FIU Undergraduate Research Journal

Volume 1
Issue 1 *Spring 2023*

Article 3

Inducible Resistance to Pyrethroid Insecticide is Lacking in Adult Aedes aegypti Mosquitoes

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Parente, M., 2023. "Inducible Permethrin Resistance Lacking in *Aedes aegypti* Mosquitoes." *FIU Undergraduate Research Journal*, 1(1), pp. 10–12.
DOI: https://doi.org/10.25148/URJ.010322

FIU | Undergraduate Research Journal

Inducible Permethrin Resistance Lacking in *Aedes aegypti* Mosquitoes

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Mosquitoes have evolved increased resistance to pyrethroid insecticides including permethrin, and studying their metabolic mechanisms of resistance is the window to human counteraction. If early exposure to insecticides can upregulate certain detoxification genes, this creates lower rates of mortality in a single mosquito's lifetime. Yellow fever mosquitoes (*Aedes aegypti*) were exposed to a sublethal dosage of permethrin and mortality rates at a later LC_{50} dose exposure were recorded. Mortality rates of induced mosquitoes were not lower than the mortality rates of unexposed (control) mosquito groups. If early exposure did not increase mortality, either evidence for inducible same-generational resistance remains to be seen in *Aedes aegypti*, or other factors were responsible for under-stimulating inducible resistance that were not acknowledged in the experimental design. The experiment may be replicated with adjusted test intervals to find the exact interval at which the upregulated proteins are still active and can confer resistance.

Keywords: *Epigenetics*, *mosquito*, *permethrin*, *resistance*, *inducible resistance*, *Aedes aegypti*

Malaria *mosquitoes* in the genus *Anopheles* have earned a reputation as the most lethal animal on Earth. They are followed closely by the yellow fever mosquito, *Aedes aegypti* (*Ae. aegypti*), which also carries dengue, chikungunya, and Zika. As a mosquito-vectored disease, dengue is second in frequency to only malaria, with between 50 and 390 million infections each year resulting in 22,000 deaths globally (NIAID, 2016). No vaccines exist for dengue, Zika, or chikungunya.

Females of most mosquito species must feed from a vertebrate blood source to acquire protein needed for egg production. Much like other disease-vector arthropods, *Ae. aegypti* has become a human-habitat specialist reproducing most readily in warm, humid, urban regions where humans are readily accessible (Brown et al., 2013; McBride et al., 2014). The ranges of *Ae. aegypti* and dengue overlap significantly across Africa, Central and South America, and Asia, which are all regions known for their humid, tightly packed cities (Mattingly, 1957). *Ae. aegypti* is now expanding into more temperate regions throughout the United States (Johnson et al., 2017). Wherever transmission rates are high, arthropod-borne viruses become genetically diverse and difficult to target with vaccines (Çavdaroğlu et al., 2021; Katzelnick et al., 2020;).

Many local governments and private homeowners alike apply insecticides in efforts to control these disease vectors (Clifton et al., 2019; Research Outreach, 2020). This experiment was conducted at Florida International University in Miami-Dade County, a county that employs aerial spraying over residential areas to target non-disease vector mosquitoes such as black salt marsh mosquitoes (*Aedes taeniorhynchus*), but also the vector *Ae. aegypti* during a 2016 response to increased local Zika transmissions (Aerial Mosquito Spraying, n.d.).

Literature Review

Permethrin

A popular insecticide for *Ae. aegypti* population control is permethrin, which interacts primarily by disrupting the gating kinetics of voltage-gated sodium channels (Imamura et al., 2000). Permethrin is a synthetic analog of pyrethrin, a natural insecticide compound in the *Chrysanthemum cinerariaefolium* (Bulgarella et al., 2020).

Permethrin functions as a neurotoxin by binding to the neuronal voltage-gated sodium channels and keeping them open, blocking repolarization and causing paralysis until the organism dies, either by dehydration or loss of circulation (Haddi et al., 2017; Tomlin, 2006;). Permethrin is favored as an insecticide because of its high effectiveness, low mammalian toxicity, thermodynamic stability, and inexpensive cost to create and ship (Nasuti et al., 2008). We chose permethrin as the insecticide for this experiment because of these factors, as well as its popularity in *Ae. aegypti* resistance experiments and as an insecticide in South Florida (Centers for Disease and Control, 2020).

Metabolic Resistance

Metabolic resistance prevents toxins from reaching voltage-gated sodium channels by increasing the activity of detoxifying enzymes (Imamura et al., 2000). Metabolic resistance mechanisms in *Ae. aegypti* have been identified in permethrin-resistant populations, especially in South America, Central America,

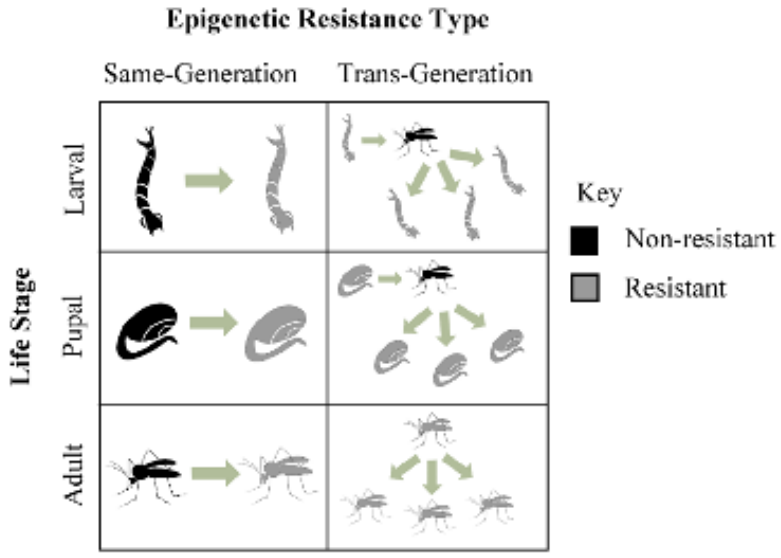
Southeast Asia, and Africa. Widespread application of permethrin, the lack of resistance surveillance to inform pesticide management programs, and short generation times of the vector are likely factors contributing to this issue (Estep et al., 2018; IRAC, 2021; Marcombe et al., 2012; Moyes et al., 2017;). The consequences of increased mosquito resistance to insecticides are catastrophic: not only are rebounds of disease projected, but hardier mosquitoes require deadlier and deadlier insecticides to control them. Using higher concentrations of permethrin or alternating to novel insecticides may have unpredictable, harmful effects on human health and the environment despite the benefit of reducing disease vector populations (Rigau-Pérez et al., 1998).

Although multiple mechanisms are well-characterized for pyrethroid resistance in insects, we focused on metabolic resistance because studies of other insects, including mosquitoes, showing it can be subject to epigenetic control (Oppold et al., 2015; Ingham et al., 2019). In contrast to natural selection which acts over many generations, the potential enhancement of metabolic resistance pathways by epigenetic upregulation of select genes could increase insecticide resistance within one organism's lifetime (Bonduriansky & Day, 2009; Liew et al., 2020;). This has already been shown in the mosquito *An. gambiae* (Ingham et al., 2019). Mechanisms of metabolic resistance include mutations, amplification, and transcriptional enhancements in the genes for cytochrome P_{450} monooxygenases, esterases, and glutathione-S-transferases (GSTs) (Hemingway et al., 2004). No studies to date have documented epigenetic upregulation of the genes for detoxifying enzymes in response to insecticide exposure in *Ae. aegypti*.

The goal of this study is to probe for the presence of inducible permethrin resistance in *Ae. aegypti*. Multiple life stages and generation types lead to a matrix of possible epigenetic resistances against insecticides as illustrated by **Figure 1**. Experiments may be conducted on larvae, pupae, or adults, and used to examine effects within the same generation or across generations from parent to offspring.

Figure 1

Epigenetic Resistance Matrix



Note. The columns are titled according to generation type: 'Same-Generation' or 'Trans-Generation.' The rows are titled according to life stage: 'Larval,' 'Pupal,' and 'Adult.' For same-generation resistance, early exposure to insecticide causes the non-resistant larva, pupa, or adult mosquito to become resistant in that same life stage. For trans-generation resistance, early exposure to insecticide at a given life stage yields an adult mosquito, which then yields offspring that are resistant in the same life stage its parent was exposed at.

After exposing young adult *Ae. aegypti* mosquitoes to sublethal dosages of permethrin, their rates of mortality at a future lethal dose may be compared to the mortality rates of unexposed controls. If mosquitoes exposed earlier in life demonstrate lower rates of mortality after a later insecticide exposure, we would be demonstrating evidence for the potential for same-generational resistance in the adult stage of *Ae. aegypti* mosquitoes, filling in the bottom left cell of this matrix.

Methods

Husbandry

Ae. aegypti of the Orlando lab strain were kept in an environmental room at 26-28°C and 50-70% humidity. Eggs were hatched using the deoxygenated water method, with Hikari First Bites fish food, which stimulate hatching larvae more reliably than fish pellets. Larvae were reared in polycarbonate trays (32x17x10 cm), 200 per tray, and fed one Tetramin Tropical fish pellet daily until pupation. Pupae were placed in small plastic cups with deionized water and subsequently placed within Bugdorm DP100B mesh cages to eclose. Adults had access to a vial of 10% sucrose solution with a cotton wick.

Both the Lethal Dose 50 (LC_{50}) calibration and Inducible Resistance experiment were conducted on female mosquitoes between two days after eclosure (E2) and five days after eclosure (E5) to best represent the times at which mosquitoes were young and most likely to respond (Davis, 1984; Tallon et al., 2019).

Chemical Reagents

Stock permethrin solution was made up of acetone and diluted 1% by weight powdered permethrin (Sigma-Aldrich: Pestanal™). From this stock, the desired concentrations of 0.000166% and 0.000025% permethrin (defined by mass) to be used for the Inducible Resistance Bioassay were diluted into capped scintillation vials and wrapped with Parafilm to prevent evaporation. Solutions were stored under fume hood within tightly closed polycarbonate trays.

LC_{50} Calibration Bioassays

We calibrated a baseline LC_{50} curve using WHO-standard procedures to determine the highest sublethal dose of permethrin in acetone, and therefore, the LC_{50} in the specific laboratory strain used for this experiment (WHO, 2016). Calibration was necessary because the WHO-standard LC_{50} for *Ae. aegypti* has a very narrow and error-prone window of accuracy, and without calibration the standard LC_{50} may be inaccurate for the Orlando laboratory strain, leading to potentially inaccurate mortality results.

On E2, adult females were aspirated all at once into individual transfer tubes (shortened 3D-printed and acrylic WHO tubes) for rapid anaesthetization. The mosquitoes were placed, still in the transfer tubes, over a constant low stream of CO_2 anaesthetizes mosquitoes without incurring significant damages or changes to mortality. We exposed mosquitoes to permethrin using the direct application method (**Figure 2**) (Agramonte et al., 2017). We used a Hamilton Repeating Dispenser to pipette 2µL of the sublethal dose permethrin solution onto the thorax (aiming for the postnotum) of the CO_2 anaesthetized mosquito. Mosquitoes were transferred to holding tubes lined with filter paper and a 1 mL Eppendorf tube of 10% sugar solution with cotton wick.

Each replicate contained unique mosquitoes to other replicates, providing biological replicates as opposed to technical. No mosquitoes were tested twice across replicates.

To find the LC_{50}, we tested mortality of mosquitoes exposed to a log series of permethrin concentrations: 0.001%, 0.00055%, 0.0003%, 0.000166%, 0.0001%, and 0% control, at 8, 8, 10, 8, 8, and 8 replicates respectively, chosen to reflect the span of test-LC_{50} concentrations in *Ae. aegypti* studies (Kubik et al., 2021; Whiten & Peterson, 2015).

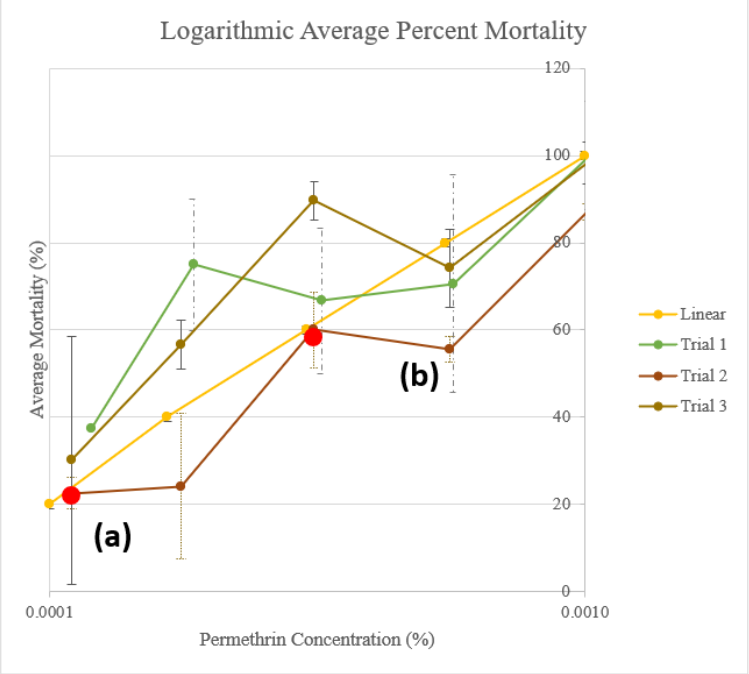
Figure 2

Direct Application Method for Pesticide Exposure

Note. A low stream of CO_2 diffused from below quickly knocks down active mosquitoes. Mosquitoes were anesthetized for 2-3 minutes of CO_2 at a time to prevent neurological damage. A 10µL Hamilton Repeating Dispenser was used to pipette solutions; two separate syringes for permethrin solutions and acetone control were used to prevent cross-contamination.

Figure 3

Preliminary LC_{50} Calibration Results



Note. LC_{50} Curve: Average mortality after exposure to permethrin at various concentrations. From this data we determined (a) the sublethal pre-exposure dose of 2 µl 0.000025% and (b) LC_{50} dose 2 µl 0.000166% permethrin. Error bars represent standard error of the mean, represented by the red dots. Different colors represent different trials (green for Trial 1, red for Trial 2, brown for Trial 3), as well as the "linear" best fit curve (yellow). Data points were offset by a minimal x-value to aid in legibility of the error bars.

Table 1

LC_{50} Data

% perm	Replicates	*N* mosq	*N* mort	Average Mortality %
0.001000	8	108	102	95.1256
0.000549	8	72	52	66.7778
0.000302	10	145	118	72.1101
0.000166	8	118	53	51.9289
0.000100	8	150	43	30.0811
0	3	60	0	0

Mean mortality after exposure to permethrin at various concentrations. A total of 694 female *Ae. aegypti* were tested with the 5 experimental permethrin concentration solutions, and 60 females were tested with the Control solution, indicated by the *N* mosq. column. Five to 20 mosquitoes were exposed in each replicate, indicated by *N* Replicates, while the sum of mortalities for each concentration's replicate is indicated under *N* mort. Fewer females were used in the Control group due to husbandry constraints at the time of the experiment. However, the 0% mortality obtained in the Control group gives us confidence that the procedures for anesthesia and direct application of permethrin caused no mortality.

The permethrin concentration of 0.00017% yielded a mean mortality of 51.9% (±12.47%) across 8 replicates. This concentration was chosen for the LC_{50}. The concentrations of 0.00055% and 0.00030% deviated from linear logarithmic results; fewer mosquitoes died in the 0.00055% dose than in the 0.00030% dose, despite being at a higher concentration of permethrin (**Table 1**). Potential explanations for error are reviewed in the **Discussion**. Additionally, the sublethal dose (i.e., the highest dose at which no mosquitoes die) was not found in the initial LC_{50} experiment. A subsequent, smaller-scale experiment was performed to narrow down concentrations further (**Table 2**).

Table 2

Sublethal Dose Follow-up Experiment

Trial (% permethrin)	Number of Replicates	Mortality (%)
0.0001	4	6.70
0.00005	4	4.73
0.000025	4	0
0.00001	3	0
Control	1	0

Mean mortality after exposure to permethrin at various concentrations. A total of 167 female mosquitoes were used for the experimental doses, and 15 females were used to validate the procedures in the Control group. Ultimately, 0.000025% permethrin was chosen as the sublethal dose, as it consistently yielded 0% mortality across 4 replicates, amounting to 38 total surviving mosquitoes. Fewer replicates were used for this experiment due to husbandry constraints at the time of the experiment.

Inducible Resistance Experiment Bioassays

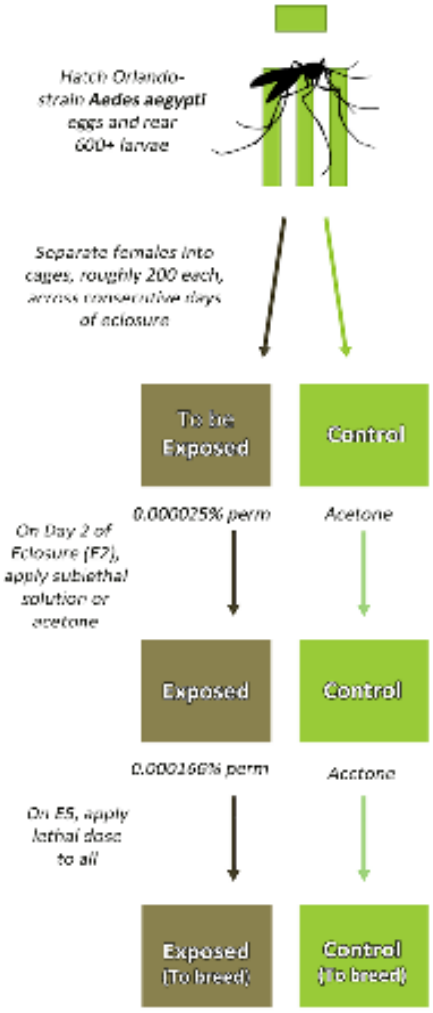
Two days after eclosure (E2), adult females were respirated into transfer tubes designed for rapid anaesthetization. These tubes were separated into control and pre-exposed groups. The pre-exposed group

was exposed to the sublethal 2 µL dose of 0.000025%, while the control group was only exposed to the acetone carrier.

Both groups received the LC_{50} dose of 2µL of 0.000166% permethrin (**Figure 4**). We replicated this experiment across multiple days following a progressive mosquito maturation schedule. Placing the pupa within one empty cage and moving un-pupated larvae to the next empty cage, allowing them to emerge as adults at their staggered biological schedules, helped compensate for somewhat asynchronous larval development. Each cage was labeled with the date at which the pupae emerged, allowing this experiment to be performed across successive days, each day containing a cage of E2 (and later E5) adults on which to experiment. Mortality for E2s and E5s was counted after a 24-h recovery period. Equipment (tubes, tubs, mesh caps, cages, etc.) was cleaned thoroughly after each use with 75-90% ethanol and an Alconox solution.

Figure 4

Experimental Design



Note. as described in **Inducible Resistance Experiment Bioassay**.

Statistical Analysis

For the preliminary LC_{50} calibration experiment, mosquitoes that eclosed over consecutive days were separated by day of eclosure (E0) (i.e., *Day 1*, *Day 2*, *Day 3*) and kept in separate cages to allow three replicate trials when mosquitoes were the right age. Controls showed no mortality, so the replicates were pooled. Mortality was divided by total number of mosquitoes tested at that dose, generating a proportion of mortality. We calculated 95% confidence intervals of the mortality proportion for each experimental group.

Results

Mortality of the pre-exposed mosquitoes did not differ significantly from that of controls, based on wide overlap of the 95% confidence intervals. From this data, we see no evidence of induced resistance to permethrin in pre-exposed adult *Ae aegypti*. Mortality level of both groups was higher than the ~0.5 mortality expected of controls exposed to the LC_{50} dose. Potential explanations for heightened mortalities are reviewed in the **Discussion**.

Table 3

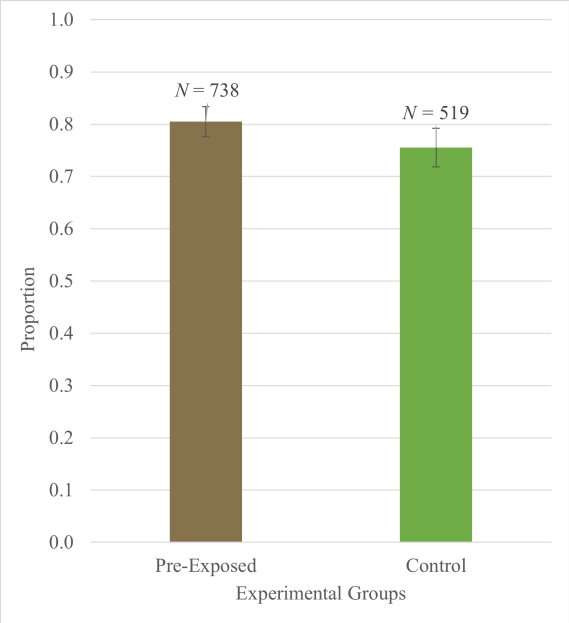
Results from Exposure of Pre-exposed and Control Females to the Calculated LC_{50}∫ dose of Permethrin

Type	N mosq.	N E5 died	Prop. died	CI-95	CI-95 lower	CI-95 upper
Pre-exposed	738	594	0.805	0.029	0.776	0.833
Control	519	392	0.755	0.037	0.718	0.792

Note. Overlap of the 95% confidence intervals indicate no significant differences in mortality of the two groups.

Figure 5

Mortality after Exposure to Permethrin at E5 between Pre-exposed and Control Groups



Note. 1,237 mosquitoes total were tested. An overlap of 95% confidence intervals demonstrates no significant difference between groups. The expected result of lower mortality in the pre-exposed group did not occur. Instead, the pre-exposed group experienced 5% higher mortality than the control.

Discussion

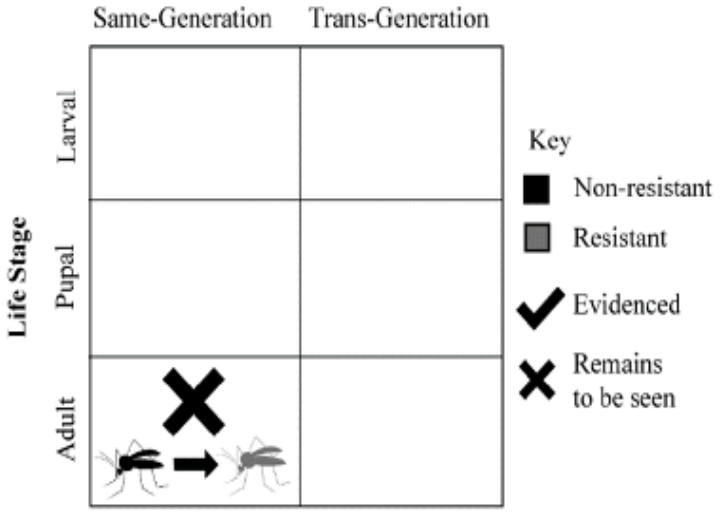
Results of this experiment suggest that sublethal doses of permethrin did not confer resistance to permethrin in the same lifetime. The expected result was a lower mortality of mosquitoes exposed early to permethrin. To the contrary, we found an insignificantly lower mortality in the exposed group. Both proportions were near 0.75-0.80, a value significantly higher in the control group than the predicted 50%

mortality after exposure to the LC_{50} (**Figure 5**). Several factors may be responsible for the higher-than-expected mortality. Error in the creation of the permethrin solutions may have created higher concentrations than anticipated. Acetone carrier from the solution may have evaporated in the short moments that the vial was open (despite extensive airtight sealing with Parafilm for storage) or over the days of the experiment. Over 5 days, 0.06g of acetone evaporated from a control vial, which likely also occurred in the permethrin-in-acetone solution vials, leading to higher concentrations of permethrin during the application of the lethal dose. Despite these potential errors, the 0% mortality rates at the sublethal dose for the controls, and the similarity of the pre-exposed and control groups validate the experimental procedure itself.

Our results provided no evidence in support of epigenetic resistance in adult *Ae. aegypti*, perhaps suggesting insufficient upregulation of their metabolic resistance pathways or another epigenetic mechanism that may be investigated in future studies. Despite not having identified the specific epigenetic mechanism involved, the lack of evidence in support of same-generational inducible resistance in adult *Ae. aegypti* mosquitoes fills in the bottom left cell of the Epigenetic Resistance Matrix (**Figure 6**). Sublethal exposure of larvae or pupae might still induce resistance in adults. In the world of disease vector control, further investigations cementing the lack of inducible *metabolic* resistance in *Ae aegypti* would be good news: one less mechanism by which these mosquitoes can confer resistance to insecticides. This generates hope for a slightly slower rate of resistance overall across populations, given that cross-generational effects may be passed down heritably in the epigenome.

Figure 6

Results as Applied to the Epigenetic Resistance Matrix



Note. Our experiment analyzed the quadrant represented by same-generation inducible resistance in the adult life stage, and our results suggest that this resistance remains to be seen.

Future studies of *Ae. aegypti* should search for both short-term induction and transgenerational induction of pyrethroid resistance. Perhaps a one-day interval between sublethal and lethal exposure would induce significant resistance. Peak transcription in *Anopheles* takes nearly 4 hours, but the induced protein itself is likely to persist longer (Pérez-Zamorano et al., 2017). A transient effect could have caused inducible resistance for a 3-day window.

Ultimately, little is known of the relationship between the epigenome of *Ae. aegypti* and their metabolic resistance mechanisms, but the long-term consequences of insecticides on human health and on the environment make for a promising avenue of research.

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