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## Protocols for testing the toxicity of novel insecticidal chemistries to mosquitoes.

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**TITLE:**

Protocols for Testing the Toxicity of Novel Insecticidal Chemistries to Mosquitoes

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Mosquito, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, larvicide, adulticide, endectocide, bioassay, toxicity, LC<sub>50</sub>

**SHORT ABSTRACT:**

Protocols are described for assessing the toxicity of chemistries to immature and adult mosquitoes for development as new classes of larvicides, adulticides and endectocides. The protocols enable high-throughput testing of multiple chemistries at single-point dose and subsequent evaluation via dose response assay to determine toxicity on contact or via ingestion.

**LONG ABSTRACT:**

New classes of insecticides with novel modes of action are needed to control insecticide resistant populations of mosquitoes that transmit diseases such as Zika, dengue and malaria. Assays for rapid, high-throughput analyses of unformulated novel chemistries against mosquito larvae and adults are presented. We describe protocols for single point-dose and dose response assays to evaluate the toxicity of small molecule chemistries to the *Aedes aegypti* vector of Zika, dengue and yellow fever, the malaria vector, *Anopheles gambiae* and the northern house mosquito, *Culex quinquefasciatus*, on contact and via ingestion. As an example, we evaluated the toxicity of

amitriptyline, a small molecule antagonist of G protein-coupled receptors, via larval, adult topical and adult blood-feeding assay. The protocols provide a starting point to investigate insecticide potential. Results are discussed in the context of additional experiments to explore product applications and mechanisms for delivery.

## INTRODUCTION:

Mosquitoes transmit the causative agents of infectious diseases that impact human health globally<sup>1</sup>. The three most important mosquito genera affecting human health are *Aedes*, *Anopheles*, and *Culex*. Species of *Aedes* mosquitoes vector the arboviruses that cause Zika, dengue, yellow fever and chikungunya. *Anopheles* species vector malaria parasites and *Culex* species transmit West Nile virus and filarial nematodes<sup>2</sup>. The World Health Organization (WHO) has called for the eradication of ten Neglected Tropical Diseases (NTD) by 2020<sup>3</sup> and identified mosquito control as the most viable strategy. Insecticides are powerful tools to control mosquitoes and reduce disease transmission<sup>4,5</sup>. However, the emergence of insecticide-resistant mosquito populations threatens the continued control of many diseases<sup>6-8</sup>. The Innovative Vector Control Consortium (IVCC) has launched the first dedicated effort to develop replacement insecticides for mosquito control<sup>9</sup>. New insecticides with novel modes of action (MoA) are needed to meet WHO goals and achieve disease control. The discovery and development of new insecticide classes with novel MoAs require whole organism assays for rapid, high-throughput analysis of chemistries and in comparison to industry standards<sup>10</sup>.

Multiple assays are available to evaluate the repellency and toxicity of insecticides to larvae and adult mosquitoes, and some are suitable for the evaluation of novel formulated or unformulated chemistries. The WHO protocol<sup>11</sup> is a contact assay used to test products against fourth instar larvae. Published studies also describe a variety of larval assays used to test the toxicity of small molecule chemistries to larvae of *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*<sup>10,12-16</sup>. The Centers for Disease Control and Prevention (CDC) bottle bioassay is used to assess insecticide resistance in field caught populations of adult mosquitoes via evaluation of lethal concentration (LC) and lethal time (LT) in comparison to a standard diagnostic dose of a commercial insecticide<sup>17</sup>. The WHO susceptibility test offers another approach to evaluate insecticide resistance in adult mosquitoes whereby mosquitoes are exposed to insecticide-impregnated paper inside glass vials for 1 h and mortality is assessed at 24 h<sup>18</sup>. Other assays spray formulations inside sealed cages and record mosquito knockdown and death at desired timepoints<sup>19</sup>. Topical application to the mosquito thorax has been used to evaluate potency of different pesticides<sup>20</sup>. The Liverpool Insect Testing Establishment (LITE)<sup>21</sup> employs Standard Operating Procedures to evaluate the bioactivity of chemistries to adult mosquitoes. The LITE adult topical assay applies a small volume of test product to the thorax of adult mosquitoes with mortality scored at 48 h. The assay enables quantification of the dose received per individual mosquito<sup>21</sup>. The LITE tarsal assay evaluates the toxicity of test product to adult mosquitoes resting on a chemically treated surface and is used to identify chemistries with potential for development as indoor residual sprays (IRS) or use in insecticide treated bed-nets (ITNs) to control *Anopheles* mosquitoes.

The WHO and CDC assays have limitations for testing novel, unformulated chemistries, and the goal of the CDC bottle bioassay is evaluation of susceptibility over short assay periods (two hours) rather than toxicity<sup>22,23</sup>. In addition, assays for evaluating the toxicity of chemistries delivered in the blood meal and potential for development of systemic-acting products (*i.e.*, endectocides) are lacking. Here, we describe assays for testing the mosquitocidal activity of novel chemistries that are soluble in water or one of several organic solvents against the larvae and adults of *Aedes*, *Anopheles* and *Culex*. First, we demonstrate a larval assay previously described by our group<sup>10,12,13,16</sup>, and performed here as (1) a screen to rapidly evaluate multiple chemistries at single point dose, and (2) a dose response assay to determine the lethal concentration (*e.g.*, LC<sub>50</sub>, LC<sub>75</sub> or LC<sub>90</sub>) of a single chemistry. Next, we describe two adult assays for determination of lethal dose (LD). The first of these is an adaptation of the LITE protocol for testing of topically applied, novel chemistries against adult mosquitoes in comparison to a positive control. The second is a feeding assay for the evaluation of chemistries delivered systemically via the blood meal.

## PROTOCOL:

Note: All strains and reagents required for the work described in the following protocols, and suppliers are listed in the **Table of Materials**.

### 1. Culture of Mosquito Larvae and Adults

Note: Insecticide susceptible strains of mosquitoes are available from the Malaria Research and Reagent Reference Repository. Recommended strains are as follows: *Aedes aegypti* Liverpool (LVP) strain, *Anopheles gambiae* Kisumu (KISUMU1) strain and *Culex quinquefasciatus* Johannesburg (JHB) strain.

1.1) Culture mosquito larvae from eggs on a 12 h day/12 h night cycle at 28 °C and 75-85% relative humidity (RH) in 25 cm x 40 cm plastic trays (~ 400 larvae per tray) as described by Nuss<sup>29</sup>. Feed the larvae ground gerbil (*A. aegypti* and *An. gambiae*) or flake fish food (*C. quinquefasciatus*). For larval assays, collect larvae at the third larval (L3) instar stage.

1.2) Rear adult mosquitoes from pupae that have been transferred to plastic cups and place inside 20 L plastic cages in an insectary under the conditions described above. Maintain mosquitoes on 20% sugar solution as described elsewhere<sup>29</sup>. Collect adults at 4-5 days post emergence.

### 2. Larval Contact Assay (Single Point Dose or Dose Response Assay)

Note: The assay can be conducted with chemistries that are soluble in water or dimethyl sulfoxide (DMSO), provided that the final concentration of DMSO in test wells does not exceed 1%. Alternative solvents may be an option, but it is essential to first confirm that the final concentration does not cause more than 10% mortality at 72 h post exposure. The assay may be performed as a single point dose or dose response assay. If performing the latter, it is recommended to test a range of concentrations (minimum of five), spanning the expected LC<sub>50</sub>.

2.1) Label the wells of a clear 24-well tissue plate as shown in **Figure 1**.

2.2) Prepare a stock solution of the test chemistry in a 1.5 mL tube.

2.2.1) Weigh each chemistry using an analytical balance, and depending on solubility characteristics, re-suspend in sterile ddH<sub>2</sub>O, DMSO or other suitable organic solvent of choice (see **Figure 2**).

2.3) Determine the final concentration of the test chemistry that will be evaluated and prepare serial dilutions from the stock solution accordingly, using the appropriate solvent.

Note: The concentration and volume of chemistry and solvent can be calculated using the formula  $C_1 \cdot V_1 = C_2 \cdot V_2$  where  $C_1$ =concentration of sample 1,  $C_2$ =concentration of sample 2,  $V_1$ =volume of sample 1 and  $V_2$ =volume of sample 2. See the example calculations shown in **Figure 2** and Table 1.

2.3.1) Prepare a 10 mM stock solution and serial dilutions as required to obtain desired test concentrations (**Table 1B**).

2.4) Using a wide-bore plastic transfer pipette, transfer five L3 larvae to the well of a tissue culture test plate; L3 larvae can be recognized by length (~ 2.5 – 3.5 mm) and head capsule width of ~ 0.025 mm<sup>24</sup>. Gently remove the water with a 1 mL pipette and replace with the desired volume of ddH<sub>2</sub>O (see below). Repeat to obtain n = 4 technical replicates per treatment (*i.e.*, 20 larvae total per treatment).

2.5) Add the appropriate volume of test chemistry to each of the four replicate wells in the 24-well test plate and gently swirl the plate to ensure uniform mixing of chemistry. Place the plate in a test or growth chamber under constant conditions (*e.g.*, 25 °C and ~ 75-85% RH is recommended, and a 12 h light/12 h dark cycle if possible).

Note: For reproducibility between experiments, ensure subsequent assays are performed under the same environmental conditions.

2.6) Record the number of dead/non-responsive larvae in each well at 30 min, 1, 1.5, 2, 3, 24, 48 and 72 h post-exposure (or alternative time points) on the score sheet (**Table 2**). Gently tap the side of the plate. If no movement is observed, gently touch the larva with a sterile toothpick. Score larvae that do not respond to tapping/touch as “dead”.

Note: Other morphological and behavioral phenotypes may be observed and can be recorded.

2.7) Calculate the percent larval mortality per treatment. Graph results as a histogram or logarithmic curve using software of choice such as (*e.g.*, Graph-pad Prism 6 or similar) and calculate Lethal Concentration (LC) values relative to the control.

2.8) Correct for control mortality using the modified Abbott's formula if desired, as follows:

$$\text{Mortality (\%)} = (X - Y) * 100 / (100 - Y),$$

where X = the percent mortality in the treated sample, and Y = the percent mortality in the control.

2.9) Repeat the assay using a separate batch of mosquitoes to obtain n = 3 or more biological replicates.

### 3. Adult Topical Assay (Single Point Dose or Dose Response Assay)

Note: The adult topical assay is conducted using acetone as a solvent. Alternative solvents may be an option, but it is essential to first confirm that the final concentration does not cause more than 10% mortality at 48 h post exposure. The assay may be performed as a single point dose or dose response assay and is used to determine lethal dose (LD). If performing the latter, it is recommended to test a range of concentrations (minimum of five), spanning the expected LD<sub>50</sub>.

3.1) Determine the number of adult female mosquitoes required to complete the assay.

Note: A point dose assay with a single chemistry will require a minimum of 90 mosquitoes (30 for the positive control, 30 for the acetone-only negative control and 30 for the test chemistry).

3.2) Culture 3 to 5-day-old adult female mosquitoes and move to a separate 20 mL plastic bucket using an aspirator (Figure 3).

3.3) Label 9 oz paper cups with the name and concentration of the test chemistry. Set aside 10 cm x 10 cm mesh squares and rubber bands for each cup (see Figure 4).

3.4) Select an existing insecticide as a positive control (e.g., the synthetic pyrethroid, bifenthrin) and prepare a 1% stock solution (10 µg/µL) in acetone.

Note: Calculations should take into account the purity of the chemistry. For example, for a chemistry that is 99% pure, dissolve 10.1 mg in 1000 µL of acetone to obtain a 1% solution. Seal with paraffin film and store at -20 °C.

3.5) Using the same procedure as above, prepare a stock solution of the test chemistry.

Note: Many test chemistries are highly labile, and solutions should preferably be made fresh each time the bioassay is performed.

3.6) Prepare serial dilutions of the positive control and test chemistries from stock solutions as shown in Figure 5 using 20 mL glass vials previously rinsed with acetone.

3.7) Purge a 1 mL glass syringe with acetone, fill with acetone and secure in the micro-applicator adjusted to deliver a volume of 0.25 µL.

3.8) Working in batches of ten, remove 3 – 5-day old adult female mosquitoes from cages using an aspirator and anesthetize for up to five min at 4 °C in a fridge or on ice. Next, transfer mosquitoes to a Petri dish and place on ice for up to 10 min.

3.9) Working quickly, remove a single female with fine tweezers, and apply 0.25 µL of test solution to the dorsal thorax using the syringe micro-applicator. Confirm delivery of chemistry by observation using a dissecting microscope to ensure each mosquito receives the appropriate volume of test chemistry.

3.10) Transfer the mosquito to a labeled paper cup resting on ice, and repeat nine times to obtain n = 10 treated mosquitoes. Seal the mosquitoes in the cup with a mesh square secured with a rubber band, and transfer to a plastic tub or growth chamber under constant conditions of 28 °C and 75-85% RH (on a 12 h day/12 h night cycle is preferable).

3.11) Repeat the above experiment twice to obtain n = 3 technical replicates (*i.e.*, 30 mosquitoes total) per treatment or control group.

3.12) Repeat steps 3.7 – 3.10 first with the test chemistry and then the positive control.

Note: Another useful control to include if sufficient mosquitoes are available is a “blank” of 30 anesthetized mosquitoes that do not receive either test chemistry or solvent.

3.13) Record the number of “dead/non-responsive” mosquitoes at 30 min, 60 min, 2, 24 and 48 h post-exposure (or alternative time points) using the score sheet (**Table 3**). Score mosquitoes as “dead/non-responsive” if they show lack of movement, defined as laying on one side or on the back and with the inability to fly.

3.14) If desired, correct for control mortality using the modified Abbott’s formula as follows:

$$\text{Mortality (\%)} = (X - Y) * 100 / (100 - Y),$$

where X = the percent mortality in the treated sample, and Y = the percent mortality in the control.

3.15) Display results as a histogram or an exponential curve. For a dose response assay, calculate the LD<sub>50</sub>, LD<sub>75</sub> or LD<sub>90</sub> value for the test chemistry relative to the control.

3.16) Repeat the assay at least twice using separate batches of mosquitoes to obtain n = 3 or more biological replicates.

#### 4. Adult Blood-Feeding Assay (Single Point Dose or Dose Response Assay)

4.1) Collect approximately 150 4-5-day old adult female mosquitoes and transfer to a separate cage. Repeat to obtain 6 cages. Remove the source of sugar 1-24 h prior to the feeding assay.

4.2) Prepare a stock solution of chemistry as described above, accounting for the purity of the chemistry (a typical stock solution is 80 mM in water or salt buffer), and subsequently prepare serial dilutions in water/buffer as required.

4.3) Add 40  $\mu$ L of each of the dilutions to 960  $\mu$ L of defibrinated rabbit blood to obtain the desired test concentration. Apply membrane film to a feeding unit and seal with a rubber ring. Transfer 1 mL of blood via pipette and attach the feeding unit to a heating unit.

4.4) Gently swab the membrane surface with freshly made 10% lactic acid solution and place the feeding unit into the cage of adult mosquitoes. Cover the cage with a dark cloth or black trash bag and allow mosquitoes to feed for one hour.

4.5) Repeat steps 4.2 – 4.4 for the remainder of test solutions and the negative (blood-only) control.

4.6) On completion of feeding, place buckets at 4 °C in a refrigerator for 5 min to anesthetize mosquitoes. Quickly count and record the total number of female mosquitoes in each bucket. Remove male mosquitoes and non-fed female mosquitoes, and count and record the total number of fed and partially fed mosquitoes by examination of the abdomen.

4.7) Retain partially and fully fed female mosquitoes, aiming for a minimum of 50 blood-fed mosquitoes per dose.

4.7) Transfer buckets to a plastic tub or growth chamber under constant conditions of 28 °C and 75 – 85% RH (on a 12 h day/12 h night cycle is preferable).

4.8) Record the total number of dead/non-responsive mosquitoes at 0.5, 1, 1.5, 2, 24, 48 and 72 h (or alternative time-points as desired) using the score sheet (**Table 4**).

4.9) On the third day post blood feeding, introduce an egg cup into the cage and collect eggs over a 72 h period. Count the total number of eggs produced per treatment under a dissecting microscope.

4.10) Calculate the percent mortality and fecundity as a function of the total number of fed mosquitoes for each treatment group and the control. Correct for control mortality using the modified Abbott's formula if desired.

4.11) Display toxicity and fecundity data as a histogram plot or exponential curve (if performing a dose response assay) using software of choice. If performing a dose response assay, calculate the LD<sub>50</sub>, LD<sub>75</sub> or LD<sub>90</sub> value for the test chemistry relative to the control.

4.12) Repeat the assay at least twice using separate batches of mosquitoes to obtain n = 3 or more biological replicates.

#### REPRESENTATIVE RESULTS:

**Figure 6** shows the percent mortality of L3 *Ae. aegypti* following exposure to five doses of the dopamine receptor antagonist, amitriptyline in comparison to the water only (negative) control at 24, 48 and 72 h. In this example, the data collected at each of the time-points display a typical logarithmic curve and reveal a dose-dependent effect of amitriptyline on larval mortality. The LC<sub>50</sub> value decreases over the time course of the experiment and can be calculated using the 72 h data (*i.e.*, midpoint of the logarithmic curve).

**Figure 7** shows the percent mortality of 3 – 5-day-old adult females *Ae. aegypti* following exposure to a 400 µM dose (10 µg/µL) of the dopamine receptor antagonist, amitriptyline and in comparison to the synthetic pyrethroid, bifenthrin-positive control (technical grade; 500 pg/µL), acetone-only negative control and untreated mosquitoes (blank). In this example, both amitriptyline and bifenthrin cause significant adult mortality relative to the negative control. Note that adult mortality decreases over time, presumably due to metabolic detoxification of test chemistries.

**Figure 8** shows the percent mortality (left y-axis) of 3-5 day old adult female *Ae. aegypti* fed a blood meal treated with one of four doses of the dopamine receptor antagonist, amitriptyline, and the fecundity (right y-axis; average egg count/female) for the first gonotrophic cycle post exposure in comparison to control mosquitoes (blood-fed only; negative control). The data reveal that there is no significant effect of amitriptyline on adult mortality relative to the control but demonstrate that amitriptyline significantly increases fecundity at the highest dose (400 µM).

#### FIGURE AND TABLE LEGENDS:

**Figure 1. Set-up of the larval dose response assay.** Image showing the set-up of the larval dose response assay performed using *Aedes aegypti* L3 larvae in 24-well plate.

**Figure 2. Schematic showing procedure for set up of the (A) single point dose or (B) dose response larval assay in 24-well plate.** Following transfer of five L3 larvae to wells, excess water is gently removed via pipette and replaced with fresh, sterile ddH<sub>2</sub>O. Next, stock and serial dilutions of the test chemistry are prepared and added to a final volume of 1 mL/well.

**Figure 3. Culture of adult mosquitoes.** Image showing 20 L plastic bucket used for culture of adult mosquitoes and use of an aspirator to remove mosquitoes.

**Figure 4. Adult topical assay performed using 4-5 day old female *Aedes aegypti*.** Following treatment with chemistry or solvent, adult mosquitoes are placed in paper cups and transferred to a test chamber for the duration of the assay.

**Figure 5. Stock and serial dilutions.** Schematic diagram showing the procedure for preparation of (A) stock and (B) serial test solutions for the adult topical assay.

**Figure 6. Representative data from a larval contact dose response assay.** Representative data from a larval contact dose response assay showing percent mortality of *Aedes aegypti* L3 larvae following exposure to the dopamine receptor antagonist, amitriptyline at 24, 48 and 72 h. Each data point represents mean in  $\mu\text{M} \pm \text{SEM}$ . Data represent  $n = 3$  biological replicates.

**Figure 7. Representative data from an adult topical dose response assay.** Representative data from an adult topical dose response assay showing toxicity of the dopamine receptor antagonist, amitriptyline ( $10 \mu\text{g}/\mu\text{L}$ ) to 3-5 day old female *Aedes aegypti* relative to the synthetic pyrethroid, bifenthrin ( $500 \text{ pg}/\mu\text{L}$ ) positive control) and negative control (acetone vehicle only) at 2, 24 and 48 h. Data represent  $n = 3$  biological replicates. Error bars indicate SEM.

**Figure 8. Representative data from an adult ingestion assay.** Representative data from an adult ingestion assay showing toxicity of the dopamine receptor antagonist, amitriptyline at  $100 \mu\text{M}$ ,  $200 \mu\text{M}$  and  $400 \mu\text{M}$  dose to 3-5 day old adult female *Aedes aegypti* and impact on fecundity (expressed as the average total egg count per female over the first gonotrophic cycle). Data represent  $n = 3$  biological replicates  $\pm \text{SEM}$ .

**Table 1. Example calculations for *Aedes aegypti* L3 larval (A) single point dose assay and (B) dose response assay.**

**Table 2. Example score sheet used to record data from the mosquito larval dose response assay.** Control, water-only or water with 1% DMSO or other solvent only. Typical doses used to test small molecule antagonist chemistries such as amitriptyline are  $25 \mu\text{M}$ ,  $50 \mu\text{M}$ ,  $100 \mu\text{M}$ ,  $200 \mu\text{M}$  and  $400 \mu\text{M}$ .

**Table 3. Example score sheet used to record data from the adult topical dose response assay.** Typical doses used to test small molecule antagonist chemistries such as amitriptyline are  $12.5 \text{ ng}$ ,  $25 \text{ ng}$ ,  $50 \text{ ng}$  and  $100 \text{ ng}$  chemistry/individual mosquito.

**Table 4. Example score sheet used to record data from the adult blood-feeding assay.** Typical doses used to test small molecule antagonist chemistries such as amitriptyline are  $50 \mu\text{M}$ ,  $100 \mu\text{M}$ ,  $200 \mu\text{M}$  and  $400 \mu\text{M}$ .

## **DISCUSSION:**

Insecticides are powerful tools to combat mosquito-borne diseases such as malaria<sup>25</sup>, dengue and Zika<sup>26</sup>. Extensive insecticide use has produced mosquito populations that are resistant to chemical control, leading to the development of insecticide resistance, which is considered the

single biggest threat to continued disease control. The last decade has seen a dramatic increase in populations of *Anopheles* spp. resistant to synthetic pyrethroids (SPs) used in bed nets in Africa<sup>7</sup>. Populations of *Aedes albopictus*, a vector of dengue and Zika, have been reported in Florida and New Jersey that are resistant to organophosphates<sup>6</sup>. Similarly, resistance to DDT and pyrethroids has been documented in populations of *Anopheles* and *Aedes* spp. in Colombia<sup>27,28</sup>. There is urgent need to develop new larvicides and adulticides that bind to diverse orthosteric or allosteric sites on known molecular targets or that disrupt new targets (*i.e.*, novel MoA chemistries)<sup>29,30</sup> and have minimal environmental impact. There is also increasing recognition of the potential around endectocides for mosquito control<sup>31,32,33</sup>.

To facilitate the rapid development of such products, we describe a series of protocols designed to evaluate the toxicity of small molecule chemistries to mosquito larvae and adults via two delivery routes - contact and ingestion. Protocols such as the WHO susceptibility test and the CDC bottle assay are in common use for evaluating the toxicity and repellency of existing insecticidal formulations<sup>1,2</sup>. However, these assays have limitations for analyses of unformulated novel chemical entities (NCEs) and are not amenable to testing large numbers of chemistries in moderate or high throughput.

Here we describe a standard larval assay to rapidly evaluate NCEs in single point dose that can scale for high throughput analyses. We also describe an adaption of an assay developed by the LITE<sup>21</sup> to evaluate the contact toxicity of small molecule chemistries to adult mosquitoes. Finally, we describe an ingestion assay to evaluate toxicity of NCEs delivered via the blood meal to adult female mosquitoes and explore endectocide potential. These larval and adult assays can be performed at a range of doses in order to determine LC and LD values, respectively, and both larval and adult contact assays can be performed in comparison to one or more existing chemical formulations. The adult topical assay also enables the calculation of LD values per individual mosquito. The protocols can be performed using species of *Aedes*, *Anopheles* and *Culex*<sup>16,29</sup> and could be modified to accommodate the specific biological characteristics of a species, if desired. In our experience, mosquitocidal chemistries typically exhibit broad activity across these three genera<sup>16,29</sup>, but there is value to assessment of species selectivity.

The assays are a starting point to rapidly screen multiple chemistries for insecticidal activity. Those chemistries that exhibit toxicity in one or more assays could be selected for further analyses via secondary and tertiary assays. Examples include the LITE tarsal assay and sugar feeding assays<sup>34,35</sup>. The selection of additional assays is typically determined by considerations around anticipated market and application, with results providing insights to possible product delivery modes. In the representative results shown here, we explore the toxicity of amitriptyline, an antagonist of mammalian and invertebrate dopamine receptors that has been evaluated for potential as a novel MoA insecticide<sup>10,12,13,16</sup>. Amitriptyline exhibited toxicity to larvae and adults of *Ae. aegypti* in the  $\mu$ g range suggesting larvicidal and adulticidal activity, and providing rationale for an exploration of an amitriptyline-based chemical series to identify analogues with high potency. No significant effect on mortality or fecundity was observed in *Ae. aegypti* exposed to amitriptyline in the blood meal. While the stability of the chemistry in defibrinated blood and effective dose received per mosquito is not known, notable limitations of the assay, the data

suggest that amitriptyline and other GPCR-active compounds may have little potential as endectocides, at least in unformulated state.

Precautions to limit biological variation between assays are essential. All assays should be performed under standard conditions of temperature and humidity, preferably in an insect growth chamber, in order to ensure reproducibility. It is imperative to standardize procedures for scoring phenotypic end-points as these can be highly subjective, leading to significant variation in data recorded by different laboratory personnel. The larval assay is best suited to evaluation of chemistries that are soluble in water, although the assay can be performed using organic solvents such as DMSO provided that the final concentration is less than 1% per well. We recognize several limitations to the adult topical assay. First, it is necessary to work rapidly when preparing serial dilutions and delivering doses as the carrier (acetone) is highly volatile and evaporation may produce variation in the amount of chemistry delivered. Second, care should be taken to limit the period of anesthesia as this can contribute to mortality. It should also be noted that solvents such as acetone can produce a rapid “knock-down” effect in the first several hours of the topical assay, which should not be confused with effects due to the test chemistry. We note that the volume of the mosquito blood meal varies between individuals, complicating an assessment of the dose received per adult in the adult ingestion assay. Finally, assay data should not be considered in cases where the mortality in the negative control exceeds 10%.

Products that act synergistically are increasingly recognized for their potential to extend the utility of existing products and provide control of insecticide resistant pest populations<sup>15,16,36</sup>. Larval assays have been used to examine synergism between the formamidine pesticide, amitraz and a variety of chemistries that disrupt invertebrate octopamine receptors<sup>14,15</sup>. The larval assay and adult topical assay described are suitable for evaluating synergistic or additive effects between combinations of test chemistries. We note that synergists such as piperonyl butoxide (PBO) can be incorporated into each of the three assays described here.

The above-described assays provide a standardized experimental series for evaluation of unformulated, small molecule drugs against aquatic and terrestrial mosquito life stages. These assays are specifically designed to evaluate toxicity and offer several advantages over existing assays in common use. Importantly, the assays are amenable to automation and can be performed at industrial scale to enable evaluation of thousands of test compounds. Lastly, as the field considers the development of products that act via new MoAs, including via disruption of novel molecular targets and biochemical pathways, it will be essential to score multiple phenotypic endpoints in addition to those such as mortality and paralysis. The above assays permit such investigations, thus providing a step towards tomorrow’s highly innovative insecticidal technologies.

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## DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

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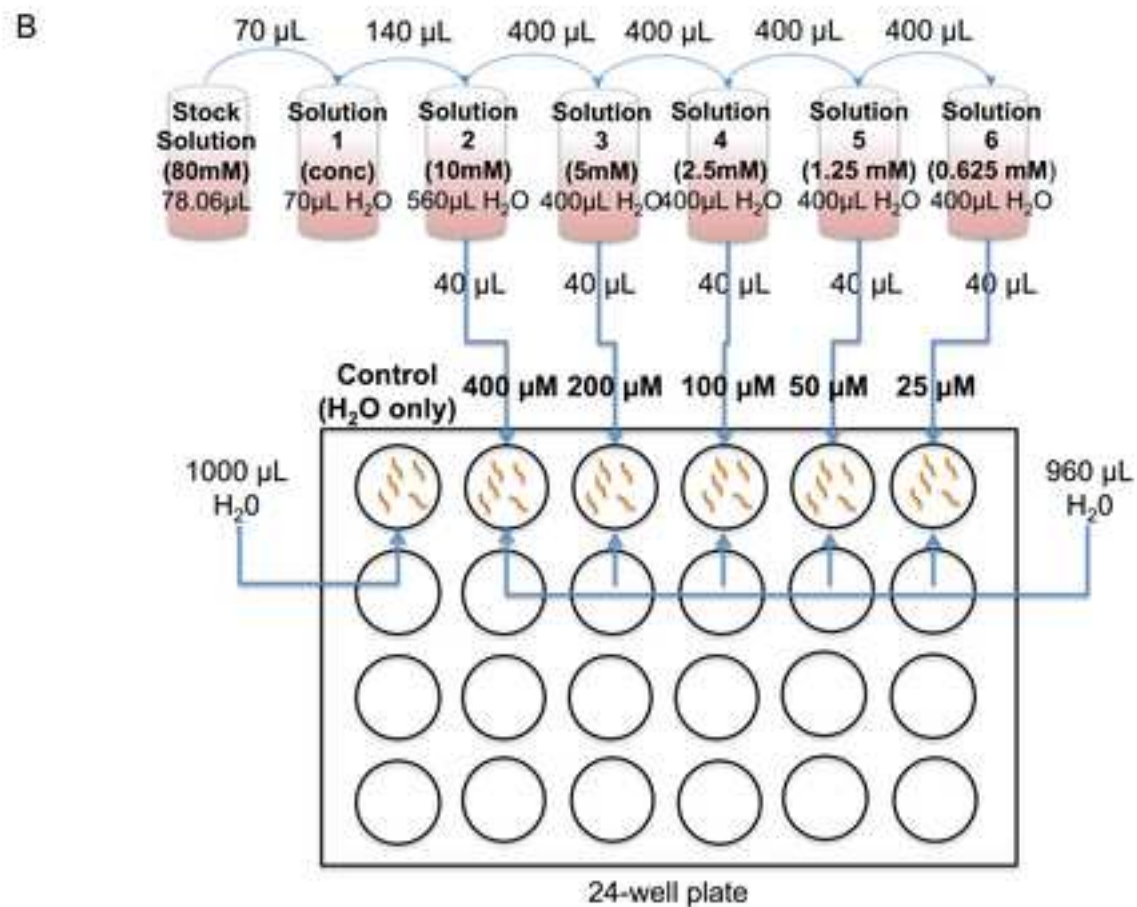
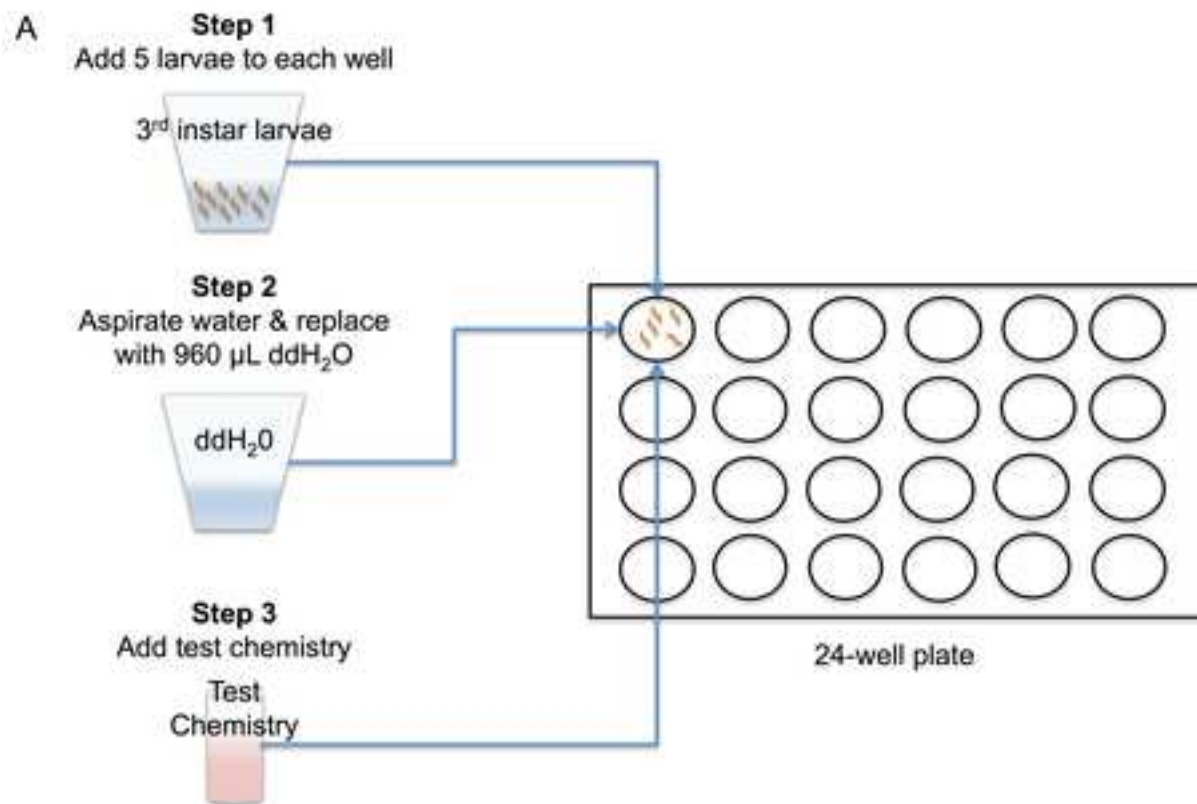


Figure 3

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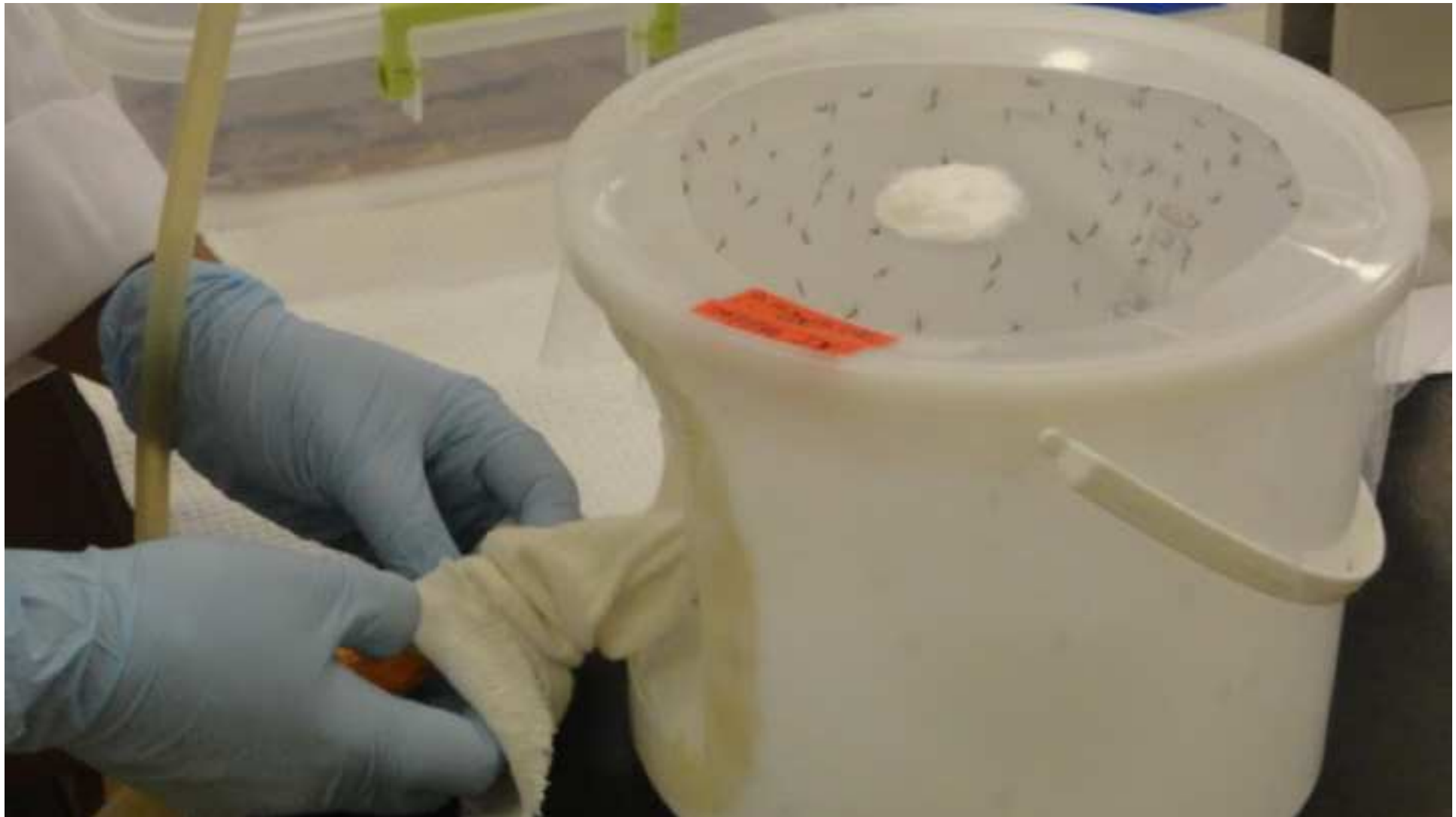


Figure 4

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**A.**

Dilute 40  $\mu\text{L}$  of Solution 1 in 960  $\mu\text{L}$  of acetone



1. **Stock Solution**  
10  $\mu\text{g}/\text{mL}$



2. **Working Stock**  
400  $\text{ng}/\mu\text{L}$

**B.**

**Solution 3**

Dilute 500  $\mu\text{L}$  of  
Solution 2 in 500  $\mu\text{L}$   
of acetone

**Solution 4**

Dilute 500  $\mu\text{L}$  of  
Solution 3 in 500  $\mu\text{L}$   
of acetone

**Solution 5**

Dilute 500  $\mu\text{L}$  of  
Solution 4 in 500  $\mu\text{L}$   
of acetone

**Solution 6**

Dilute 500  $\mu\text{L}$  of  
Solution 5 in 500  $\mu\text{L}$   
of acetone

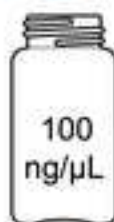


200  
 $\text{ng}/\mu\text{L}$

0.25  $\mu\text{L}$



50  $\text{ng}/\text{mosquito}$

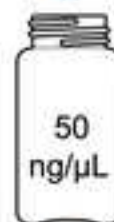


100  
 $\text{ng}/\mu\text{L}$

0.25  $\mu\text{L}$



25  $\text{ng}/\text{mosquito}$

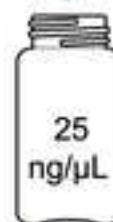


50  
 $\text{ng}/\mu\text{L}$

0.25  $\mu\text{L}$



12.5  $\text{ng}/\text{mosquito}$



25  
 $\text{ng}/\mu\text{L}$

0.25  $\mu\text{L}$



6.25  $\text{ng}/\text{mosquito}$

Figure 6

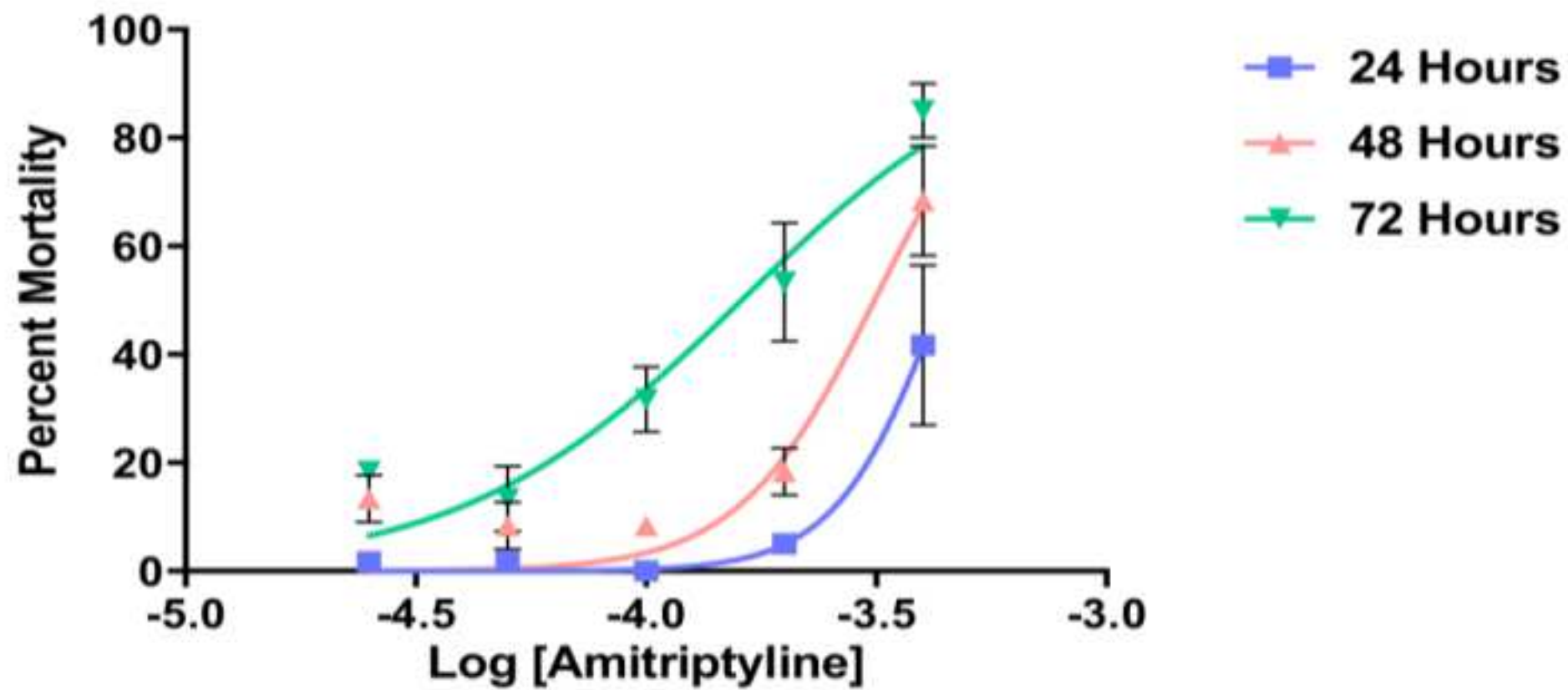


Figure 7

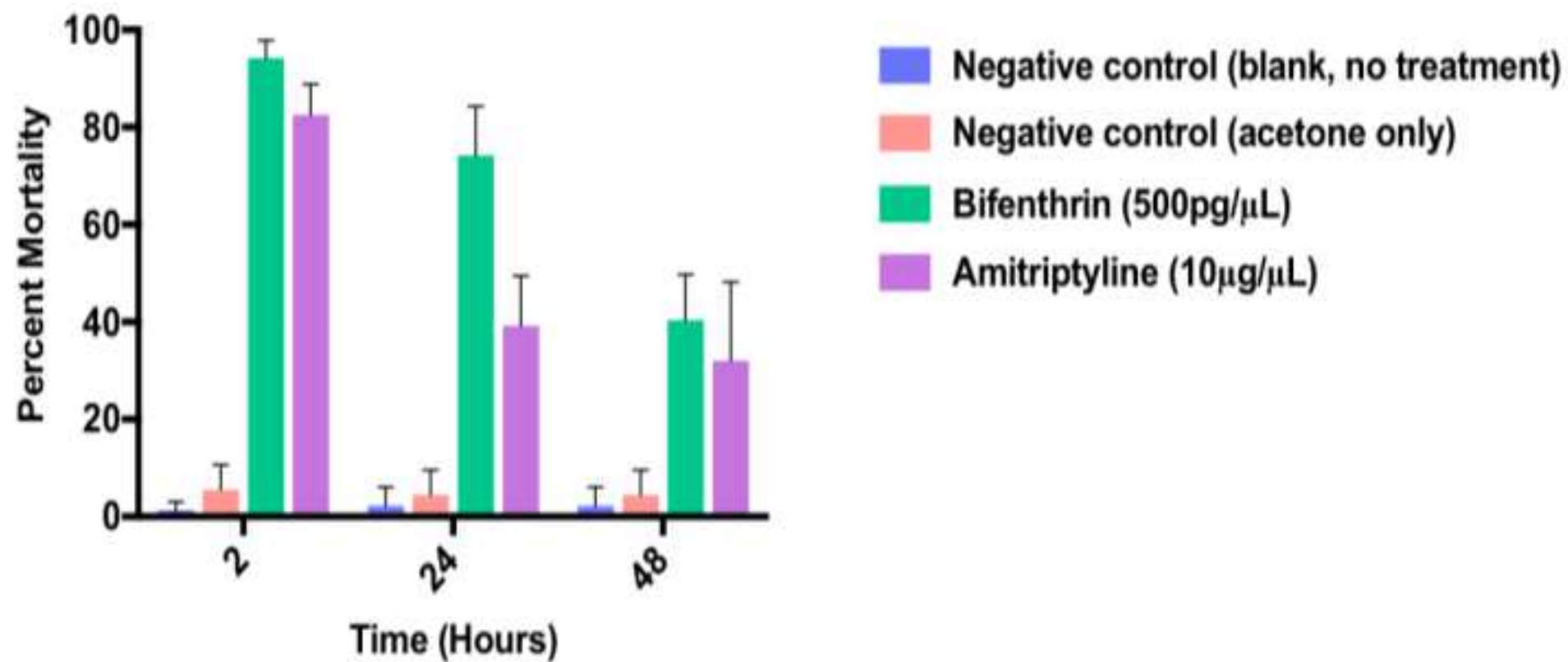
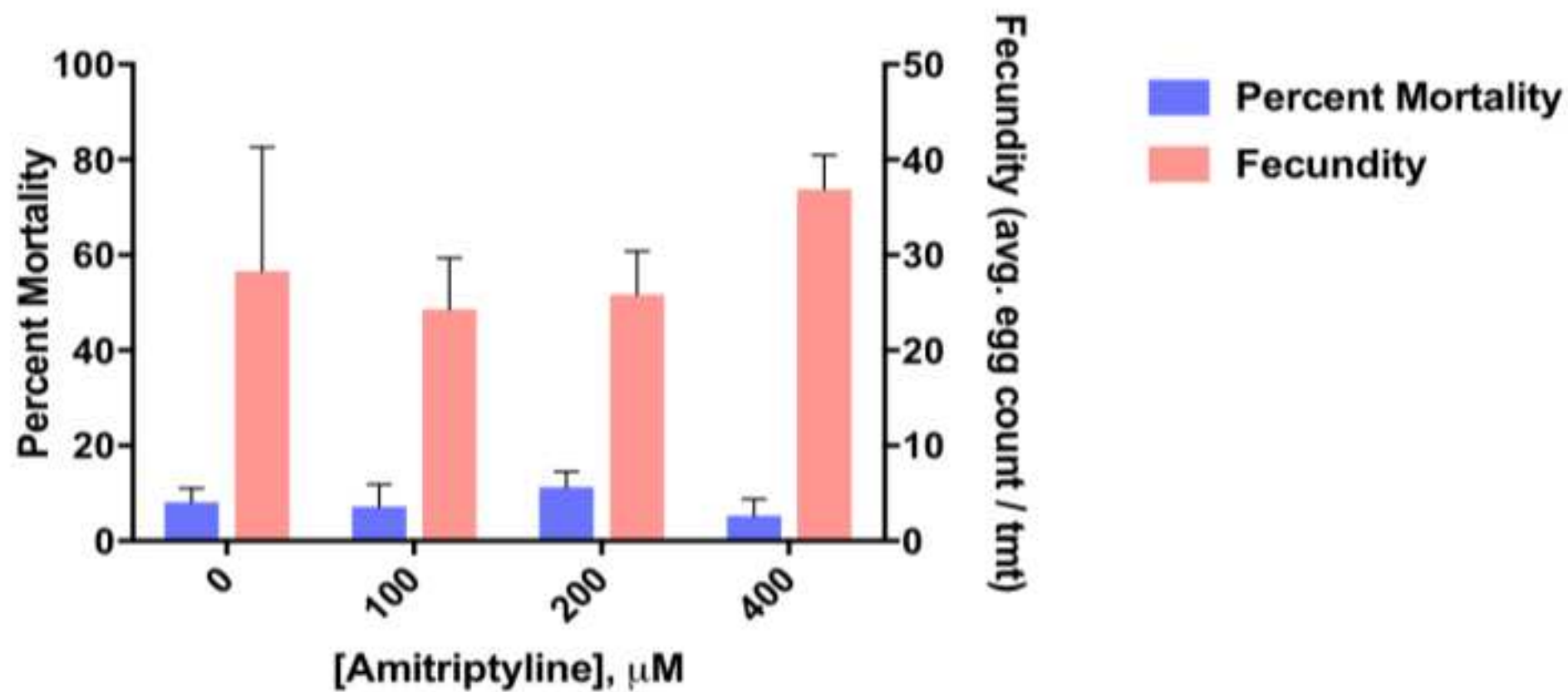


Figure 8



A. Single point dose assays with three chemistries					
Chemistry	Concentration (400 uM)				
Amitriptyline	<b>80 mM Stock:</b> 2 mg of amitriptyline X 98% (purity of powder) X 0.001 g/1 mg X 1 mol /313.86 g X 1 L/0.08 mol X 10 <sup>6</sup> μL/L = 78.06 μL of water. <b>Serial Dilutions:</b> Dilute stock 1:2 with ddH <sub>2</sub> O and then 1:4 to achieve 10 mM. Add 40 μL of 10 mM stock to well containing 960 μL of ddH <sub>2</sub> O to achieve a final concentration of 400 μM/well. Prepare serial dilutions of 10 mM stock 1:2 to achieve 5 mM, 2.5 mM, 1.25 mM and 0.625 mM stocks.				
Cis-(z)-flupenthixol	<b>80 mM Stock:</b> 2 mg of cis(z)flupenthixol X 98% (purity of powder) X 0.001 g/1 mg X 1 mol/507.44 g cis(z)flupenthixol X 1 L/0.08 mol X 10 <sup>6</sup> μL/L = 48.28 μL of water. <b>Serial Dilutions:</b> As above.				
Amitraz	<b>80 mM Stock:</b> 2 mg of Amitraz X 0.001 g/1 mg X 1 mol/293.4 g of amitraz X 1 L/0.08 mol X 10 <sup>6</sup> μL/1 L = 85.21 μL of DMSO (note: Amitraz is only soluble in DMSO). <b>Serial Dilutions:</b> As above.				
B. Dose response assay					
Chemistry	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5
	400 μM	200 μM	100 μM	50 μM	25 μM
e.g., Amitriptyline, cis-(Z)-flupenthixol or amitraz	40 μL	40 μL	40 μL	40 μL	40 μL
	10 mM stock	5 mM stock	2.5 mM stock	1.25 mM stock	0.625 mM Stock

<b>Mosquito L3 Larval Assay</b>						
<b>Date initiated:</b>						
<b>Investigator:</b>						
<b>Species/Strain:</b>						
<b>Assay Type:</b> Single point dose/dose response						
<b>A. Mortality (No. dead mosquitoes)</b>						
<b>Time (hours)</b>	<b>Dose 1</b>	<b>Dose 2</b>	<b>Dose 3</b>	<b>Dose 4</b>	<b>Dose 5</b>	<b>Control</b>
0.5						
1						
1.5						
2						
2.5						
3						
12						
24						
48						
72						
<b>B. Percent Mortality</b>						
<b>Time (hours)</b>	<b>Dose 1</b>	<b>Dose 2</b>	<b>Dose 1</b>	<b>Dose 4</b>	<b>Dose 1</b>	<b>Control</b>
0.5						
1						
1.5						
2						
2.5						
3						
12						
24						
48						
72						

Mosquito Adult (3-5 day old) Topical Assay						
Date initiated:						
Investigator:						
Species/Strain:						
Assay Type: Single point dose/dose response						
A. Mortality (No. dead mosquitoes)						
Time (hours)	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Control
0.5						
1						
1.5						
2						
2.5						
B. Percent Mortality						
Time (hours)	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Control
0.5						
1						
1.5						
2						
2.5						

<b>Mosquito Adult (3-5 day old) Ingestion Assay</b>						
<b>Date initiated:</b>						
<b>Investigator:</b>						
<b>Species/Strain:</b>						
<b>Assay Type:</b> Single point dose/dose response						
<b>A. Number Fed Mosquitoes</b>						
	<b>Dose 1</b>	<b>Dose 2</b>	<b>Dose 3</b>	<b>Dose 4</b>	<b>Dose 5</b>	<b>Control</b>
<b>Fed</b>						
<b>Not Fed</b>						
<b>Total</b>						
<b>% Fed</b>						
<b>% Not Fed</b>						
<b>B. Mortality (No. dead mosquitoes)</b>						
<b>Time (hours)</b>	<b>Dose 1</b>	<b>Dose 2</b>	<b>Dose 3</b>	<b>Dose 4</b>	<b>Dose 5</b>	<b>Control</b>
<b>2</b>						
<b>24</b>						
<b>48</b>						
<b>72</b>						
<b>C. Percent Mortality</b>						
<b>Time (hours)</b>	<b>Dose 1</b>	<b>Dose 2</b>	<b>Dose 3</b>	<b>Dose 4</b>	<b>Dose 5</b>	<b>Control</b>
<b>2</b>						
<b>24</b>						
<b>48</b>						
<b>72</b>						

Name of Material/ Equipment	Company	Catalog Number
Insecticide susceptible mosquito strains	Malaria Research and Reagent Reference Repositor	
Acetone	Mallinckrodt Chemical	CAS 67-64-1
Amitraz	Sigma-Aldrich	CAS 33089-61-1
Amitriptyline	Sigma-Aldrich	CAS: 549-18-8
Bifenthrin	Sigma-Aldrich	CAS: 51529-01-2
Cis-(Z)-flupenthixol	Sigma-Aldrich	CAS: 51529-01-2
Defibrinated rabbit blood	Hemostat	DRB0100
Dimethylsulphoxide (DMSO)	Sigma-Aldrich	MKBF2985
Hemotek membrane feeding system	Hemotek Ltd	Serial no. 1303
Micro-applicator	Burkard Manufacturing Co.	-
24-well cell culture plate with lid	Corning Incorporated	3526
Advantage rubber bands	Alliance Rubber Co.	-
Glass syringe (1 ml)	Becton Dickinson and Co.	512004
Disposable scintillation vials (20 ml)	Fisher Scientific	74505-20
Tulle fabric, white	Walmart	-
Paper cups	Dixie Consumer Products LLC.	PF15675/13D
Petri dishes (150 mm)	Corning Life Sciences	-
Transfer pipettes	Fisher Scientific	13-711-7M
Stereo microscope	Olympus	SZ6045
Tweezers (fine)	Fontax	-

### Comments/Description

<https://www.beiresources.org/MR4Home.aspx>; Recommended strains: *Aedes aegypti* Liverpool (LVP) strain, *Anopheles gambiae* Kisumu (KISUMU1) s

Use for dilutions and control

Requires dilution in DMSO

Can be diluted in acetone

Synthetic pyrethroid used as positive control

Pharmacologically tested as disruptor of dop-like receptors

Blood source for adapted mosquitoes to artificial feeding

Organic solvent used to dissolve amitraz

Used for feeding mosquitoes

Tool needed for topical application experiments

-

Used to seal the paper cups

Needed for the micro-applications. Glass is better than plastic

Glass vials prevent evaporation

-

Used to keep adult mosquitoes in adult topical assays

Used to maintain the mosquitoes "slept" on cold without direct contact with ice

Used to sort larvae

Used to score larval assays and perform micro-applications

Used to handle adult mosquitoes

[train and Culex quinquefasciatus Johannesburg \(JHB\) strain](#)



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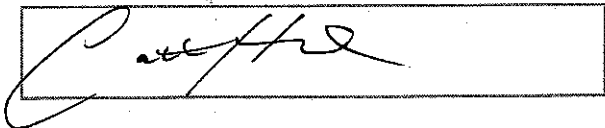
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**JoVE57768 Response to Reviewer Comments****“Protocols for testing the toxicity of novel insecticidal chemistries to mosquitoes”****Brito-Sierra et al.,**

We thank the JoVE Scientific Editor and reviewers for insightful reviews and helpful comments regarding the above submission. Overall, the reviewers had a favorable response, stating *“this experimental approach...is a very valuable one, and represents a sensible way to streamline the process of identifying potential new active ingredients against mosquitoes, for which there is an increasingly desperate need”*, *“It is a very good manuscript providing very clear protocols for testing novel insecticide leads against mosquitoes”*, and *“the manuscript and protocols are very well written and clearly presented and the paper represents a very useful addition for scientists working in the field”*. Both reviewers recommended a number of changes that have been addressed in the revised manuscript. Here we outline the revisions we have made to the manuscript and our point-by-point response to reviewer comments. We believe that these changes have resulted in a significantly improved manuscript and hope that our submission is now acceptable for publication in the JoVE.

**Changes recommended by the JoVE Scientific Editor**

Protocol detail.

*We believe that sufficient detail is provided to answer the question “how is the step performed” for each and every step outlined in the protocol. Where this detail is not described in the protocol, the reader is directed to published works as per JoVE format.*

Protocol highlight.

*Our protocol is within the ten-page limit and we have highlighted 2.5 pages of “filmable” text that provide a cohesive narrative and logical flow. Notes and non-filmable materials are not highlighted/recommended for filming.*

Discussion.

*The Discussion includes points 1-5 (modifications and trouble shooting, limitations, significance with respect to existing methods, future applications, and critical steps) as requested.*

Figures and Tables.

*Figures and Tables are original, referenced and called out in order of appearance.*

References.

*References comply with JoVE standards.*

Commercial language.

*Reference to commercial products has been removed except in the case of the Hemotek unit as the protocol is specifically designed to incorporate this device.*

Abbreviations.

*These are defined at first use.*

Units.

*Standard abbreviations and symbols are used.*

## **Reviewer Comments**

### **Reviewer #1:**

#### Major Concerns:

The three bioassays selected by the authors give a good indication of activity of a compound against *Aedes* and *Culex* mosquitoes which would help to identify active ingredients which may be worth developing into vector control products. However, in the case of *Anopheles* I feel a test for tarsal contact efficacy is missing, and since this is the main way in which *Anopheles* mosquitoes encounter insecticides this is a limitation of the testing pathway. Compounds may not be active on cuticular contact or even on ingestion where they would be on tarsal contact, and so useful compounds may be missed. I feel this limitation should be addressed in the Discussion at least, or ideally with the addition of a tarsal contact assay in the manuscript and video, but otherwise this is a strong manuscript presenting a useful methodology, and recommend publication.

*The reviewer raises an important point. However, the goal of the protocols is to provide a preliminary series of assays and initial insights to topical toxicity. We note that there are many secondary and tertiary assays that could be performed depending on assay results, including the tarsal assays mentioned by the reviewer. We also note that the tarsal assay has been developed by the LITE to investigate chemistries for development as indoor residual sprays and incorporation into bednets effective against *Anopheles* mosquitoes, and is available on request. We are reluctant to reproduce an assay developed by others and instead refer the reader to the LITE site for further information. Lastly, our goal was development of protocols to broadly assess activity across genera, rather than products specific for *Anopheles*. To provide clarity for the reader, we have addressed this point in the revised manuscript.*

While it is a good idea to use a worked example, G protein-coupled receptors, to illustrate the methodology and produce example results, the way that this is presented is not very clear, and at some points this causes confusion. For example, are the concentrations mentioned in the methodologies specific to the receptors or suggested as good starting points for bioassays on all compounds? The results presented also seem to be slightly unusual, with mortality appearing to decrease over time after topical application (which should be addressed in the manuscript, maybe the axis should be relabelled KD/mortality?), and a lack of dose response apparent in the blood feeding assay. I would suggest two possible approaches to improve clarity. A) keeping this example to illustrate the testing pathway, but more explicitly going through the results and the decision making regarding its potential as an insecticide against mosquitoes, with a consideration of what the best mode of delivery might be. B) presenting more illustrative example data sets for each assay which show the results which one would more usually expect to see, different clear examples for each bioassay rather than one compound tested with each methodology.

*Our experience with multiple classes of small molecule chemistries suggests (see publications 10, 12, 13, 16 and 29) that the concentrations recommended provide an appropriate starting point for any new chemistry, including GPCR-targeting compounds. We believe this point is addressed in the manuscript. The reduction in mortality over time is common with many chemistries we have evaluated and reflects (a) the phenotypic endpoint selected (paralyzed insects are scored, but may recover over the assay period), and (b) possible metabolism of chemistry and subsequent recovery of the mosquito. This point is acknowledged in the representative data section. At the suggestion of the reviewer, we have re-labeled the axes. However, we note that "knockdown" is a term applied to a phenomenon observed with SPs and DDT and believe it would be misleading to convey that the bounce back effect is related to this phenomenon when there is no evidence that this is the case (except potentially in the case of bifenthrin). The issue of dose received in the blood feeding*

*assay is addressed in the revised Discussion. We have elected to retain the example provided. At the recommendation of the reviewer, we have added text to the Discussion to help guide the reader through typical decision-making steps and subsequent considerations of delivery mode.*

#### Minor Concerns:

The Abstract could be rewritten to give the same information in a clearer, more streamlined way by removing some repetition, and the structure of the manuscript of presenting a methodology and then an example analysis of one potential compound as a demonstration of the pathway would be more clearly explained.

*Addressed. The Long Abstract has been revised.*

The testing pathway is proposed as effective for *Aedes*, *Anopheles* and *Culex* mosquitoes, three very different genera of mosquitoes, yet this is not addressed. The methods could be made more effective if tailored to the genera, for example performing the blood feeding assay on *Anopheles* in the afternoon to maximise the feeding rate, or giving an indication of whether different results might be expected from a given assay.

*Addressed in the revised Discussion.*

The authors note that to obtain ~200 adults for a bioassay, 7 trays of 400 larvae need to be reared, which seems to suggest extremely inefficient rearing, lack of synchronous emergence, or high mortality. If this statement is kept in it would be better explained, but might be better removed as this will very much differ between facilities depending on rearing methods.

*We apologize - this sentence was included in error and has been removed from the submission.*

Why is the rearing temperature 28 degrees C but the testing temperature 25 degrees?

*The temperatures reflect standards used for (a) rearing of mosquitoes (typically 27-28°C for the species in question) and (b) recommended conditions for larval toxicity assays (typically performed at 25°C in a test chamber) and adult assays (typically performed at the same temperature as rearing). These are conditions widely adopted across the field. This point has been addressed in the revised protocol. We note that toxicity assays could be performed at a range of temperatures to evaluate effect on performance of chemistries.*

The blood feeding assay calculates doses based on the concentration of active ingredient in the hemotek reservoir, available to the mosquito, without attempting to quantify the uptake by an individual female, which would be informative. It is also not clear from the text, but should be from the video, that different concentrations of stock solutions should be made up, but the same volume of each stock added to each hemotek reservoir to obtain different doses. Information on how to determine whether females have blood fed, and what to do about those that partially feed, would be helpful, and may differ between genera.

*The reviewer makes valid points regarding uptake of chemistry (a limitation of feeding assays now acknowledged in the revised Discussion), different concentrations of stock (now addressed at 4.3) and confirmation of blood feeding (addressed at 4.6).*

In the topical application methodology, the removal of individual females from the petri dish for application seems to add an unnecessary manipulation of the mosquitoes and operational step. I would suggest applying the insecticide to each mosquito directly, while they lie on the petri dish.

*Unfortunately the micro-applicator requires use with a microscope to confirm delivery of small volumes of chemistry to the anesthetized mosquito. This equipment is static. While we appreciate the recommendation of*

*the reviewer and the potential advantages of this approach, working in the petri dish is not possible given the current assay design. However, we note that the assay could be modified for purpose by investigators.*

The mention of synergists does not fit the manuscript - synergism is an important issue, but without mention in the Introduction, or inclusion in the methods, it is not clear why it is suddenly mentioned in the Discussion. Certainly, it would be useful to describe how synergism can be investigated using the three methodologies proposed.

*We respectfully disagree with the reviewer on this point. Synergists are widely used in combination with insecticides, and of increasing interest to a pest control industry exploring options to extend the utility of existing product formulations. JoVE requires that manuscripts point to additional/future applications of the protocol (see comments from the Scientific Editor above). Our reference to synergists was an attempt to address this point. The three assays described are amenable to inclusion and evaluation of synergists such as PBO.*

Is the maximum concentration of solvent in the larval assay 10% as mentioned in the methodology, or 2% as mentioned in the discussion?

*We thank the reviewer for identifying this discrepancy. This has been corrected in the revised Protocol and Discussion.*

I could not find the protocol for topical application on the LSTM website (reference 21).

*We apologize – the protocol has been removed from the site. SOPs are available on request from LITE. We have revised the link, and direct readers to the LITE website.*

## **Reviewer #2:**

### **Major Concerns:**

IN CASE there is some time and resources to also add/include a similar feeding assay for sugar baits, thatr would be great as would further strengthen the paper, which could then stand a more comprehensive "gold standard". But if this is not applicable for addition in this manuscript, it is also fine - it is a suggestion/ recommendation for improvemen, but not condition for acceptance.

*We thank the reviewer for their suggestion regarding additional assays. We note that there are multiple secondary and tertiary assays, including tarsal and sugar bait assays that could be explored following the primary assays described here. Unfortunately these are beyond the scope of the current submission - focused as stated on a range of initial assays to determine toxicity in moderate/high throughput. We are considering a complementary publication with the JoVE to showcase secondary and tertiary assays for multiple applications.*

### **Minor Concerns:**

Lines 86-87, please check, goal of CDC assays the evaluation of repellency, is that correct?

*Addressed.*