temperature or light exposure). If the solutions have expired or are suspected to have degraded, remake the solutions and ensure proper storage conditions are followed.

Inconsistent mortality between replicates/days

The time of the day when insects are exposed to the insecticide could affect the level of resistance expressed, especially for metabolic resistance (Balmert et al., 2014). Repeat this protocol during the same window of time each day to avoid time-of-day as a potential variable contributing to changes in mortality. Other potential factors contributing to inconsistent mortality between replicates include i) specimens being differentially reared between experiments. Ensure all specimens are of the same age range, reared at the same temperature and at similar densities and food availability. ii) Insecticide concentrations (of some solutions) degrade over time or become more concentrated due to acetone evaporation. Remake the solutions and ensure proper storage conditions. iii) Inconsistent mortality scoring. Ensure the same person scores mortality or develop clear protocol to be used consistently across the team. Use blind scoring to reduce bias in mortality scoring.

Insects sticking to the surface of the sorting tray:

Acetone reacts to plastics used in this protocol, such as Petri dishes. If using acetone on Petri dishes or similar plastic surfaces, the specimen will likely adhere to the surface. This adhesion can be avoided by lining the sorting tray with weighing paper or using a non-plastic sorting tray. Additionally, condensation on the surface of plastic in the sorting tray or holding cups can lead to insects adhering to the condensation or the specimen may be too cold and potentially freeze to the surface. Adjust the knockdown method to reduce condensation while not allowing the specimens to become too cold/frozen (e.g., place weighing paper between the specimens and the plastic sorting tray).

R analysis errors:

Once the mortality data is collected, a variety of complications may occur during analysis. The most common reason an R code cannot complete the actions for the data file is that the data format does not match the code (e.g., column headings and/or empty cells). If more serious complications arise, refer to the R help pages built into Rstudio (R Core Team, 2021).

Limitations of the above-described topical application method:

Insecticide absorption via topical application method does not mimic natural exposure: Topical application on the primary body is not the natural way of insecticide absorption. In the field, insects mostly absorb insecticides through their legs over the length of time they are in contact with the insecticide-treated surface or on their wings through small aerosol particles (Cooperband et al., 2010; Richards et al., 2020), rather than a rapid exposure on the ventral surface. However, the direct application of a known insecticide

dose will accurately establish phenotypic response to insecticides, needed for genetic and evolutionary studies or comparisons of insecticide susceptibility across space or time. Therefore, this approach is beneficial for testing technical resistance but will not directly measure practical resistance (the efficacy of the actual intervention tool in a field setting (Namias et al., 2021)). However, it is important to note that the current standard methods (e.g., WHO tube tests and CDC bottle bioassays) also cannot capture or mimic aerosol insecticide-exposure in the field.

Topical application assays can only assess contact absorption insecticides:
This method is intended for use with insecticides that work through contact and absorption of the insecticide and not for use with oral insecticides, such as boric acid commonly used in attractive toxic sugar baits (Barbosa et al., 2019).

Significance of the method:

The topical application method expands on well-established standards for insecticide bioassays by calculating the lethal dose (not concentration) and measuring technical (not practical) resistance (Namias et al., 2021). Given below are the advantages and disadvantages of this method over existing insecticide susceptibility assays.

Lethal dose calculation:
This method determines the lethal dose of the insecticide, rather than the lethal concentration that the CDC and WHO bioassays use to establish the discriminating dose (Lissenden et al., 2021). The lethal dose is more meaningful because it is a quantified

amount of insecticide known to elicit mortality, whereas the lethal concentration does not consider how much insecticide the organism actually acquires. When using the lethal dose calculation, differences between sex- or size-dependent susceptibility profiles can be more accurately observed and quantified, making this measurement even more versatile.

Technical resistance:

This method assesses technical resistance, which is resistance as measured under standardized controlled environments. Such measurements are good for surveillance of the spread of insecticide resistance and linking phenotypic resistance with potential markers (Namias et al., 2021). Because of the decreased variation in mortality resulting from the topical application bioassay, it allows for better identification of new resistance markers. However, due to the unnatural exposure of insecticides to the mosquito, this assay is not suitable for the estimation of efficacy of a specific intervention in a specific population. Other assays are needed for measurements of such practical resistance (Namias et al., 2021) .

Specimen adaptability:

This method can be practiced on other important arthropods such as crop pests (e.g., Colorado potato beetle), house pests (e.g., cockroaches and bed bugs), or pollinators (e.g., bees) with simple changes to the knockdown approach and/or insecticide dose, volume, and/or concentration (as described above). The ease of adaptability can help analogize insecticide resistance research across different research fields. The use of an LD_{50} value

opposed to a lethal concentration that kills 50% of the specimens (LC_{50}) allows accurate comparison across species.

Cost:

Similar to CDC bottle bioassays and WHO tube tests, costs to run the topical application assay are minimal (see the **Table of Materials**). The essential pieces of equipment are the syringe (approximately $70US) and the dispenser (approximately $100US), which are reusable across assays.

Number of specimens needed:

A minimum of 20–25 specimens should be used per topical application assay cup and a minimum of five insecticide concentrations is recommended to be tested per experiment, with a minimum of three replicates recommended for the procedure. Overall, this results in a minimum of 300–375 specimens needed for a complete test, which is comparable to the number of specimens needed if performing resistance intensity tests using WHO tube tests or CDC bottle bioassays. However, if reduced variability is achieved with the topical application bioassay, the same number of specimens may lead to more statistical power to compare susceptibility data across space or time.

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The authors declare no conflicts of interest.

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CHAPTER 2

A COMPARISON OF INSECTICIDE SUSCEPTIBILITY BIOASSAYS

Abstract

Insecticide resistance remains a major public health problem as it drives the spread of vector-borne diseases. Mosquitoes are responsible for over 700,000 human deaths a year, and resistance is spreading faster than new insecticides are being made. The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have developed insecticide susceptibility assays that are widely used to test for insecticide resistance; however, their methods result in high variability in the mortality data, making it difficult to draw reliable conclusions on insecticide susceptibility in a given area or compare across time and space. The variation in mortality from the topical application bioassay, a method infrequently used, has not been quantified or compared to the other two bioassays. Here, I compare the CDC bottle bioassay and topical application bioassay using deltamethrin (DM) on an inbred laboratory insecticide resistant *Aedes aegypti* strain (further data will be collected to determine the variation within the WHO tube test). The results show that the topical application bioassay exhibited the least amount of variation in mortality data, followed by the CDC bottle bioassay. The relationship between DM concentration and mortality was highly significant for the topical bioassay ($X^2 = 35.5$, $p = 2e\text{-}08$), but surprisingly, no significant relationship was found between DM concentration and mortality for the CDC bottle bioassay ($X^2 = 5.9$, $p = 0.053$). A significant difference in variance in mortality was found for the normalized mortality data between the CDC and topical dose-response data ($F = 5.8253$, $p = 0.006549$), while no significant difference in variance was detected in

the LC_{50} experiments between the CDC and topical assays ($F = 2.2233$, $p = 0.1454$). This shows that the topical bioassay may be a more reliable assay to determine the resistance phenotype in a mosquito population, and that CDC data may not be as reliable as expected. This comparison of variability between bioassays will allow vector control specialists to better compare resistance data from location to location and further improve resistance management strategies.

Introduction

Insecticide resistance is a continuing evolutionary problem that has led to the perpetuation of mosquito-borne diseases, including Zika, West Nile, chikungunya, and malaria (World Health Organization, 2020). Unfortunately, resistance to insecticides is occurring faster than new insecticides are made, and resistance management strategies (RMS) are therefore urgently needed. To perform surveillance for resistance in mosquito populations, insecticide susceptibility tests are regularly used, with the CDC bottle bioassay (Brogdon & Chan, 2010) and WHO tube test (World Health Organization, 2016b) being the most commonly used, even though they experience high variability in mortality results (Lissenden *et al.*, 2021). This variability occurs because insecticide exposure in these bioassays is not controlled, leading to more noise within the data. For instance, the CDC bottle bioassay may underestimate resistance as it only assesses knockdown, not mortality, and may especially fail to detect metabolic resistance because it does not take the mosquito recovery period into account since ‘mortality’ is only assessed after 30 minutes of exposure (metabolic resistance enables mosquitoes to detoxify an insecticide over time) (Owusu *et al.*, 2015). Additionally, it is not known how much insecticide is picked up by the mosquitoes during the CDC bottle bioassay because

there is no assay currently developed that can do this on the individual mosquito level. However, the CDC bottle bioassay does provide ease of use in the field as it does not require much equipment, only an aspirator, bottles, and insecticides. The drawback to the WHO tube test is that it provides an untreated resting place at each end of the cylinder tube where the mosquitoes could be unexposed to the insecticide for a period or the entire duration of the test (Figure 6). This would be a major concern when testing insecticides that act as a deterrent to the mosquito, or to test mosquitoes that exhibit behavioral resistance (which causes the mosquitoes to avoid an insecticide) (Namias *et al*., 2021). Because of the lack of a sensitive assay to quantify insecticide uptake per mosquito, it is also not known how much insecticide is picked up by the mosquitoes during this bioassay. Nonetheless, the WHO tube test has been shown to exhibit less variation compared to the CDC bottle bioassay (reviewed in Lissenden *et al*., 2021). A highly infrequently used insecticide susceptibility test, the topical application bioassay, has not yet been compared to the two previously mentioned bioassays using *Aedes aegypti* mosquitoes. This topical assay may exhibit the least amount of variation as it involves treating individual mosquitoes directly with a consistent volume and concentration of insecticide via a syringe, therefore, theoretically, each mosquito is exposed to the same insecticide dose (Jensen *et al*., 2022). Furthermore, this method also involves weighing the mosquitoes in pools, which allows for calculating the mass-relativized dose per mosquito by taking the variation of mosquito mass into account (Waits *et al*., 2017). Since the CDC bottle bioassay and WHO tube test are used interchangeably to detect resistance within a mosquito population, and since they might measure resistance slightly differently (CDC through knockdown and WHO through mortality), it is important to

know how they compare to each other (as well as to the topical bioassay). Few studies compare the two popular bioassays, with the main discussions only including Bagi *et al*., 2015, Lissenden *et al*., 2021, Messenger *et al*., 2017, and Owusu *et al*., 2015. One study did compare results from the CDC bottle bioassay and topical application bioassay using permethrin on *Ae. albopictus*, which showed that the CDC bottle bioassay and topical bioassays do not always agree on resistance results (Waits *et al*., 2017). In fact, this study showed that the CDC bottle bioassay failed to detect significant differences in permethrin susceptibility for certain mosquito strains, which could be due to differences in insecticide uptake, among other possibilities further discussed below. Not many studies have used the topical bioassay to assess insecticide resistance, with the few including Chang *et al*., 2014, Brito-Sierra *et al*., 2019, Burgess *et al*., 2020, Estep *et al*., 2018, Kostromytska *et al*., 2018, Parsons *et al*., 2022, and Pridgeon *et al*., 2008. This comparison between the CDC bottle bioassay, WHO tube test, and topical application bioassay should be made in order to identify how analogous the different methods are and how much variability exists between each method.

To help fill this knowledge gap, **I have collected the first data on a comparison of the variability between the CDC bottle bioassay, WHO tube test, and topical application bioassay using the insecticide deltamethrin (DM) on an inbred laboratory *Aedes aegypti* strain.** This study will help evolutionary biologists, entomologists, and policy makers to 1) better understand the mechanisms of each bioassay, 2) interpret results from the different bioassays (e.g., low mortality rates detected in CDC bottle bioassays might not mean there is no resistance since CDC bottle bioassays have been shown to fail to detect resistance as seen in Waits *et al*., 2017), 3)

understand how insect mass plays a role in the resistance phenotype, and 4) better inform RMS by providing more accurate and useful resistance data at a given site. **I hypothesized that the topical application bioassay exhibits the least amount of variation in mortality between replicates since each mosquito is exposed to a controlled dose of insecticide, corrected by weight of mosquito pool.**

Materials and Methods

Mosquito species tested. The *Aedes aegypti* MC1 (Maricopa County) strain was used in this study, which was kindly provided to us by Dr. Michael Riehle from the University of Arizona. The eggs of this pyrethroid resistant strain were collected in the Phoenix area of Maricopa County by the Riehle lab in 2018, and this strain has been continuously reared in the Huijben lab since 2019. MC1 is a homozygous resistant strain and possesses two point mutations: V1016I and F1534C (Figure 7). The V1016I mutation is located within the sixth segment of the second domain of the voltage gated sodium channel (VGSC) at position 1016 that causes a valine to isoleucine substitution (Stenhouse *et al*., 2013), and this mutation confers resistance to both permethrin and deltamethrin insecticides (Vera-Maloof *et al*., 2020). The F1534C mutation is located within the sixth segment of the third domain of the sodium channel at position 1534 that results in a phenylalanine to cysteine substitution (Mack *et al*., 2021). F1534C confers resistance to permethrin and other pyrethroids, in addition to organochlorides (Du *et al*., 2016; Estep *et al*., 2018; Stenhouse *et al*., 2013; Vera-Maloof *et al*., 2020), but it does not likely confer resistance to Type II pyrethroids unless combined with another *kdr* mutation (Du *et al*., 2016).

All mosquitoes tested were 2 – 5 days old, female and non-blood fed since blood feeding can affect susceptibility phenotype (Machani *et al*., 2019). Additionally, mosquitoes were reared under standard rearing conditions and were kept in incubators set to 27°C, 80% RH, and a 12:12 hour photoperiod. For more information on the standard rearing process for *Ae. aegypti*, see the Food and Agriculture Organization of the United Nations guidelines (2017).

Insecticide preparation. Deltamethrin solutions were prepared using the gravimetric method (instead of the volumetric method) in order to know the true concentration of each DM dose being used. The gravimetric method uses the mass of the insecticide and solvent (in our case, acetone) to determine the actual concentration of our solution. For further details on how to carry out this method, see Jensen *et al*., (2022).

Insecticide susceptibility tests. Four replicates of 4 – 6 different deltamethrin concentrations were performed to construct dose-response curves for each bioassay. These concentrations were chosen by performing previous bioassays that resulted in mortality ranging from 0 – 100%. Once the LC_{50} (LD_{50} for topical) had been determined for each method, 10 replicates of each LC_{50} (or LD_{50}) were performed to quantify variance in mortality between each method (five more replicates still need to be collected for the topical bioassay and will be completed in April 2022). All bioassays were performed between 8:30am and 4:30pm and were performed at room temperature (21 ± 2 °C, 23 ± 3% RH). In all bioassays, a mosquito was considered dead/knocked down if unable to hold itself upright or fly in a coordinated motion. All CDC bottles, WHO tubes, and topical plastic cups were labeled with random IDs for blind mortality assessment.

CDC bottle bioassays: Procedures were followed as described in Brogdon & Chan, (2010). To create dose-response curves, five glass Wheaton® 250 ml bottles were individually coated with a different DM concentration and one bottle coated with only acetone for the control. To coat the bottles, DM insecticide solutions were allowed at least 1 hour to come to room temperature, and 1 ml of insecticide solution (or acetone) was pipetted into the bottles. The bottles were capped and maneuvered so that insecticide covered all parts of the bottles and caps. The bottles were then uncapped and placed on a bottle rotator (Cole-Parmer®) for 15 min to allow the insecticide to evenly coat the bottles and the acetone to evaporate. The bottles were stored uncapped in the dark for a minimum of 1 hour and a maximum of 23 hours until use in the assay. Twenty – twenty-five 2–5-day old non-blood-fed female mosquitoes were aspirated into the bottles, and the mosquitoes were exposed in the bottles for 30 min. The number of mosquitoes knocked down or alive was recorded at 30 min (whichever was easiest to count depending on the amount dead or alive). A minimum of four replicates at each concentration for the dose-response curves were performed, in addition to multiple replicates of LC50 experiments.

WHO tube tests: Procedures were followed as described in the standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests (2022). To prepare the insecticide treated papers, filter paper (Whatman™ No. 1) was cut into 12 x 15 cm dimensions. DM concentrations were prepared by mixing the insecticide with acetone and olive oil following the WHO SOP mentioned above (Note: The WHO SOP recommends using silicone oil with deltamethrin, but olive oil was used instead as it was less viscous and easier to pipette). The DM solutions were then pipetted onto the

individual papers until they were fully covered with the insecticide solution. The control paper was treated with acetone and olive oil only. Papers were allowed to dry in a fume hood for 24 hours and subsequently stored in a 4 °C fridge, individually wrapped in aluminum foil. When ready to use, each paper was placed into individual plastic exposure tubes from the WHO tube test kit (purchased from the Universiti Sains Malaysia – Vector Control Research Unit). Untreated filtered paper (cut in the same dimensions) was placed into individual holding tubes. Twenty – twenty-five 2-5 day old non-blood-fed female mosquitoes were aspirated into a holding tube. The holding tube was closed shut with the gate, and mosquitoes were allowed to acclimatize for 1 hour. After 1 hour, the exposure tubes (containing the treated papers) were connected to their respective holding tube. The gate (now located in between the holding and exposure tubes) was opened and mosquitoes were allowed to move from the holding tube to the exposure tube on their own for 30 seconds. After 30 seconds, the exposure tube was held upright at an angle and the holding tube was tapped to coax the mosquitoes into the exposure tube. Air was exhaled through the mesh in short bursts as an additional method to move the mosquitoes from the holding to exposure tube. After most (or all) mosquitoes were transferred to the exposure tube, the gate was closed, the holding tube was detached from the exposure tube, and mosquitoes were maintained in the exposure tubes for 1 hour. After 1 hour, knockdown was recorded. Then, the holding tubes were re-attached to the exposure tubes and the mosquitoes were transferred back to the holding tubes using the same coaxing method described above. After the final transfer, the exposure tubes were detached from the holding tubes, and each tube of mosquitoes was provided a cotton ball soaked with 10% sucrose that mosquitoes were able to drink from through the mesh side of the tube.

Mosquitoes were placed in an incubator at 27 °C and 80% RH, and mortality was recorded 24 hours later. Papers were used up to six times following the WHO guidelines. Two replicates of dose-response curves have been collected, and two more replicates will be performed in April 2022 (in addition to 10 replicates of the calculated LC_{50}).

Topical application bioassays: Before beginning the bioassay, the DM insecticides were removed from the freezer, were inverted five times, and were allowed to sit at room temperature for at least 1 hour. 2-5 day old non-blood-fed female mosquitoes were aspirated out of a cage into falcon tubes, which were immediately capped and placed on ice. Mosquitoes remained on ice for at least 30 minutes before dosing occurred. After the mosquitoes were sufficiently knocked down, they were poured onto a tray (which sat on top of the ice), and, using forceps, they were picked up and placed into small plastic cups (also sitting atop the ice). This was done until six cups were filled with 25 mosquitoes each – this provided enough mosquitoes to test five different DM concentrations and one control at once during the bioassay. After the mosquitoes were sorted into their cups, each cup of mosquito was weighed on a scale, and immediately placed back on the ice. After weighing, the Hamilton syringe (Hamilton™ 80465, Fisher Scientific, Waltham, MA) was rinsed with acetone and then filled with the acetone control solution (Note: mosquitoes were always treated working up from the lowest to highest DM concentration to prevent cross-contamination). The first cup of mosquitoes was poured out onto the plastic tray (sitting atop the ice) and a cotton swab was used to separate out the mosquitoes for ease of dosing. Each mosquito was then individually treated with 0.5 µl of control or insecticide solution. After dosing, the mosquitoes were poured back into their respective plastic cup, and the cup was covered

with mesh secured with a rubber band. A cotton ball soaked with 10% sucrose solution was provided on top of the mesh. Mosquitoes were then placed in an incubator at 27 °C and 80% RH, and mortality was assessed 24 hours later. A minimum of four replicates at each concentration were performed, in addition to five replicates of two different LD50 experiments. Five more replicates at each LD50 concentration will be collected in April for a total of ten replicates at each LD50 concentration.

Data analysis. The CDC LC_{50} (lethal concentration that kills 50% of the mosquitoes) and the topical LD_{50} (lethal dose that kills 50% of the mosquitoes) were calculated by performing probit (probability unit) analysis on the dose-response data following previously published methods (Dunford *et al*., 2015; Smith *et al*., 2019). This was done by transforming corrected mortality data into probit values and performing linear regression between the insecticide concentration and the transformed mortality data. Due to the nature of probit analysis, mortality values of 0 or 1 (0% or 100% mortality, respectively) were removed before completing the transformation. Thus, the positive and negative controls are not included in figures 10, 11, and 12. After probit analysis, a Chi-square test was performed to assess the fit of the linear models. To assess whether different assays had significantly different variance, the Levene's test was used on the LD_{50} and LC_{50} variance estimates, as well as the dose-response data. If the Levene's test shows a significant difference in variance between the bioassays, pair-wise comparisons using the Bonferroni correction will be performed to determine which assays exhibit significantly different variance between each other. All dose-response mortality data was normalized by calculating the mean mortality at each concentration and subsequently subtracting the observed mortality from the mean mortality value. These differences (also called residuals)

between the observed and mean mortality were compared to bioassay type in the Levene's tests, and this was done for the experiments that did not include 0 or 100% mortality in the insecticide-treated groups.

Variance estimates at LD_{50}. After dose-response experiments were completed, the LC_{50} and LD_{50} were calculated so that they could be tested and variation within one concentration between replicates could be calculated. The LC_{50} and LD_{50} mortality results were chosen for the Levene's test so as to capture the highest amount of variation possible (e.g., if the LC_{90} was chosen, that would not leave much room for variation to occur above the LC_{90}). Experiments were repeated at these concentrations but had to be repeated at other concentrations that were more likely to actually give 50% mortality due to the range of mortality results that exists around the calculated LC_{50} and LD_{50} values. Five experiments of the LC_{50} over five different days for CDC (five replicates per day), and two experiments of the LD_{50} over two days for topical (with five replicates per day) were performed in order to quantify the variance that occurred around these concentrations. For the two topical bioassay experiments, only two concentrations were tested (five replicates at each concentration), and five more replicates of each concentration will be collected in April 2022.

In the dose-response experiments for the MC1 resistant *Ae. aeygpti* strain, the CDC bottle bioassay exhibited the greatest amount of variation (Figure 8) and the topical bioassay exhibited the least amount of variation (Figure 9). Not enough data has been collected on the WHO tube test to assess variation accurately (Figure 10), but the rest of this data will be collected throughout April 2022. Dose-response concentrations were chosen based on the diagnostic dose (which is 2X the concentration that kills 99% of a

susceptible strain) given by the CDC and WHO guidelines (CDC bottle bioassay diagnostic dose for *Ae. aegypti* = 10 μg/bottle for 30min mortality assessment, WHO tube test diagnostic dose for *Ae. aegypti* = 0.03% for 1hr mortality assessment). No formal diagnostic dose exists for *Ae. aegypti* and the topical bioassay; however, deltamethrin was tested on a mosquito strain with the same *kdr* mutations in the study by Jensen *et al*., (2022), and dose-response data for my experiments were chosen based on concentrations used within that study.

Results

In all bioassay experiments, concentrations much higher than the diagnostic doses given by the guidelines had to be used in order to obtain complete dose-response curves, suggesting that the MC1 may exhibit a high level of resistance to deltamethrin. Mortality in the untreated control bottles for the CDC bottle bioassays was 0% in all experiments, a maximum of 8% in two of the topical bioassays, and a maximum of 4% in the WHO tube tests. Abbott's formula was used to correct mortality relative to the mortality observed in each control (Abbott, 1987). The relationship between DM concentration and mortality was highly significant for the topical bioassay (X^2 = 35.5, p = 2e-08) (Figure 11), but surprisingly, no significant relationship was found between DM concentration and mortality for the CDC bottle bioassay (X^2 = 5.9, p = 0.053) (Figure 12). A significant difference in variance in mortality was found for the normalized mortality data from the CDC and topical dose-response data (F = 5.8253, p = 0.006549), while no significant difference in variance was detected in the LC_{50} and LD_{50} experiments for the CDC and topical assays (F = 2.2233, p = 0.1454). The LC_{50} calculation for the CDC bottle bioassay

was 4.84 μg/bottle (95% CI: 4.59 - 5.08), while the calculated LD_{50} for the topical bioassay was 0.393 ng/mg (95% CI: 0.340 - 0.475).

As mentioned above, these calculated concentrations did not result in exactly 50% mortality once tested; therefore, a range of concentrations expected to give 50% mortality were tested in order to capture the mortality range that exists around the calculated LC_{50} and LD_{50} values. The WHO tube test LC_{50} mortality data will be collected in April, and full analysis of the WHO tube test data will be performed the first week of May (Figure 13).

Discussion

The objective of this study was to compare the variation in mortality data from the CDC bottle bioassay, WHO tube test, and topical application bioassay. The most variation was seen within the CDC bottle bioassays, followed by the topical application bioassay (Figure 14) (the rest of the WHO tube test data will be collected in April 2022). In fact, the variation within the CDC bottle bioassay was so high that no significant dose-response relationship could be established. This is important to note because, in the field, multiple replicates of CDC bottle bioassays are not often performed (due to lack of mosquitoes), and therefore, classifying a mosquito population as susceptible or resistant based on one assay (that is prone to high variation) could lead to inaccurate results. For instance, if one CDC bottle bioassay is performed in the field at the diagnostic concentration, and the results show < 90% mortality (which would be considered confirmed resistance via the CDC guidelines), the resulting mortality may be due to the fact that the mosquito population is indeed resistant, or it could just be the result of

chance. This should be taken into consideration when reviewing CDC bottle bioassay literature, and the importance of sample size for this assay is discussed later below.

Some of the variation within the CDC bottle bioassay could be due to mosquitoes resting on the surface of the bottle for different amounts of time, which would cause the mosquitoes to pick up different amounts of insecticide. This could also be the case for the WHO tube test, in addition to the possibility that some groups of mosquitoes could have spent a great amount of time resting on the untreated surfaces of the WHO tubes. Additionally, if the CDC bottles are not coated properly or consistently, mortality results could be more variable due to the inconsistencies in homogeneity of the insecticide coating. Even though only one user prepared all of the bottles in this study, it is unclear how well deltamethrin binds to glass and how evenly it was distributed throughout each bottle. A similar issue could also be seen in the WHO tube test, where, if the insecticide-impregnated papers are not prepared consistently, mosquitoes could be exposed to varying amounts of insecticide. The 3D structure of the paper does not help either since the insecticide is able to penetrate the paper, leaving an unknown amount of insecticide on the surface to which the mosquitoes are actually exposed. However, the points mentioned above would not be an issue for the topical bioassay since each mosquito is directly treated with an identical volume and concentration of insecticide.

The variation seen within the CDC bottle bioassays could also be due to the fact that it does not control for mosquito weight (this also applies to WHO tube tests). The topical bioassay does, however, take this into account by calculating the mass-relativized dose per mosquito, and it was found that there was a substantial difference in weight between different days of testing the topical bioassay (e.g., some cups of mosquitoes

weighed ~ 40 mg while some cups from different days weighed ~ 70 mg). This incorporation of mosquito mass allows for the calculation of the LD_{50} (versus LC_{50}), which is more meaningful because it accounts for larger and smaller mosquitoes within each test batch. This is an important parameter to measure as mass correlates with insecticide susceptibility because mosquitoes require a different dose based on their size (and different mosquito groups can differ substantially in weight) (Owusu *et al*., 2017).

Additionally, mortality assessment was quite different between each bioassay, and this could have contributed to the observed variation within each method. When assessing "mortality" (i.e., knockdown) in the CDC bottle bioassay, it was sometimes difficult to determine if a mosquito was knocked down or not because they would often experience tremors due to their hyper-excitable state (which occurs via the deltamethrin binding to the sodium channels), leaving room for much subjectivity. When assessing mortality in the WHO tube tests, mosquitoes would sometimes get caught between the papers and the tubes, making it difficult to determine if the mosquitoes died from being trapped or from the insecticide. For the topical bioassay, there was some static in the plastic cups that caused some of the mosquitoes to stick to the sides of the cups, so much care was needed to determine if the mosquitoes were sticking to the sides because they were dead or because of the static (Note: tapping the sides of the cups would often knock the mosquito to the bottom of the cup, where mortality was easier to assess; however, paper cups could be used in the future to prevent static). Overall, the variation in this study is unlikely to be caused by mortality assessment procedures since only one user performed the assessments, but this would a bigger issue when comparing data between different studies where different individuals had their own method of assessing mortality.

Having a clear definition of alive and dead before assessing bioassay mortality is of upmost importance (e.g., classifying a mosquito as dead if it is unable to upright itself, stand, and fly in a coordinated motion).

The preparation for each bioassay is also different. The topical bioassay entails anesthetizing the mosquitoes on ice before sorting and dosing – if some mosquito groups spent more time on ice than the others, they might have a different reaction to the insecticide (though this is highly unlikely as Jass *et al*., (2019) showed that *Ae. aegypti* are particularly adaptable when exposed to cold). The CDC and WHO assays require very minimal mosquito manipulation, as the mosquitoes are directly aspirated out of their cage into their respective bottles and tubes. Another factor that may contribute to variation in the WHO and topical bioassay is the setup of the bioassay with the sucrose cotton ball sitting on top of the mesh-covered cup or tube. This setup could select for more resistant mosquitoes, because the mosquitoes have to be strong enough to reach the top in order to ingest sucrose. Therefore, maybe some mosquitoes are dead at 24 hours because they did not get enough fluids or because the insecticide killed them. This could be tested by placing the sucrose cotton ball at the bottom of the cup.

Finally, a drawback to the CDC bottle bioassay is that, due to the high variation exhibited in the results, a larger sample size is needed to account for the variation. This is difficult to do in the field since it is not always possible to collect enough mosquitoes and may require multiple trips to different houses just to get enough mosquitoes aspirated off the walls of the houses. This study shows that 150 mosquitoes may not be a large enough sample size for the CDC bottle bioassay, and this should be taken into account when performing and analyzing field experiments. The topical bioassay, on the other hand,

does not experience enough variation to require a larger sample size. WHO tube test data will be collected and analyzed to determine the needed sample size for accurate statistical analysis.

Because of the variation that occurs day-to-day outside of laboratory control (further discussed below), it was difficult to calculate the true LC_{50} for the CDC bottle bioassay. The supposed LC_{50} (6.85 μg/bottle) was calculated after the dose-response data was collected, but when that concentration was tested, it turned out to be closer on average to the LC_{80} (with mortality ranging from 68 – 96% within that experiment). The LC_{50} was recalculated after more data was collected, and a lower concentration (5 μg/bottle) was then tested that was expected to be closer to the actual LC_{50}, and instead was closer on average to the LC_{70} (with mortality ranging from 24 – 96% within that experiment). A similar issue occurred when testing the calculated LD_{50} (0.393 ng/mg) for the topical bioassay. When the calculated LD_{50} was tested, the results were closer on average to the LD_{30} (with mortality ranging from 24 – 44% within that experiment). Mortality never reached 100% in these LC_{50} and LD_{50} experiments, therefore still providing data on the variation between the replicates, but still showed how challenging it can be to determine accurate lethal concentrations for the CDC and topical assays under controlled conditions. This could be a result of the steep dose-response curve that is observed when testing pyrethroid insecticides. Any variation that occurs around the LC_{50} can result in an extreme change in mortality results since mortality increases quickly along the curve as insecticide concentration increases. Because of this trend seen in pyrethroid dose-response curves, it could be seen as a limitation to test the LC_{50}'s since the most variation is expected to be seen around that concentration, and this variation

could be due to insecticide type and not the assay itself. To avoid this, concentrations near the ends of the dose-response curve could be tested.

For the LC_{50} and LD_{50} experiments of the CDC and topical bioassays, there was less variation seen within each day of testing compared to the days when complete dose-response curves were constructed, as seen in the results of the two Levene's tests. This could be a result of environmental differences (maybe the lab was colder or warmer one day), or differences within the mosquito groups, such as weight, genetic variation, and age. With age, for instance, each group contained mosquitoes with ages ranging from 2 – 5 days, and it is possible that some groups contained more 2-day old mosquitoes than others, which resulted in them being more susceptible due to their cuticle not being as tough compared to the older mosquitoes. The opposite could also be true, where maybe some groups contained more of the older mosquitoes, thus causing them to be more resistant compared to the younger mosquitoes. These differences in the laboratory-reared mosquitoes are likely smaller compared to field mosquitoes, where field mosquitoes would not have access to optimal and consistent rearing conditions; therefore, these differences would play a larger role when testing field-collected populations.

It is clear that each bioassay is very different from one another, and the details of each method must be taken into account when deciding which is best to use. For instance, it is important to consider whether it is best to assess knockdown or mortality. If wanting to test how many mosquitoes might become knocked down when exposed to a certain insecticide concentration, then measuring 30-minute knockdown in a CDC bottle bioassay can provide that information. This might be useful data when assessing to what extent long-lasting insecticidal bed nets (LLINs) knock down mosquitoes. However, if it

is seen as a threat that mosquitoes can recover from being knocked down (e.g., when metabolic resistance in common), then assessing 24-hour mortality may be important so that an insecticide that actually kills the mosquitoes can be determined and implemented into the vector-control treatment strategy. Additionally, if the goal is to observe the phenotypic response of mosquitoes after they have picked up insecticide through tarsal contact (the natural route of exposure), then the CDC and WHO assays would be more useful compared to the topical bioassay since, in the field, mosquitoes are not exposed to insecticides the way they are during the topical bioassay (though a larger sample size may be needed to account for the high variation). However, if the goal is to know how mosquito populations are changing over space and time, then the assay that exhibits the least amount of noise (i.e., variation) would be best to use. For instance, if the goal is to examine the relationship between the genotype of a mosquito and its resistance phenotype, then the topical application bioassay might be more useful as it ensures each mosquito is treated with an identical volume and concentration of insecticide, ensuring that the resulting phenotype is more likely due to the organism's genotype. This gap between genotype and phenotype is further discussed below.

It is also crucial to keep intensity of resistance in mind when performing these bioassays. Bagi *et al.*, (2015) provides a useful example: Mosquitoes collected from site A may yield a 50% mortality rate in a susceptibility test, while mosquitoes collected from site B may yield an 85% mortality rate. This does not mean that resistance is less of a threat in site B, because if the 15% that survive at that site are extremely resistant, they will be able to live longer even when exposed to treated surfaces and thus have the potential to transmit more disease. On the other hand, if the 50% that survive at site A

possess a low level of resistance, then they are more likely to be killed by further exposure to insecticides, and therefore may not pose a great threat to the public. To ensure this is not overlooked, bioassays have been modified to take the intensity of resistance into account (Bagi *et al*., 2015). The CDC bottle bioassay, for instance, has additional time points that extend beyond the diagnostic time (DI) (the time it takes to kill all susceptible mosquitoes at a given concentration), where knockdown can be measured at 45, 60, 75, 90, 105, and 120 minutes. The longer it takes for mosquitoes to be knocked down, the higher intensity of resistance can be expected to exist within that population (Messenger *et al*., 2017). Another approach is to test concentrations that are 2X, 5X, and 10X stronger than the diagnostic dose (DD), which is 2X the concentration it takes to kill 99% of a susceptible mosquito strain. A similar method is performed in the WHO tube tests, where the guidelines suggest assessing the intensity of resistance by testing concentrations that are 5X and 10X stronger than the DD. Once resistance has been detected, these higher concentrations can be tested to determine the intensity of resistance, which is useful information when deciding when it is best to switch to a different insecticide. However, very little data is published on the strength of resistance in mosquito populations, and it is necessary to agree upon a standardized method to quantify resistance intensity in order to accurately assess the resistance phenotype of a population (Bagi *et al*., 2015).

Nonetheless, even with the additional information of resistance intensity, and regardless of the amount of variation that exists within a bioassay, susceptibility tests can only be so informative when it comes to determining the success or failure of vector control strategies in the field. Some bioassays have been developed that try to more

closely mimic field settings, such as the cone and tunnel assays, but these studies are often performed with mosquitoes that are reared in optimal laboratory conditions (Namias *et al*., 2021). These technical resistance assays (which measure resistance under standardized, controlled environments) are useful for detecting resistance within a mosquito population, but practical resistance assays (which determine the response of mosquitoes to actual interventions under natural conditions) may be more effective in determining control success or failure (Namias *et al*., 2021). For instance, the experimental hut trials (where LLINS and indoor residual spraying (IRS) are tested) most closely imitate the field setting by allowing mosquitoes to enter especially designed experimental huts containing an intervention and a human volunteer to attract mosquitoes (as they would in local dwellings) and mortality and deterrence due to the intervention product can be calculated the next day (Sherrard-Smith *et al*., 2018). However, these experiments are costly and time consuming, in addition to there being few existing sites where these trials can be conducted. A more possible scenario would be to, for example, test a LLIN using larger cages surrounding the area of the net to be tested (instead of the small cones in the cone assay) to allow the mosquitoes to seek refuge from the exposure. Another possibility is to treat several natural water bodies with larvicides, randomly place mosquito larvae at all life stages into floating mesh containers within the water, and assess their phenotypic response to the treatments. This would allow the larvae to experience naturally occurring environmental conditions such as changes in water temperature and salinity. These types of practical resistance experiments could inform policy decisions such as switching to a new insecticide class when the previously used treatment is no longer effective (Namias *et al*., 2021).

The importance of technical resistance assays should not be overlooked, however, as data is lacking on the link between genotype and phenotype within disease vectors. The Window of Selection (WoS), a theoretical framework that gives insight into the impact a certain insecticide concentration has on genotype, can help identify the intensity of selection for resistance at various insecticide concentrations (South *et al*., 2020). This framework has been used for many years in antibiotic resistance research (Drlica & Zhao, 2007), but the WoS was only recently introduced into insecticide resistance studies (South *et al*., 2020) and requires detailed dose-response curves based on technical resistance data. If studied more widely in diploid organisms, this framework could help vector control specialists pick insecticide concentrations that would be more effective based on the vector population's genotype and fitness costs. More research needs to be done on the WoS for organisms heterozygous for the resistance allele, as well as on how selective pressures could influence the amount of time that the "window" is open, in order to know how to best incorporate this framework into future studies. Part of this gap could potentially be filled using technical resistance assays to, for example, determine the link between genotype and phenotype for heterozygous organisms. Thus, the need for technical resistance experiments persists and should not be neglected.

Limitations. Although this study was successful at comparing variation between the CDC bottle bioassay and topical application bioassay (and soon the WHO tube test), there were limitations to the experimental design. First, only one (resistant) strain was tested, and this study should be repeated on a susceptible mosquito strain to serve as a control to show that the resulting variation could apply to other strains as well. The original plan was to complete this study on both a resistant and susceptible *Ae. aegypti*

strain, but due to time constraints, both could not be completed, and the resistant strain was chosen so that variability in a strain with *kdr* mutations could be quantified. Second, the bioassays were executed in only one location, meaning the variation that would be observed from location to location was not captured; therefore, the variation reported here is likely an underestimate of the true variation reported in the field. However, this could also be seen as a strength because the results in this study truly reflect the variation seen within the assay and not as a result of differences between multiple users/locations. Third, *Ae. aegypti* was used as the model organism (which does not spread malaria). It would be ideal to use *Anopheles* mosquitoes since they are the actual malaria disease-vector, but due to restrictions in working with non-native mosquitoes in our facility, I was not able to get permission to work with a malaria-transmitting vector. Though there are undoubtedly differences between *Anopheles* and *Aedes* mosquitoes, the *kdr* mutations are very analogous in both species and occur in almost identical places within the VGSC, meaning that the genetics between both species are very similar. Additionally, because the nature of this study was to quantify variability between insecticide susceptibility tests, using *Ae. aegypti* still served as a useful model organism because of its genetic similarities to *Anopheles* mosquitoes. Fourth, the experiments were performed within a wide 8 hour time period (between 8:30am – 4:30pm) which could have impacted results since time of day does have an effect on insecticide susceptibility due to the mosquito's metabolism (Balmert *et al.*, 2014). Finally, delayed mortality could have been assessed in all three bioassays to see if variation decreased over time post-exposure. Further studies should be done to determine 1) the effect of user bias on insecticide susceptibility tests, 2)

the variation in delayed mortality results, and 3) the variation that exists within a susceptible mosquito strain.

Conclusion. This study provided the first comparison of the variation between the CDC bottle bioassay, topical application bioassay, and WHO tube test. The overall goal of vector control and RMS is to decrease the emergence and spread of resistance while simultaneously reducing disease transmission, and the many bioassay tools at our disposal will only be useful if they are used in the correct settings. This study seeks to draw attention to the advantages and disadvantages of various bioassays and vector control tools and calls for a greater discussion around the usefulness of technical and practical resistance experiments.

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CHAPTER 3

CONCLUSION

The objective of my studies throughout my masters was to develop new and improved theories and methods that will help win the fight against the emergence and spread of resistance. To do this, I optimized the topical application bioassay for mosquitoes and fruit flies with some colleagues and drew attention to the method's usefulness by publishing our manuscript in JoVE, as well as recording a video showing how to perform the bioassay. I then took this study a step further and designed my individual research project where I performed the first comparison of the topical application bioassay to two commonly used insecticide susceptibility tests: the CDC bottle bioassay and WHO tube test. It is clear from that study that all three bioassays are extremely different from each other, and each has its own advantages and disadvantages. Careful consideration should be given to the goal of the resulting bioassay before choosing which method to use. If the goal is to fill the gap between genotype and phenotype, then the topical application bioassay is best to use as each organism is treated with an identical volume and concentration of insecticide. However, if the goal is to determine how a mosquito reacts to picking up insecticides on a material surface, then the CDC or WHO assays may be best. Further studies should quantify the amount of insecticide a mosquito absorbs during each bioassay, as this would help determine if the variability seen in the assays is due to differences in insecticide absorption. Additionally, this study needs to be repeated on a susceptible mosquito strain to serve as a control strain, and having multiple users perform the bioassay could allow user variation to be included in the results if so desired. Finally, the value of technical versus practical

resistance assays should be considered when deciding how to move forward with vector control strategies in order to better understand the mechanisms behind vector control success or failure.

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APPENDIX A

STATEMENT OF PERMISSION FROM COAUTHORS

All co-authors have granted their permission for me to use the JoVE publication (Chapter 1) in my thesis.

APPENDIX B

FIGURES

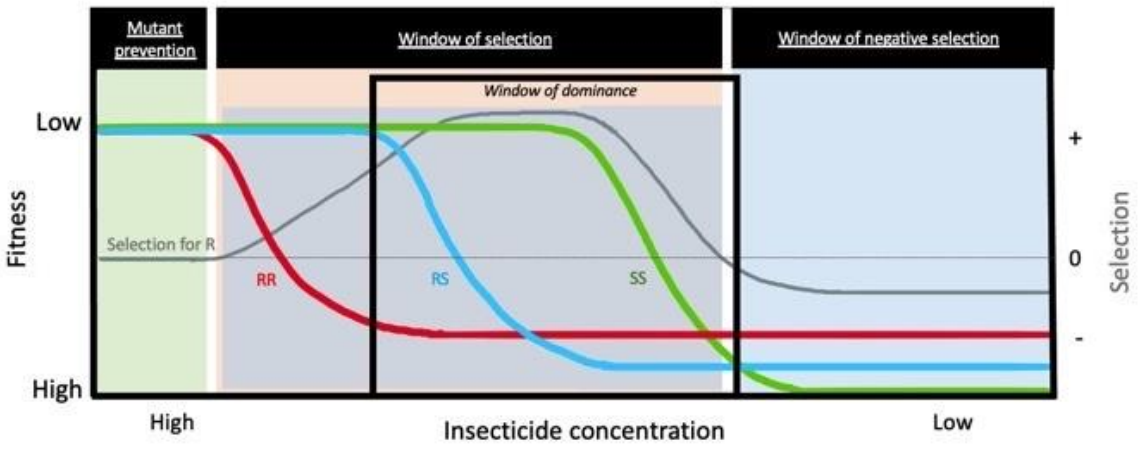

Figure 1: WoS Graph. Homozygous resistant (RR) organisms are portrayed in red, heterozygous resistant (RS) in blue, and homozygous susceptible (SS) in green. Selection for the resistance allele is depicted in the gray curve, which increases as the WoS opens but decreases as it closes. The window of dominance, which exists within the WoS, opens when RS experiences lower fitness than SS and closes when the window of negative selection opens.

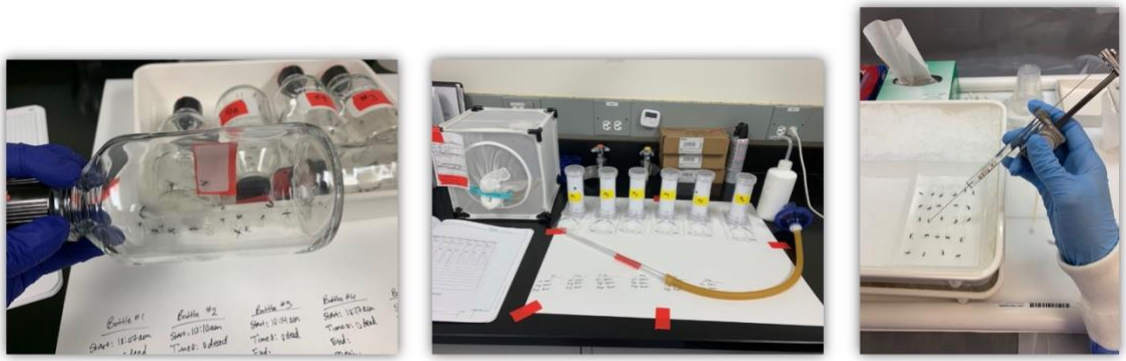

Figure 2: Susceptibility Bioassays. The CDC bottle bioassay follows methods as found in Brogdon & Chan (2010), the WHO tube test as found in the WHO SOP, and the topical application bioassay as found in Jensen, Althoff *et al*., (2022).

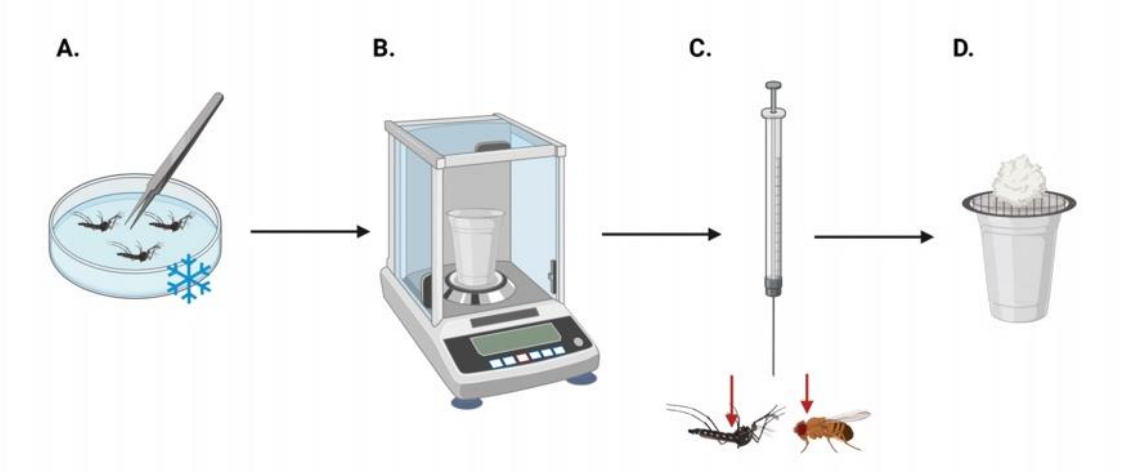

Figure 3: Topical Application Assay Protocol Diagram. Topical application assay protocol begins with (**A**) sorting specimens on ice, followed by (**B**) weighing specimens on an analytical scale, (**C**) dosing specimens with insecticide solution(s), and (**D**) 24 h waiting period post insecticide exposure with access to 10% sucrose solution *ad libitum* (via a soaked cotton ball), followed by mortality assessment. Red arrows indicate target insecticide application location for mosquitoes (left) and fruit flies (right). Note that the image is not to scale.

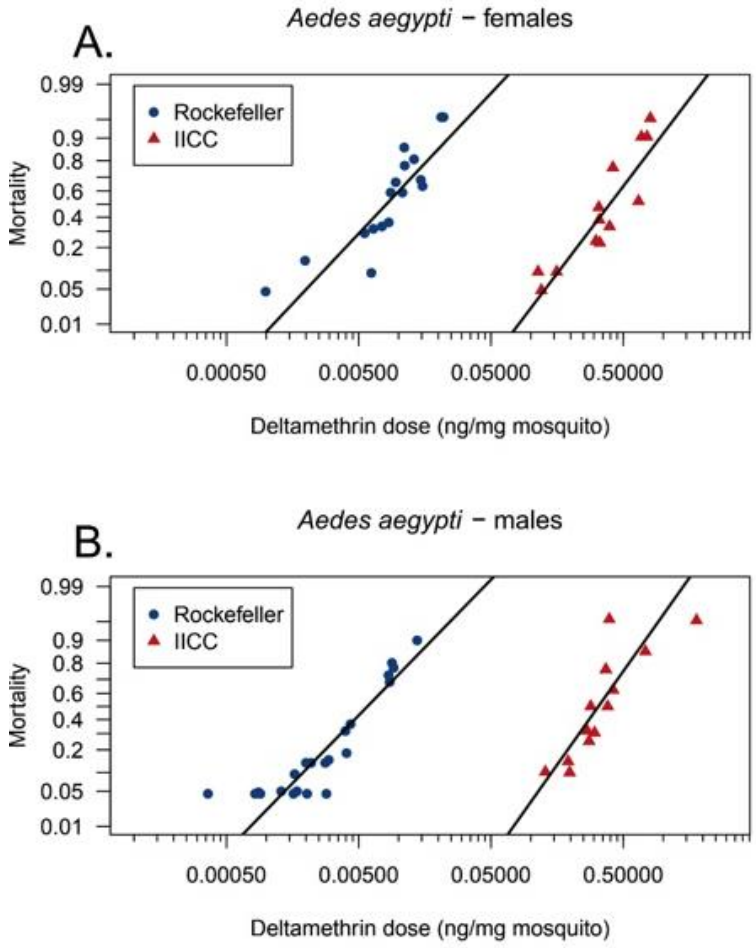

Figure 4: Representative Data of Mosquitoes Using Topical Application Bioassay. Representative dose-response data from topical application bioassay following the above protocol using deltamethrin and mosquitoes: (**A**) female *Ae. aegypti* ROCK (n = 880) and IICC (n = 550) strains, (**B**) male *Ae. aegypti* ROCK (n = 880) and IICC (n = 569) strains. Deltamethrin testing concentrations ranged from 0.00075 ng/µL to 9.68705 ng/µL, and the dose of deltamethrin applied (ng) per average mosquito mass (mg) is reflected on the x-axis. Mortality is shown as a proportion on the y-axis. The black line through each data point cluster represents the strain and sex-specific linear regression.

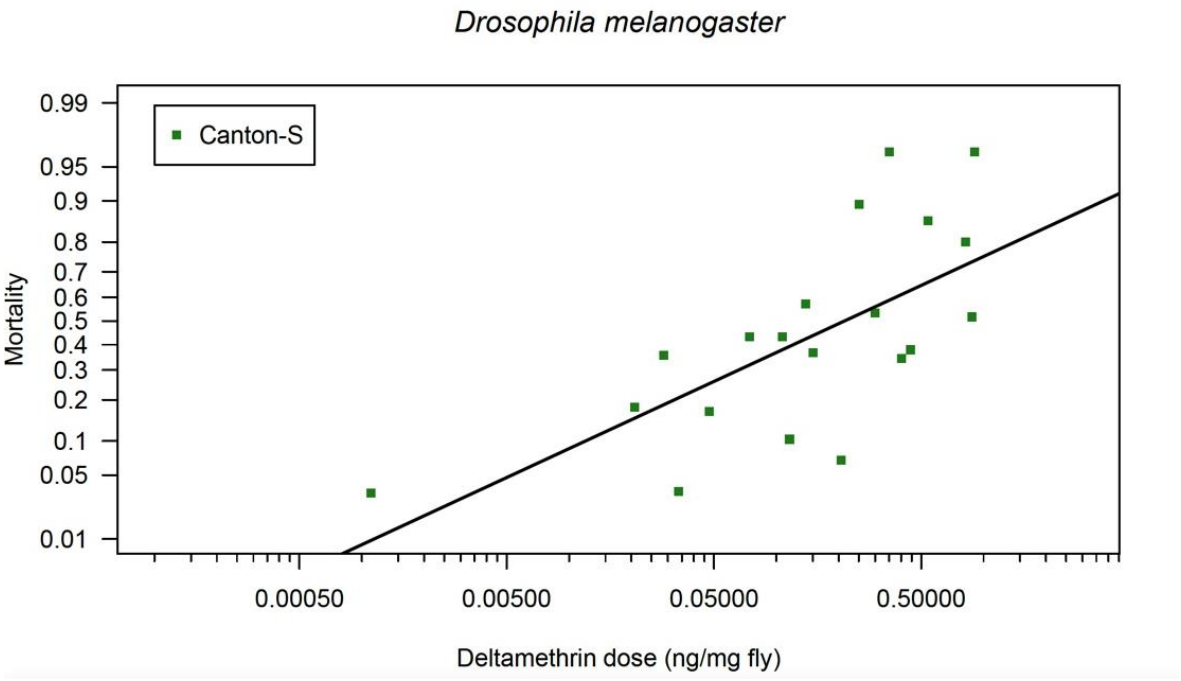

Figure 5: Representative Data of Fruit Flies Using Topical Application Bioassay. Representative dose-response data from topical application bioassay following the above protocol using deltamethrin and fruit flies: *D. melanogaster* Canton-S strain (n = 1014). Deltamethrin testing concentrations ranged from 0.00499 to 5.02876 ng/µL, and the dose of deltamethrin applied (ng) per average fruit fly mass (mg) is reflected on the x-axis. Mortality is shown as a proportion on the y-axis. The black line represents the linear regression.

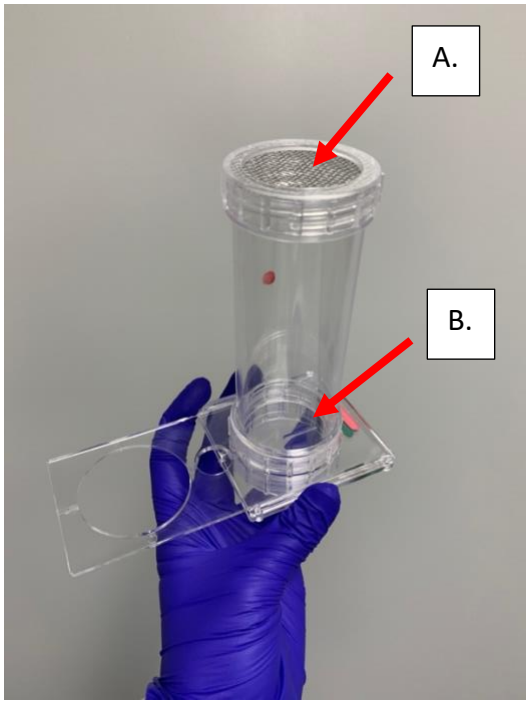

Figure 6: WHO Tube Diagram. Untreated areas of WHO tube used in WHO tube tests. A: Untreated mesh top. B: Untreated plastic bottom.

Musca aa Position	VGSC Chromosome Position	Ref. allele	Alt. allele	Aaeg aa Position	Ref. aa	Var. aa
1016	3:315983763	C	T	1012	V	I
1534	3:315939224	A	C	1554	F	C
723	3:316014588	A	T	711	S	T
410	3:316080722	C	A	408	V	L
915	3:315999297	G	T	911	I	K

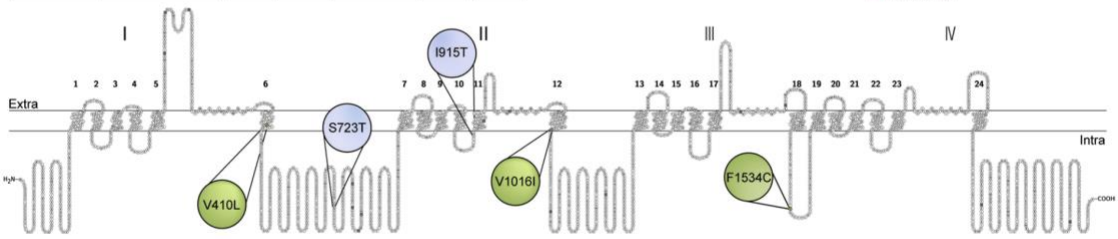

Fig. 1 Topology of the mosquito sodium channel. The *Ae. aegypti* reference sequence was translated in CLC Main Workbench, Version 7. The resulting amino acid sequence was aligned to the *Musca domestica* (ANW06229) and *Drosophila melanogaster* (AAB59195) reference protein sequences. SNP annotations were transferred to *Musca* to determine the *Musca* protein position, and structural annotations were transferred from *Drosophila* to *Aedes*. The topology of the sodium channel was illustrated using Protter version 1 [49]. The sodium channel protein contains four homologous repeat domains (I–IV). Each repeat domain has six α-helical transmembrane segments (1–6, 7–12, 13–18, 19–24). Filled circles represent the five SNPs assayed in this study. Ref. = Reference. Alt. = alternate. aa = amino acid

Figure 7: Topology of the Mosquito Sodium Channel. Image from Mack *et al.*, 2021.

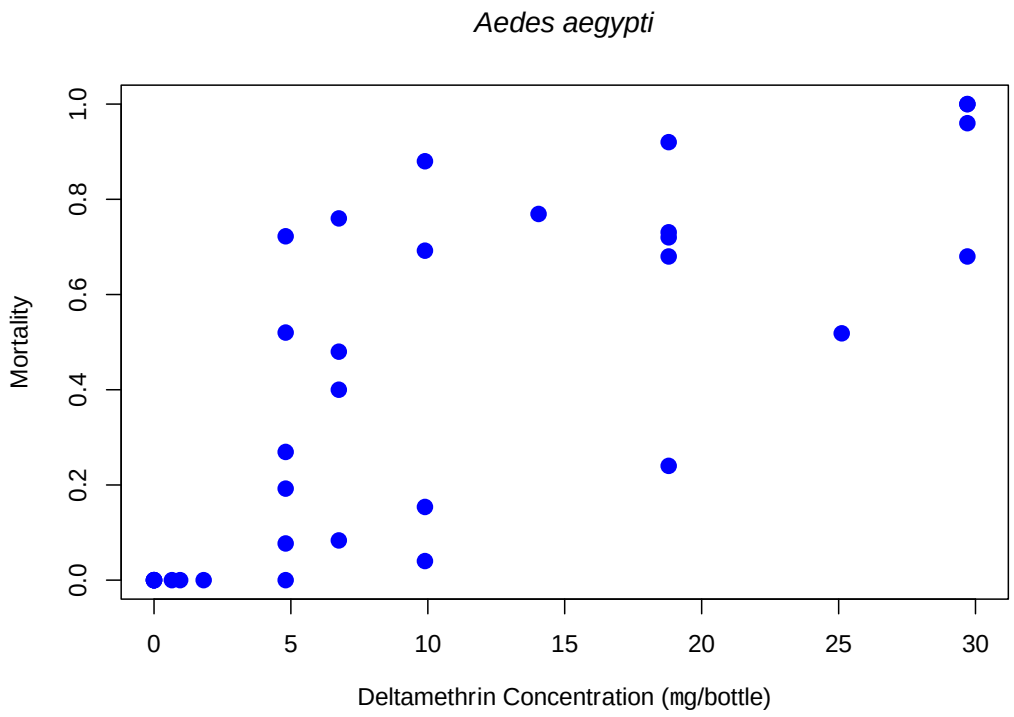

Figure 8. CDC Bottle Bioassay DM Dose-Response for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM concentration per CDC bottle (x axis). Data is not plotted on a log scale.

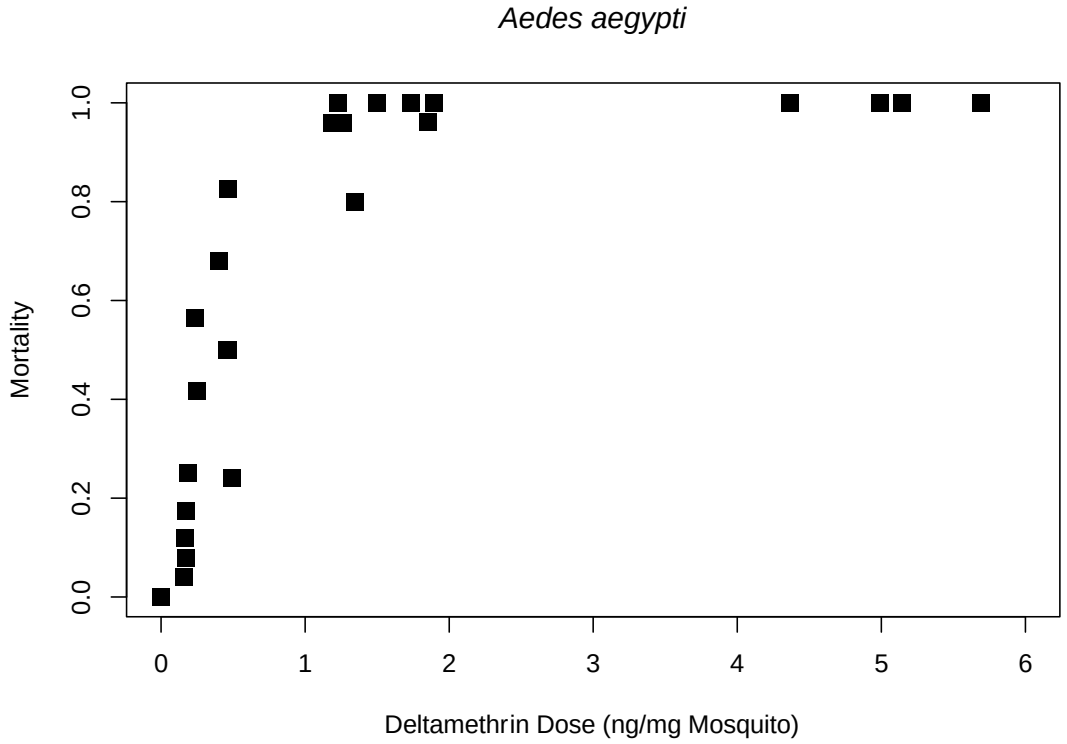

Figure 9. Topical Application Bioassay DM Dose-Response for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM dose (x axis) which takes the insecticide concentration and volume, as well as the mosquito weight, into account. Data is not plotted on a log scale.

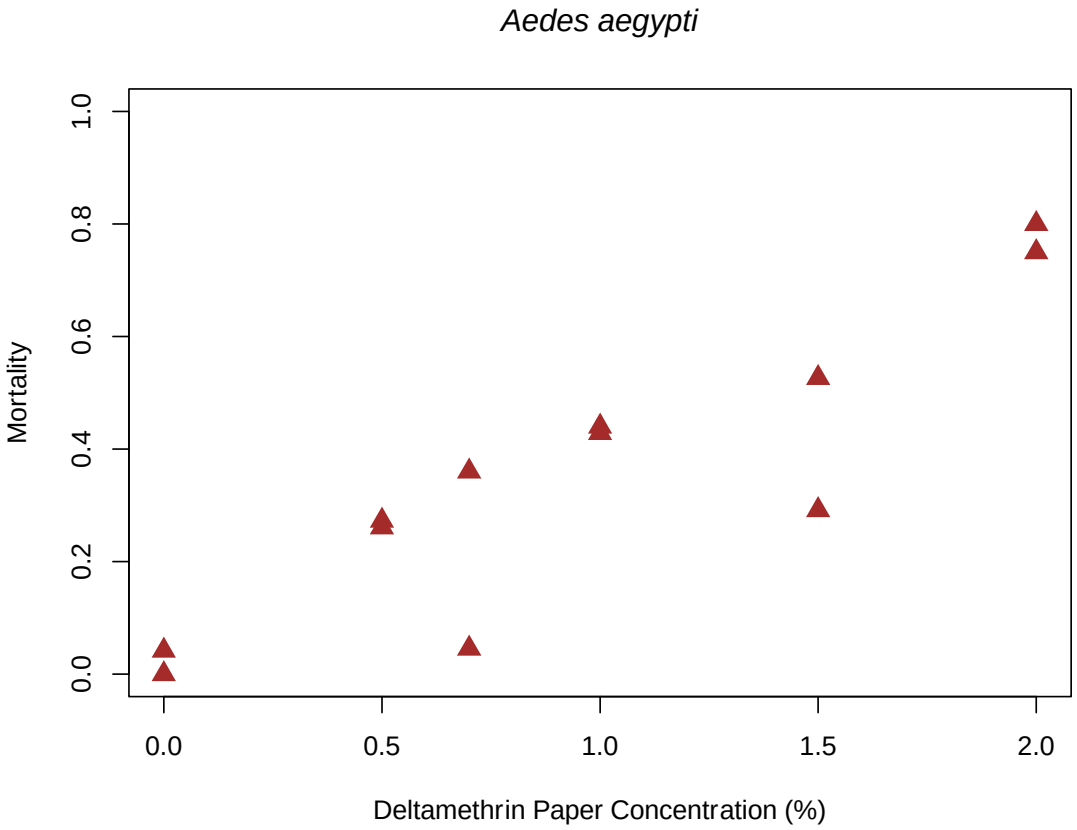

Figure 10. WHO Tube Test DM Dose-Response for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM paper concentration (x axis). Data is not plotted on a log scale.

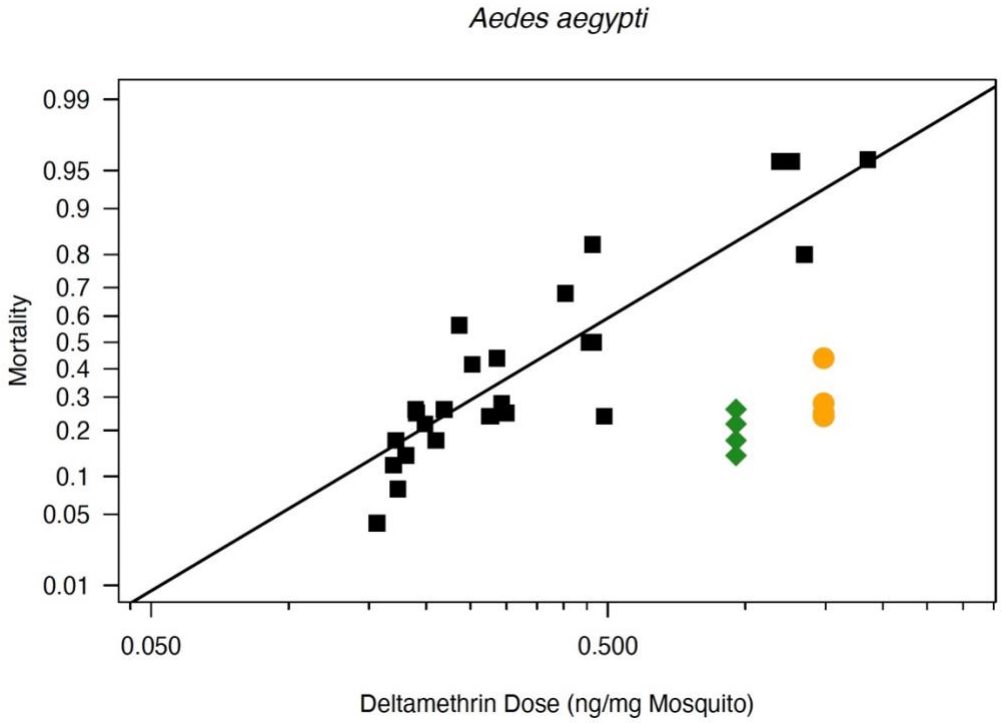

Figure 11. Topical Application Bioassay Regression Analysis for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM dose (x axis). Data is plotted on a log scale and regression analysis showed there is a significant relationship between DM concentration and mortality in the topical bioassay ($X^2 = 35.5$, $p = 2e\text{-}08$). Black squares represent dose-response data, while the LD_{50} experimental data is represented as a different shape and color for each day of testing.

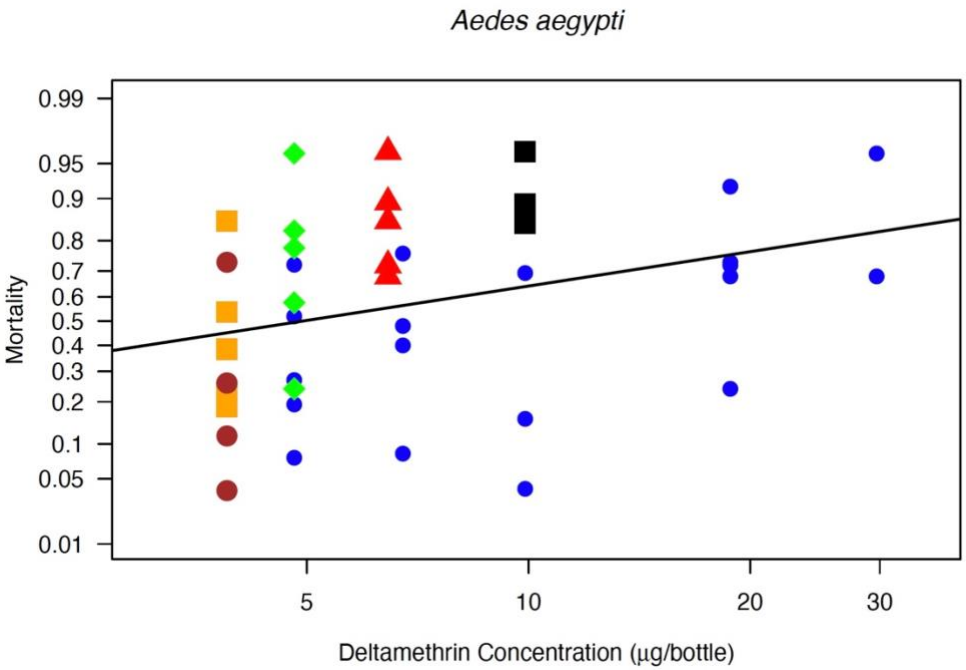

Figure 12. CDC Bottle Bioassay Regression Analysis for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM concentration per CDC bottle (x axis). Data is plotted on a log scale and regression analysis showed there is no significant relationship between DM concentration and mortality in the CDC bottle bioassay ($X^2 = 5.9$, $p = 0.053$). Blue circles represent dose-response data, while the LC_{50} experimental data is represented as a different shape and color for each day of testing.

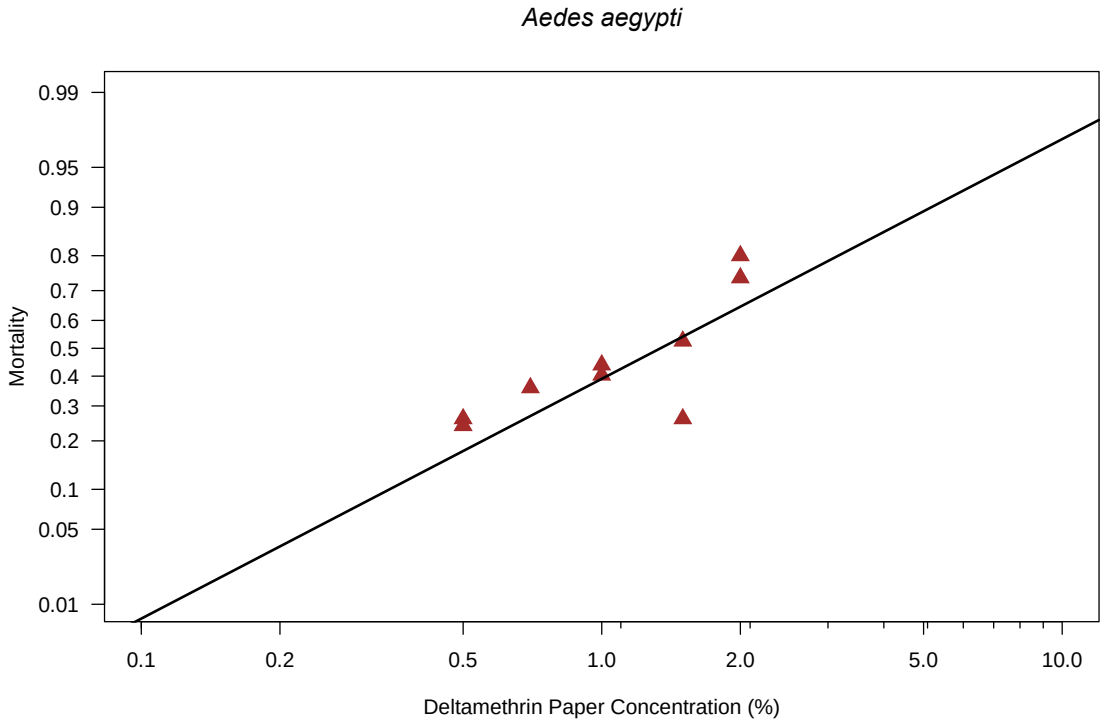

Figure 13. WHO Tube Test Regression Analysis for MC1 *Ae. aegypti* Strain. Mortality (y axis) is plotted against DM paper concentration (x axis). Data is plotted on a log scale and regression analysis will be performed once all WHO tube test data is collected.

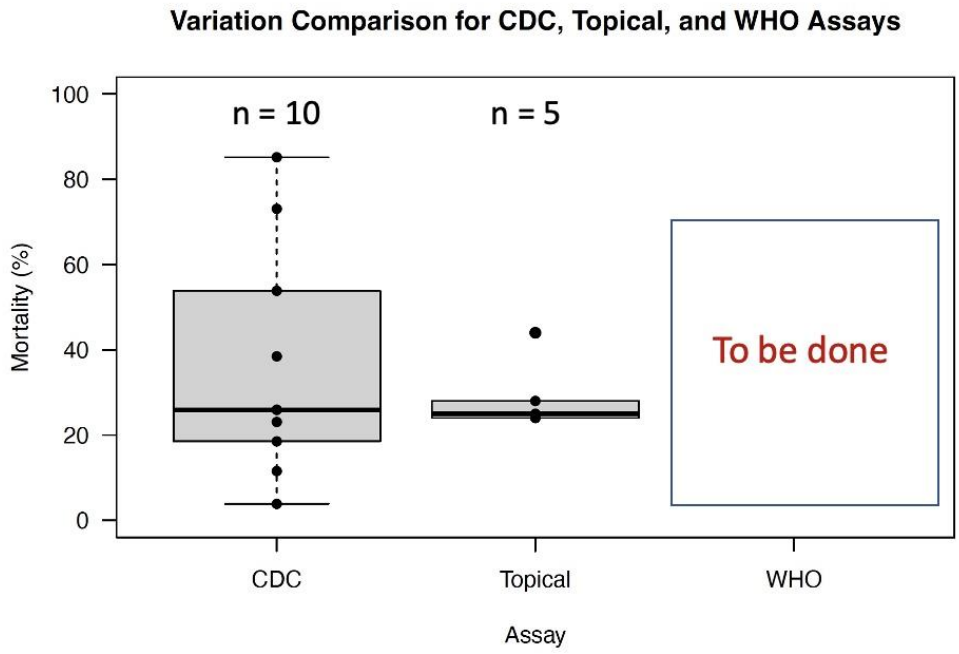

Figure 14. Variation Comparison for the CDC Bottle Bioassay, Topical Bioassay, and WHO Tube Test. The most variation in mortality was seen in the CDC assay, followed by the topical assay. Further data will be collected on the WHO tube test to quantify the variation seen within the assay.