IMPACT OF INSECTICIDE EXPOSURE METHOD ON SUSCEPTIBILITY/RESISTANCE IN *AEDES ALBOPICTUS* MOSQUITOES

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Abstract

Insecticide resistance is a concern of mosquito control programs (MCPs) whose primary function is to protect public health. Mosquitoes can develop resistance over time when exposed to sublethal doses of insecticide active ingredients (AIs). Resistance to AIs renders them ineffective as a preventive measure for the risk of mosquito-borne diseases. Mosquito exposure to insecticides during ultra-low volume (ULV) application occurs via direct liquid contact (formulated product [FP]), while barrier applications expose mosquitoes to dried residual FP. The Centers for Disease Control and Prevention (CDC) bottle bioassay (based on contact with dried residual insecticide AI) may not directly relate to operational interventions for ULV applications. Hence, the current pilot study assesses how topical/direct versus residual insecticide exposure impacts mosquito susceptibility/resistance to pyrethroid (permethrin) and organophosphate (malathion) AIs. Female *Ae. albopictus* (4–5-d old) were aspirated from a colony cage and anesthetized with cold. Mosquitoes were either treated topically with 1 µL of each AI (stocks made in acetone) or transferred to bottles containing 1 mL of residual AI as used in CDC bottle bioassays (400 µg/mL malathion; 8 µg/mL permethrin for topical and residual treatments). Control groups were treated with acetone instead of AIs (following topical and residual exposure methods). Immediately after topical exposure and 10-15 min after residual exposure, each group was transferred to separate 0.5 L cardboard cages (7 mosquitoes/cage; 2 replicate cages/group). Mosquitoes were provided 20% sucrose and placed in a 28°C incubator with 14 h light:10 h dark. Mortality was monitored/recorded for all groups 1 h, 2 h, and 24 h post-exposure. Topical exposure to malathion (50, 83, 100% mortality at 1, 2, 24 h post-exposure) showed higher mosquito mortality compared to residual exposure (0, 36, 36% mortality 1, 2, 24 h post-exposure). Both exposure methods showed high mosquito mortality for permethrin (topical: 69, 100, 100% mortality 1, 2, 24 h; residual: 71, 100, 100% mortality 1, 2, 24 h). No mortality was observed in control groups. Investigators plan to conduct a larger scale experiment using a field-collected *Ae. albopictus* population.

Introduction

Mosquito control is essential for protecting public health from vector-borne diseases. Female mosquitoes blood feed and use the protein to help develop eggs. The saliva transferred from mosquitoes to vertebrate hosts during blood feeding can contain pathogens that cause diseases like Zika, West Nile, malaria, and dengue fever (Centers for Disease Control and Prevention [CDC] 2020).

Mosquito control programs (MCPs) can serve communities by evaluating and ensuring the most effective control methods are used (Stoops et al. 2019) and educating the public about mosquito bite prevention. Of the *ca.* 200 known mosquito species in the United States (US), only 12 have been implicated in human pathogen transmission (CDC 2020). *Aedes*, *Culex,* and *Anopheles* species are the top mosquito genera classified as potential vectors for human and animal diseases in the US (CDC 2020). *Aedes* spp. have been linked to human diseases like

chikungunya, dengue, lymphatic filariasis, Rift Valley fever, yellow fever, and Zika (WHO 2020). *Aedes* spp. are generally more active during daytime hours when humans are more likely to be outside, increasing the chance of mosquito-human contact. Knowledge of mosquito biology and effective control methods can help countries decrease the number of arboviral disease cases (Brito-Sierra et al. 2019).

While insecticides can help control mosquitoes, thereby lowering the rate of vector-borne disease cases, insecticide resistance (IR) has become an increasing global issue. Mosquitoes can develop resistance over a period when they are exposed to sublethal doses of insecticide active ingredients (AIs) (Richards et al. 2018). Insecticide resistance is currently one of the top concerns of MCPs as the number of AIs are limited and the development of new chemistries are needed. When MCPs have updated, reliable information on IR in local mosquito populations, they can more effectively protect the health of the communities they serve. Some mosquito populations are more resistant than others (depending on biological, environmental, and other factors) and this trait can be passed along to future generations (Barbosa 2018). Resistance to AIs can render insecticides ineffective as a preventive and/or control measures for the risk of vector-borne diseases. The top insecticide classes used by MCPs are organophosphates (e.g., malathion) and pyrethroids (e.g., permethrin). These AIs work by disrupting mosquitoes' nervous systems, causing mortality (CDC 2020).

Different exposure methods (residual versus topical) may impact the extent to which mosquitoes react to insecticides (Waits et al. 2017). MCPs routinely use ultra-low volume (ULV) insecticides (truck-mounted machines that apply drifting droplets, targeting flying mosquitoes) to apply insecticide formulated products (FP). Formulated products contain AIs plus other ingredients (e.g., synergists) that may improve effectiveness. ULV applications are generally carried out at dusk/dawn periods due to temperature inversion dynamics (Enz et al. 2014), hence are generally ineffective for day-active mosquitoes (such as *Ae. albopictus*). Residual treatments (e.g., barrier insecticide treatments that directly soak foliage, leaving long-term [3 weeks] residual insecticide) are also commonly used and can control mosquitoes resting on foliage. The difference between mortality efficacy between topical (like ULV treatments) and residual (like barrier treatments) application methods has not been extensively researched. In the laboratory, topical applications directly expose the mosquito's body to a specific volume and dose of insecticide (Alridge et al. 2016). The same study concluded mortality rate did not differ significantly when both insecticides (permethrin and malathion) were applied to eye, abdomen, or mesothorax body parts but mortality was lower when applied to appendages (i.e., leg and wing) of adult *Culex quinquefasciatus* (Alridge et al. 2016). In residual exposures, a mosquito's entire body has the potential to encounter the AI on foliage. Another study tested *Ae. albopictus* adults and larvae resistance to permethrin, bifenthrin, and malathion using both CDC bottle bioassays and topical toxicology assays (Waits et al. 2017). When mosquitoes were exposed in a topical assay, a significant difference in susceptibility between adult and larvae *Aedes albopictus* to the permethrin AI was observed compared to residual exposure via CDC bottle bioassay which had no significant differences in insecticide susceptible laboratory strains (Waits et al. 2017). The topical assay performed better in terms of adult and larvae *Aedes albopictus* had lower levels of resistance to permethrin AI. According to the study, the application method

versus the toxicity of the tested AI (i.e., permethrin) affected levels of resistance (whether considered low, high, none) in the mosquitoes. Mortality that was 80% or higher after reaching diagnostic time (DT) and diagnostic dose (DD) for each AI used were considered resistant (Waits et al. 2017). The total mortality at end of DT determines how the results are read (i.e. low resistance or high resistance). A topical application allows a specific dose/droplet to be applied to the mosquito body by investigators and remain for the duration of the assay period. In contrast, dosage touching mosquitoes in residual application varies depending on the activity/behavior of the mosquitoes and their contact with the coated surface for the duration of the assay period (Waits et al. 2017). The same study discussed that the topical application method was preferable to the residual method because of how long the AIs remained on mosquitoes' bodies after initial contact in field environments. It was concluded the differences of mortality rates in both methods were "numerically significant but relatively small" and both methods should be utilized to further identify resistance in mosquito populations (Waits et al. 2017).

Mosquito exposure to insecticides during ULV application occurs via direct liquid contact of flying mosquitoes with FP, while barrier applications expose mosquitoes to dried residual FP on foliage and other surfaces (for up to three weeks post-application). The CDC bottle bioassay (contact with residual AI, as in barrier application) may not directly relate to operational interventions for ULV applications. Hence further research on application methods is needed to improve mosquito control assessments. Efficacy testing of the susceptibility/resistance of commonly used AIs will improve risk assessments and inform operational decisions for MCPs (Richards et al. 2018). MCPs should regularly test IR to ensure effectiveness of control efforts (Berg et al. 2021). This could be reported in regional and/or national databases such that trends in resistance for certain AIs could be tracked; however, caution is advised in using regional/national data to inform local control decisions. Efforts have been made to track worldwide resistance, the Worldwide Insecticide Resistance Network (WIN) (Moyes et al. 2017).

Typical assessments for AIs (e.g., CDC bottle bioassay) use dose-response methods, testing if AI dosage affects mosquito mortality. CDC bottle bioassays are used to evaluate lethal concentration (LC) and lethal time (LT) of AIs compared to a "standard diagnostic dose of a commercial insecticide" (Brito-Sierra et al. 2019). Assessment of AIs are a starting point to assess for dosage comparison and exposure methods for insecticide effectiveness. It is important to consider that FPs contain ingredients in addition to AIs to extend shelf life and increase effectiveness of the AI (e.g., synergists). A study that described protocols to evaluate susceptibility tests concluded adult topical assays were suitable for "evaluating synergistic" effects of FP (Brito-Sierra et al. 2019). Their results for the effectiveness of multiple assays such as: larval contact dose response assay, adult topical dose response assay, and adult ingestion assay are meant to provide insight to "product delivery modes" for further insecticide evaluation (Brito-Sierra et al. 2019). Resistance status of local mosquito populations to FP and stock solutions of AIs should be tested regularly to improve mosquito control efficacy.

The current study aimed to assess the extent to which topical (direct) versus residual (indirect) insecticide exposure impacts mosquito susceptibility/resistance to pyrethroid (permethrin) and organophosphate (malathion) AIs in *Ae. albopictus*.

Materials and Methods

Mosquito rearing. Mosquitoes were from an existing *Ae. albopictus* colony originating from Louisiana (generation F-43) and propagated in the lab using established methods (Richards et al. 2017). Eggs were placed into a pan filled with tap water. A 2:1 mixture of yeast and liver powder was fed to the larvae (Richards et al. 2017). Pupae were raised to adulthood and adults were fed 20% sucrose solution *ad libitum* and housed in an incubator at 28°C (14h light: 10 h dark).

Adapted CDC Bottle bioassay. Twenty-four hours prior to experiments, 500 mL glass Wheaton bottles were coated with 1 mL of stock solutions of permethrin and malathion (400 µg/mL malathion; 8 µg/mL permethrin) or 1 ml acetone (control), rolled, dried, and stored using established methods (Richards et al. 2018). The AI stocks (permethrin and malathion) were produced by mixing acetone (Richards et al. 2018) with technical grade AIs (ChemService - West Chester, PA). Female *Ae. albopictus* (4-5 d old) were aspirated from colony cages via mechanical aspirator. Approximately 80-85 female mosquitoes were anesthetized with cold (i.e., placed onto a metal pan on top of an ice bath). Approximately 7 female mosquitoes (one group at a time) were transferred to labeled control or treatment bottles using forceps on a hind leg. Two replicate bottles were labeled for each AI (i.e., malathion, permethrin) and control (i.e., acetone). Cardboard cages (0.5L) were labeled by AI and exposure type and adapted for mosquitoes using mesh screening on lids (Sullivan et al. 2019). After mosquitoes were placed into their respective "exposure" bottle (*ca.* 5 min), they were transferred to clean cardboard cages and (room temperature *ca*. 30-45 min post-exposure) held in an incubator set at 28°C with 20% sucrose solution *ad libitum*. Mortality rate was noted at 1 h, 2 h, and 24 h post-exposure. Each AI-specific assay consisted of 2 replicates with 7 mosquitoes/cage. Mosquitoes were provided 20% sucrose solution *ad. libitum*.

Topical application assay. The same colony of *Ae. albopictus* was used for the topical assay and anesthetized with cold using the same methods as the bottle bioassay group. Once anesthetized, approximately 21 female mosquitoes were placed in a labeled petri dish in the ice bath. Using a micropipette, 1 µL of each AI (i.e., 400 µg/mL malathion; 8 µg/mL permethrin), or acetone (i.e., control) was placed on the thorax of each mosquito (14 total for each group) before being placed into clean 0.5 L cardboard cages labeled by group and replicate. Approximately, 30-45 min post-exposure, mosquitoes were transferred to an incubator at 28°C. Mortality rate was noted at 1 h, 2 h, and 24 h post-exposure. Each AI-specific assay consisted of 2 replicates with 7 mosquitoes/cage. Mosquitoes were provided 20% sucrose solution *ad. libitum*.

Data analysis. Mortality rates were tabulated and bar graphs created to visualize results. Fisher's tests ($P < 0.05$) were used to determine any significant differences in mortality rates between AIs and control and any differences between treatment methods (topical compared to bottle exposure) (SAS Institute, Cary, NC). Mortality that reached 80% or higher by DT of 2 h at

each DD (400 µg/mL malathion; 8 µg/mL permethrin for topical and residual treatments) were considered susceptible.

Results

Mortality data for each AI (permethrin and malathion) and exposure method (topical and residual) are presented in Table 1 and Table 2. The number of mosquitoes dead at each time point was recorded and mortality percentage was calculated. No significant differences ($P > 0.05$) were observed in mortality rates between permethrin and malathion exposed mosquitoes at any time point in the topical exposure group (Table 1, Figure 1). A 100% mortality rate was calculated at 24 h time mark for all treatment groups. No mortality was observed in control groups in either residual or topical groups.

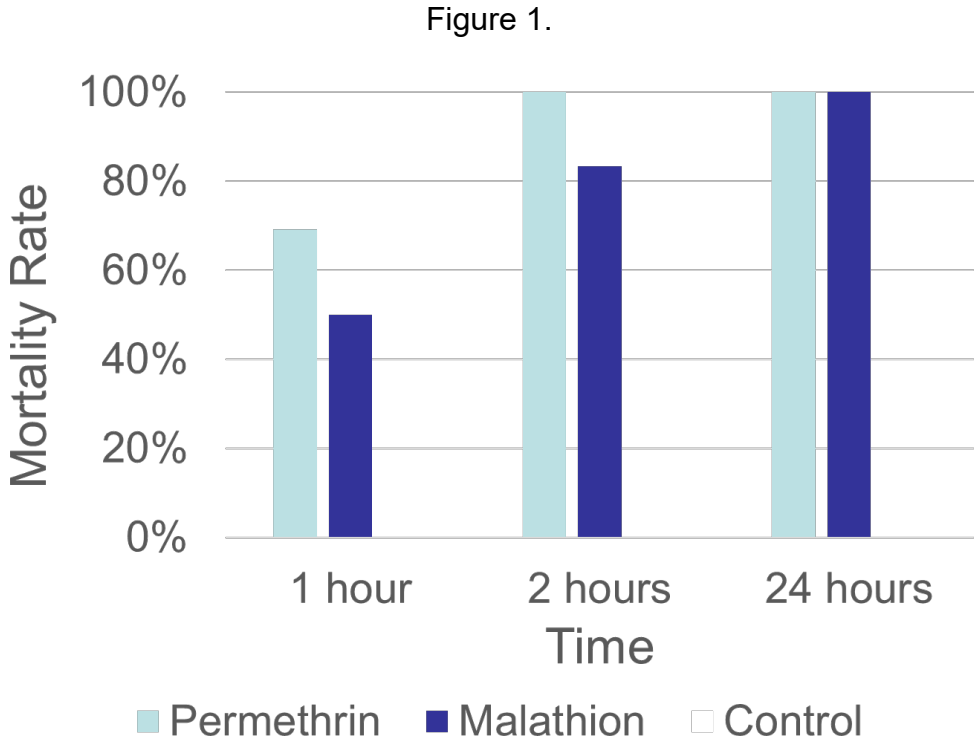
Table 1. Mortality rate topical exposure.

Time	Permethrin (no. mosquitoes)	% Mortality	Malathion (no. mosquitoes)	% Mortality	Acetone (no. mosquitoes)	% Mortality
1 hour	9	69%	6	50%	0	0%
2 hours	13	100%	10	83%	0	0%
24 hours	13	100%	12	100%	0	0%
Final assessment	-	Susceptible	-	Susceptible	-	-

Results			
	Alive	Dead	***Marginal Row Totals***
Permethrin	0	13	13
Malathion	0	12	12
Marginal Column Totals	0	25	25 (Grand Total)

The Fisher exact test statistic value is 1. The result is not significant at $P < 0.05$. No significant differences in mortality between mosquitoes topically exposed to permethrin compared to malathion.

Figure 1. Mortality rates 1, 2, and 24 hours after topical exposure for *Ae. albopictus*. No mortality was observed in the control group.



In the residual exposure group, significantly (P=0.0006) higher mortality rates were observed in mosquitoes exposed to permethrin compared to malathion (Table 2, Figure 2) for all time points grouped together.

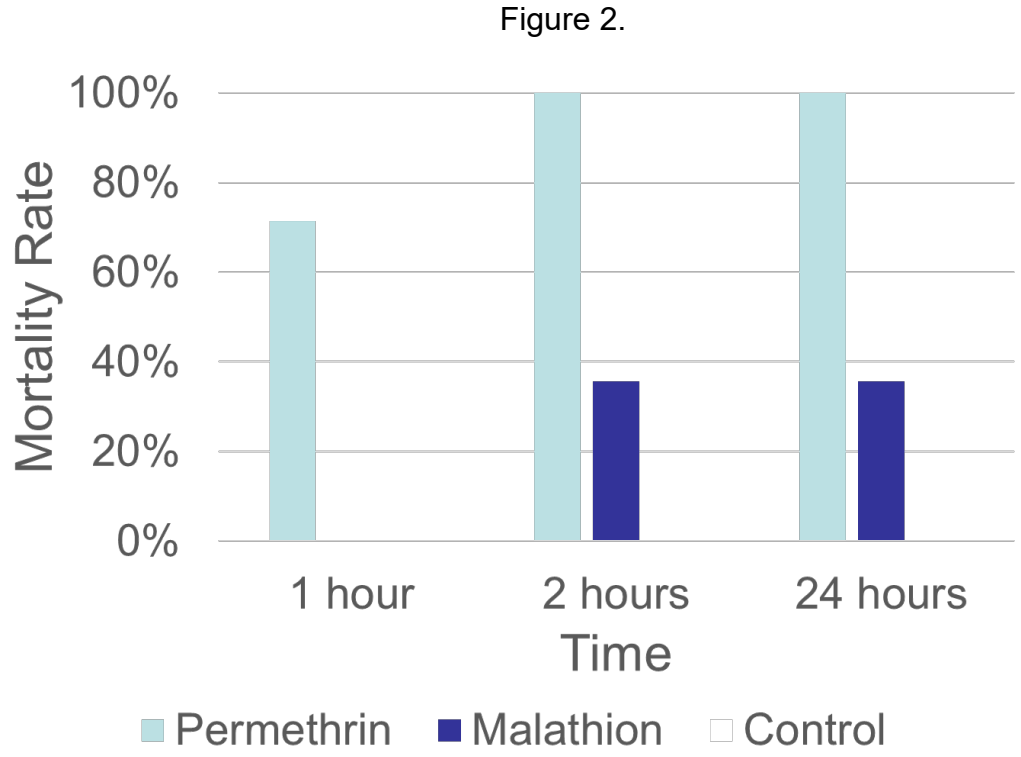
Table 2. Mortality rate residual exposure.

Time	Permethrin (no. mosquitoes)	% Mortality	Malathion (no. mosquitoes)	% Mortality	Acetone (no. mosquitoes)	% Mortality
1 hour	10	71%	0	0%	0	0%
2 hours	14	100%	5	36%	0	0%
24 hours	14	100%	5	36%	0	0%

Results			
	Alive	Dead	***Marginal Row Totals***
Permethrin residual	0	14	14
Malathion residual	9	5	14
Marginal Column Totals	9	19	28 (Grand Total)

The Fisher exact test statistic value is 0.0006. The result is significant at $P < 0.05$. Significant differences were observed in mortality between mosquitoes residually exposed to permethrin compared to malathion.

Figure 2. Mortality rates 1, 2, and 24 hours after residual exposure for *Ae. albopictus*. No mortality was observed in the control group.



Discussion

The hypothesis here was that topical (direct) versus residual (indirect) insecticide exposure would impact mosquito susceptibility/resistance to pyrethroid (permethrin) and organophosphate (malathion) AIs. In the residual malathion group, only 36% of mosquitoes died at 24 hours post-exposure. Conversely, 100% of mosquitoes exposed to malathion via the topical method died by 24 h post-exposure. Permethrin caused high mortality rates in both residual (71% mortality at 1 h, 100% at 24 h) and topical (69% mortality at 1 h, 100% at 24 h) exposure groups. In the residual exposure group, significantly (P=0.0006) higher mortality rates were observed in mosquitoes exposed to permethrin compared to malathion. No significant differences ($P > 0.05$) were observed in mortality rates between permethrin and malathion exposed mosquitoes at any time point in the topical exposure group.

Findings in this pilot study indicate that, in some cases (e.g., malathion under the conditions of this study), topical exposure may be more effective than residual exposure. This may be due to a higher degree of insecticide exposure in the group directly exposed to a liquid droplet of insecticide compared to being exposed to a dried residual (Alridge et al. 2016). In a previous study, similar results regarding efficacy of topical exposure versus residual exposure concluded that topical exposure was more effective than its counterpart (Waits et al. 2017). While this study supports part of our findings, it is important to note the aforementioned study

observed susceptibility between *Ae. albopictus* adults and larvae. The pilot study here focused on susceptibility/resistance of only adult *Ae. albopictus*. The differences noted in the previous study may be useful for conducting research on generational resistance genes in mosquito populations if they are looking at both adult and larvae groups. Generational resistance as mentioned in a different study, Modelling the impact of insecticide-based control interventions on the evolution of insecticide resistance and disease transmission, was not considered in the pilot study but it is still a researchable future topic (Barbosa 2018).

Concerning the application site for topical exposure, our results did not support nor disprove what previous studies found. For the pilot study, the insecticide for topical exposure was placed upon the mesothorax of the female mosquito. A previous study concluded that insecticides applied to appendages had lower mortality compared to areas located on the main body of mosquitoes such as eye, abdomen, or mesothorax (Alridge et al. 2016). This variable should be further evaluated as well to understand the best method for effective mosquito control. The results of the pilot study will help scientists consider what variables to test for moving forward in mosquito control. Overall, a comparison of the differences in variety of exposure methods for applying insecticides should be researched.

A larger scale experiment is planned to further evaluate these findings. Other studies could also evaluate the relationship between the efficacy of topical and residual exposure for additional AIs, FPs, and between different mosquito populations and species. Additional studies should focus on synergist effects of FP compared to technical grade AIs. Additional ingredients in FPs (e.g., synergists) are expected to affect mosquito control efficacy (Brito-Sierra et al. 2019). Current study is a general approach to testing stock solutions of AIs effectiveness independently to exposure methods.

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