**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.*