Genetic and molecular factors influencing pyrethroid response in *Aedes aegypti* from California

By

LINDSEY KAY MACK

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Entomology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Geoffrey M. Attardo, Chair

Joanna C. Chiu

Susan E. Lott

Committee in Charge

2023

Table of Contents:

Acknowledgements

First, I thank my advisor Geoff. Your excitement, curiosity, and support throughout this process was invaluable. You routinely brought me back to reality when I was overthinking things and reminded me that mistakes are the best way to learn.

Taylor, thank goodness we went through this together. Not only were you always willing to help with my experiments, but you pushed me to think about my research more deeply and from a different perspective. I truly value your friendship.

Thank you to my committee, Joanna and Susan. Your feedback and guidance have made me a better scientist, and your kindness and encouragement improved my confidence greatly.

To my parents and my siblings, Jillian and Noah, thank you for your support over the last 5 years. I'm sure you all know more than you ever expected about mosquitoes, and I am grateful you listened to me talk about my research so often.

Thank you to my friends from all stages of my life. There are too many who provided encouragement and pep talks to list here but know that I appreciate you.

Thank you to Katherine and Anton who provided my first foray into mosquito work, and for your continued support through collaboration and egg collection. I could not have completed these experiments without your help.

Finally, thank you to my dog, Penny. You got me outside when I was working too hard and provided comfort and amusement when I needed a break.

Thank you everyone, I could not have done this without you.

Abstract:

Pyrethroid resistance in the mosquito, *Aedes aegypti*, is an issue for control professionals globally. *Ae. aegypti* is a global arbovirus pest, transmitting the viruses that cause dengue fever and yellow fever among others. Resistance is developed through mutations at the target-site (the voltage gated sodium channel) or through over expression or overactivation of detoxifying enzymes like cytochrome P450s. The overall contribution of different mechanisms in combination is relatively unknown, though the specific involvement of some mutations in the target site are well understood. Additionally, specific mechanisms differ geographically. Resistance is assessed through phenotypic and genotypic analysis. To begin to understand the general contributions of different resistance mechanisms to the resistance phenotype as well as to identify previously unknown genes that may be involved in detoxification, several experiments were carried out. First, to assess whether genotypic and phenotypic analysis of resistance show similar results, as well as to investigate the likelihood that other mechanisms are at play in California, we paired these two analyses on individual mosquitoes. We determined that there were significant differences in knockdown time in individuals with identical genotypes, indicating that other resistance mechanisms are present. Next, we employed a time-course RNA-seq analysis to investigate the role of detoxifying enzymes in pyrethroid response in a strain from California. Redox homeostasis related genes as well as lipid and carbohydrate metabolism related genes all corresponded to pyrethroid treatment. Additionally, heat shock proteins experienced significant upregulation across time, indicating a role for these in response. These results indicate an overall change in the redox and metabolic environment of female mosquitoes, spurring questions of whether these changes affect her eventual offspring. To explore whether pyrethroid treatment affects the ovarian transcriptome of mothers, we devised an experiment to assess transcriptomic changes in ovaries

after exposure. We found 11 detoxification genes upregulated in the pyrethroid exposed group, included cytochrome P450s, glutathione synthestase, catalase, heat shock protein genes, and ABC transporters. In addition, we discovered shifts in genomic stability, mitochondrial function, and ribosome biogenesis. Overall, this indicates likely changes to the development rate of offspring. Further studies should focus on the association of changes to the ovarian transcriptome and the eventual resistance phenotype of the offspring. These results could indicate that insecticides are creating organisms better suited to live in a stressful environment, by mechanisms other than physical mutations.

Introduction:

Insects encounter many stressors in their environment. This can cause dramatic shifts in physiology affecting not only the current organism, but the organism's offspring as well. The response to these stressors is dynamic and multifaceted. One such stress insects encounter is insecticides, like pyrethroids.

Pyrethroid resistance poses significant challenges to pest control professionals. A wide array of taxa is resistant to this class of chemicals, including agricultural, urban, and public health pests. Of particular importance is the mosquito arbovirus vector, *Aedes aegypti. Ae. aegypti* poses a threat to health and prosperity globally. This mosquito is the primary vector of dengue, Zika, chikungunya, and yellow fever viruses, though dengue is the most widespread and damaging. In a study of 8 countries, 5 in South America and 3 in Asia, it was estimated that the total healthcare costs associated with dengue infections are as high as $1.8 billion. However, this number does not include the costs of mosquito control[1]. It should be noted that this study was published in 2008, so with inflation and worsening transmission, the number is much higher today. According to the World Health Organization, half the world's population lives in areas with endemic dengue, and 100-400 million people are infected annually. These statistics are expected to worsen (and have worsened since the early 2000s) due to climate change, habitat and land use change, and widespread insecticide resistance.

Ae. aegypti is an anthropophilic mosquito, meaning it prefers to live near humans, feed on humans, and use humanmade containers for egg-laying[2]. Their eggs are desiccation resistant and can remain viable for months while dry, lending to their invasive abilities[3]. The cross-section of insecticide resistance and invasive biology creates a significant challenge for public health officials.

These factors have led *Ae. aegypti* to invade new locations. California is one such location. In 2013, *Ae. aegypti* first established in the Central Valley but is now found as far south as the Mexico border and as far north as Redding. From population genetics analyses, it appears there were 3 separate introductions of *Ae. aegypti* into the state of California from 3 separate origins[4]. Each of these introductions display high levels of pyrethroid resistance[5]. Much is left to be known about the relative importance of various components to the overall resistance phenotype. Understanding the dynamics of this phenotype in California is important for control efforts.

The continuing threat *Ae. aegypti* poses to public health professionals and the significant burden pyrethroid resistance places on these agencies has created a need for improved vector control. To close gaps in knowledge regarding resistance testing, exposure recovery, and multigenerational effects of exposure, we performed 3 projects.

For chapter 1, the two primary tests for resistance were compared to investigate whether the results correspond. To assess this, we collected mosquitoes from 5 locations within the state, as resistance genotype is geographically variable. First, individual mosquitoes were phenotypical observed for resistance, then genotype within the pyrethroid target site was assessed. We discovered that these results were somewhat consistent, though individuals with some susceptible alleles still survived longer than individuals with entirely resistant genotypes. This indicated that other resistance mechanisms were at play and drove us to investigate genes that responded to exposure to a pyrethroid.

In chapter 2, we assessed the temporal response to a pyrethroid exposure via a high throughput gene expression analysis. Genes do not instantaneously respond to stimuli, so the use of a time course was chosen to assess a wider range of genes responding. We discovered many detoxifying genes responding over time, though there was not a definitive pattern of expression. Additionally,

genes involved in redox homeostasis and metabolism (primarily lipid metabolism) were largely upregulated. Changes continued into 24 hours after exposure, indicating lasting effects of exposure. We then assessed how these changes might affect eventual offspring.

Finally, chapter 3 investigates differences in the ovarian transcriptome between a pyrethroid treated and control group. Understanding the unintentional consequences of the use of insecticides is important in making decisions on pest control. We found that pyrethroid treatment resulted in upregulation of some detoxifying genes, which may indicate eggs are provisioned to increase tolerance. Additionally, we discovered alterations to genomic stability and probable growth rate, both of which have significant implications on the development of offspring. These results will need continued investigation to fully assess the implications of pyrethroid exposure and offspring. The findings from this research provide information and food-for-thought to vector control agencies on the use of pyrethroids in vector control.

References

1. Suaya, J. A. *et al.* Cost of dengue cases in eight countries in the Americas and Asia: a prospective study. *Am. J. Trop. Med. Hyg.* **80**, 846–855 (2009).
2. Crawford, J. E. *et al.* Population genomics reveals that an anthropophilic population of Aedes aegypti mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector. *BMC Biol.* **15**, 16 (2017).
3. Vargas, H. C. M., Farnesi, L. C., Martins, A. J., Valle, D. & Rezende, G. L. Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. *J. Insect Physiol.* **62**, 54–60 (2014).
4. Lee, Y. *et al.* Genome-wide divergence among invasive populations of Aedes aegypti in California. *BMC Genomics* **20**, 204 (2019).
5. Cornel, A. J. *et al.* Surveillance , insecticide resistance and control of an invasive Aedes aegypti (Diptera: Culicidae) population in California. *F1000 Research* 1–16 (2016).

Association between pyrethrum knockdown time and sodium channel genotypes in California *Aedes aegypti*.

Authors: Lindsey K. Mack[1*] and Erin Taylor Kelly[1*], Yoosook Lee[2], Katherine Brisco[3], Kaiyuan Victoria Shen[1], Aamina Zahid[1], Tess van Schoor[1], Anthony J. Cornel[3], Geoffrey M Attardo[1]

1. Department of Entomology and Nematology, College of Agriculture and Environmental Sciences, University of California, Davis, California
2. University of Florida – Florida Medical Entomology Laboratory, Vero Beach, Florida
3. Mosquito Control Research Laboratory, Kearney Agricultural Center, Department of Entomology and Nematology, University of California, Davis, California

*These authors contributed equally to the preparation of this manuscript.

Abstract

Background: Since their detection in 2013, *Aedes aegypti* has become a widespread urban pest in California. The availability of cryptic larval breeding sites in residential areas and resistance to insecticides pose significant challenges to control efforts. Resistance to pyrethroids is largely attributed to mutations in the voltage gated sodium channels (VGSC), the pyrethroid site of action. However, past studies have indicated that VGSC mutations may not be entirely predictive of the observed resistance phenotype.

Methods: To investigate the frequencies of VGSC mutations and the relationship with pyrethroid insecticide resistance in California, we sampled *Ae. aegypti* from four locations in the Central Valley, and the Greater Los Angeles area. Mosquitoes from each location were subjected to an individual pyrethrum bottle bioassay to determine knockdown times. A subset of assayed mosquitoes from each location was then analyzed to determine the composition of 8 single nucleotide polymorphism (SNP) loci within the VGSC gene.

Results: The distribution of knockdown times for each of the five Californian populations sampled was non-parametric with bimodal distributions. One group succumbs to insecticidal effects around 35-45 minutes and the second group lasts up to and beyond the termination of the assay (120+ minutes). We detected 5 SNPs polymorphic within California populations. One is potentially new and alternatively spliced (I915K), and four are known and associated with resistance: F1534C, V1016I, V410L and S723T. The Central Valley populations (Clovis, Dinuba, Sanger and Kingsburg) are fairly homogenous with only 5% of the mosquitoes showing heterozygosity at any given position. In the Greater LA mosquitoes 55% had at least one susceptible allele at any of the five SNPs. The known resistance allele F1534C was detected in almost all sampled mosquitoes

(99.4%). We observe significant heterogeneity in individuals with the same genotypes, confirming that the resistance SNPs alone cannot.

Conclusions: Resistance associated VGSC SNPs are prevalent, particularly in the Central Valley. Interestingly, among mosquitoes with all 4 resistance associated SNPs, we observe heterogeneity in bottle bioassay profiles suggesting that other mechanisms are important to the individual resistance of *Ae. aegypti* in California.

Introduction:

The yellow fever mosquito, *Aedes aegypti (Linnaeus 1762),* is a major vector of arboviruses such as dengue, Zika, chikungunya, and yellow fever viruses. This medically important vector was detected in California in 2013 in response to a residential service request (1). It has now been detected in 17 counties throughout the state despite aggressive surveillance and treatment efforts, presenting a significant challenge to local control agencies (1–4). The peridomestic habits of this mosquito make reduction and eradication difficult, but effective adulticides remain essential for control in the face of threats to public health.

Pyrethroid based compounds are used as adulticides, favored for their efficacy and low mammalian toxicity (5). However, the invasive populations of *Ae. aegypti* in California possess genetic mutations conferring resistance to the pyrethroid class of insecticides typically applied for vector control (6,7). Pyrethroids act on insect voltage gated sodium channels (VGSC) by binding to open channels and blocking the channel in the open conformation. This results in prolonged depolarization of the membrane and failure of neuronal function (8). This class of insecticides is partitioned into two types (I and II) based on the presence or absence of a cyano moiety at the alpha carbon (9). Point mutations within the channel domain of the protein can confer type-specific resistance by causing structural changes that reduce or eliminate the insecticides ability to bind the

channel (10). These mutations change the amino acid composition of the protein at specific locations that result in changes in charge and steric hindrance of the ion channel. These changes allow the VGSC to maintain normal function in the presence of pyrethroids.

To test for genetic markers of resistance in California, public health agencies and mosquito control districts screen for known pyrethroid resistance mutations in the VGSC gene: F1534C, V1016I, and V410L (7). These mutations are annotated based on their orthologous position in the *Musca domestica* VGSC protein (Genbank accession number: ANW06229)(11,12). V410L is located in the sixth transmembrane region of the first domain, V1016I is located in the sixth transmembrane region of the second domain, and F1534C is located in the intracellular space in the first loop of the sixth domain (Fig. 1) (10,13). Recently, another mutation (S723T) was linked to Deltamethrin resistance, though its impact on the resistance phenotype remains unknown (14). This SNP is localized in the intracellular region of the second transmembrane repeat domain. The F1534C mutation confers a low level of resistance on its own to type I pyrethroids. The V410L SNP, first described in 2017 (13), confers resistance to both type I and type II pyrethroids. The V1016I mutation on its own does not confer resistance, however in conjunction with F1534C it provides elevated insensitivity to type I and type II pyrethroids (8,15,16). Mosquitoes homozygous for V1016I, F1534C, and V410L mutations exhibit a high level of resistance to both type I and type II pyrethroids (17). As each SNP provides differing levels of protection against different classes of pyrethroids, testing for multiple SNPs in the field is relevant to screening for pyrethroid resistance(13).

Detecting and quantifying insecticide resistance in mosquitoes provides mosquito abatement groups with the tools to tailor their control strategies. The CDC Bottle Bioassay provides a convenient way to detect phenotypic resistance in adults to various adulticides (18). The CDC

Bottle bioassay involves placing 10-25 mosquitoes in an insecticide coated Wheaton bottle followed by observation of the time and proportion of knockdown for two hours after exposure. This procedure allows districts to investigate population level resistance. However, a direct analysis of the relationship between individual VGSC genotype and bottle bioassay phenotype (knockdown time) has not been completed with California *Ae. aegypti* populations. To study this relationship, we conducted phenotype assays on individual mosquitoes that were then genotyped. The *Ae. aegypti* samples were derived from multiple locations in California to explore population-level differences in response to insecticide exposure. We hypothesize that the resistance phenotype observed in *Ae. aegypti* in California is due to mutations in the VGSC, and individuals with more alternate alleles in the VGSC will have longer knockdown times.

Materials and Methods:

Mosquito Collection and Colony Maintenance

Adult mosquitoes were collected from four towns in the Central Valley of California: Dinuba, Clovis, Sanger and Kingsburg (Fig 2) as well as from the Greater LA area. Eggs from these adults were collected, allowed to develop for at least 5 days, then flooded in trays of 1 L of water and reared according to standard protocols (19). Mosquitoes were reared on a diet of ground rodent chow at 27°C under 14:10 hour (light:dark) photoperiod and adults were held at 70% relative humidity. Adults 1-3 days post-eclosion were then collected and individually exposed to a bottle bioassay to record individual knockdown time. Adult mosquitoes were fed on 10% sucrose solution *ad libitum* and did not receive a blood meal at all prior to insecticide exposure.

Individual Adult Bottle Bioassay

To determine time to knockdown for individual mosquitoes, a modified bottle bioassay was developed based on the CDC protocol (20). 250 ml Wheaton bottles (Fisher #06-404B) were coated with technical grade pyrethrum purchased from Chem Service (West Chester, PA). The pyrethrum was diluted in acetone to a concentration of 15.6 µg/ml (Lot #7581300) and bottles were coated with the insecticide following the procedure described in (21). Individual female mosquitoes were then aspirated into each bottle and observed for knockdown for up to 2 hours. Individuals were determined as knocked down when the bottle was rotated, and the mosquito could not reorient itself upright. Bottles were monitored continuously and exact time to knockdown was recorded. A susceptible lab colony, Rockefeller, was assayed as a reference for knockdown behavior in the bottle assay. For each population 80-95 adult female mosquitoes were assayed. Following knockdown, individuals were placed in the Lysis Buffer provided with the Zymo Quick DNA/RNA Miniprep kit (Cat #: D7001) and homogenized using Axygen mortar and pestles (Product PES-15-B-S1). Samples were stored at 4°C.

DNA Extraction

DNA was extracted using the Zymo Quick-DNA/RNA Miniprep kit (Product D7001) using the suggested protocol for Solid Tissue Samples. DNA concentration for each sample was tested with a Qubit instrument (Life Technologies). Approximately 4 ng/µL in a total volume of 30 µl was extracted from each whole individual.

SNP Genotyping

Our SNPs were identified from published whole genome sequences of *Ae. aegypti* (Lee et al. 2019). Sequences from the VGSC genomic locus of California mosquitoes were generated using primers reported in (22) (Genbank ID: KU728155-6). These sequences were aligned to the VGSC

locus in the corresponding region in the *Ae. aegypti* AaegL5 reference genome. The region corresponded to gene AAEL023266 (3: 315,926,360-316,405,639) in the reference genome. Polymorphic sites were screened for within the coding regions of VGSC and 8 non-synonymous SNPs were identified. One additional mutation, previously reported from other studies (23), was included in case a limited sample size of genome data missed low frequency variations within the Californian derived sequences. The final 8 SNPs screened included 3:315931548 (L to F), 3:315931672 (Q to R), 3:315983611 (A to T), 3:315939224 (F1534C), 3:315983763 (V1016I), 3:315999297 (T915K), 3:316014588 (S723T), and 3:316080722 (V410L). DNA was sent to the UC Davis Veterinary Genetics Laboratory for iPLEX assay using the MassARRAY System (Agena Biosciences, San Diego, CA) (24). The detailed information of the 8 SNPs are provided in Supplemental table X1.

Data Analysis

Statistical analysis was performed in R Version 3.5.2 (25,26). Survival analysis was performed using the survival package (27). Pairwise comparisons of survival curves were performed through a log-rank analysis. Median knockdown times were compared using Mood's median test from RVAideMemoir (28). Knockdown distribution normality was tested using the Shapiro-Wilk test in Base R. The box plot and histograms were created with ggplot2 (29). P-value thresholds were adjusted for multiple comparison correction using the Benjamini and Hotchberg method (30).

Results:

Individual Adult Bottle Bioassay

The knockdown times of all assayed mosquitoes are displayed in Figure 3. Mosquitoes that did not knockdown during the assay period were coded as such and considered using the Kaplan-Meier analysis which accounts for right-censored data. Aside from the susceptible laboratory strain, Rockefeller, the Sanger and Kingsburg populations had the lowest median knockdown times and are significantly lower than that of Clovis or Greater LA (Fig 3, Mood's Median Test, $\alpha<0.05$). The distribution of knockdown times for each of the five Californian populations sampled was non-parametric (Fig 3, Fig S1) and appear to have bimodal distributions. The distribution reveals the presence of two subsets of mosquitoes in each population with one group succumbing to insecticidal effects around 35-45 minutes and the second group lasting up to and beyond the termination of the assay. However, the relative proportions of these distributions differ between populations.

SNP Genotype by Population

Samples for genetic analysis were selected primarily from the upper and lower tertiles of knockdown times for each population. We detected 5 SNPs polymorphic within California populations (Table 1). All, with the exception of I915K, have been reported previously (13,14,31). The I915K SNP is located in a region which is alternatively spliced and its presence in the resulting transcripts has not been determined conclusively (Table 1). The V410L mutation is associated with resistance to type I and type II pyrethroids (13,14). The Central Valley populations (Clovis, Dinuba, Sanger and Kingsburg) are fairly homogenous with only 5% (12/242) of the mosquitoes tested showing heterozygosity at any given position. Alternatively, analysis of the Greater LA mosquitoes revealed that 55% had at least one susceptible allele at any of the five SNPs (Table 1, 37/67). The F1534C mutation is nearly fixed across these populations with two exceptions. One mosquito from Clovis was a heterozygote at the amino acid positions F1534C, V1016I, V410L

and S723T. One mosquito from LA was heterozygous at F1534C, and homozygous susceptible at V1016, V410 and S723 (Fig 4, Table 1). The I915K mutation was present only in the Dinuba population in heterozygotes (3/60) and these mosquitoes were also heterozygous at the V1016I, V410L and S723T positions. In the Greater LA population 15/67 were heterozygous at I915K. Of these 15, 7 were homozygous susceptible at V1016, V410 and S723. The other 8 were heterozygous at these positions (Fig 4). The sites V1016I, V410L and S723T, appear to be inherited together frequently; an individual heterozygous or homozygous at one site was correspondingly heterozygous or homozygous across all three with exceptions in the Clovis population. In the Clovis population 8 mosquitoes were heterozygous only at sites S723T and V410L (Fig 4).

SNP Genotype and Knockdown Time

Most of the assayed individuals were homozygous for the alternate "resistance" alleles. Still, some individuals with this genotype were knocked down in as little as 6 minutes after exposure to pyrethrum, overlapping with the susceptible Rock strain. More diversity of VGSC genotypes (6) was observed in the LA mosquitoes than Central Valley populations (4) (Fig 4A). This is consistent with previous reports showing little to no polymorphism in VGSC fragments in central California populations (7,32). The quantitatively different genetic variations of the Greater LA population from the Central Valley population is also consistent with a previous study monitoring both Central Valley and southern California locations (7). It is noteworthy that independent studies utilizing whole genome sequence (33), SNP chip (34), and microsatellite (34) indicated different genetic makeup separating southern CA and Central CA. The similar genetic differentiation is also observed in our VGSC genotyping dataset. Only 3/60 samples from Dinuba and 9/60 samples from Clovis had "susceptible" alleles, represented by 1 and 2 genotypes respectively. Of note, only

2 of the 309 mosquitoes tested were heterozygous at position F1534C, and those mosquitoes knocked down at 5 (Greater LA) and 18 minutes (Clovis), though it is difficult to ascertain the significance of this result with such a low frequency of this genotype. When controlling for genotype, there were still significant differences between the strains (Fig. 5). All of the Sanger and Kingsburg mosquitoes were homozygous for the resistance associated alleles. Their respective median knockdown times were 42 min (N=58) and 38 min (N=64) in the assayed mosquitoes (Fig. 5). This observation contrasts with median knockdown times observed in mosquitoes of the identical VGSC genotype from the Clovis and Greater LA populations. Phenotypic heterogeneity existing within and between groups sharing a common genotype were observed because of conducting bottle bioassays on individual mosquitoes. All bottle bioassays were performed coating each bottle with the same amount of chemical from the same pyrethrum batch and stock solution to ensure standardized and comparable assay conditions.

Kaplan-Meier Survival Analysis

Kaplan-Meier survival analysis is a non-parametric statistical test of survival that accounts for individuals that may not have experienced the "event" (in this case, knockdown) during the study period (35). Survival curve analysis of mosquitoes with equivalent genotypes reveals a range of median knockdown times that correlate with the relative proportions of susceptible versus resistant VGSC alleles. In cases where only a single individual represented a genotype, p-values were not reported (Fig. 4C). In general, individuals containing susceptible VGSC alleles do not last as long as those with the fully resistant genotype. However, some individuals carrying the homozygous resistant genotype at all alleles have knockdown times equivalent to individuals with homozygous susceptible genotypes (Fig. 4A-B). We reason this is unlikely due to methodological error, as these low knockdown times were observed across multiple populations.

Discussion:

Using the individual bottle bioassay in this study allowed us to explore the phenotypic variability of resistance in *Ae. aegypti*, and pair that phenotypic information with genotype, a previously unexplored relationship in *Ae. aegypti*. Target site mutations were prevalent in our sample populations and play an important role in resistance. Still, significant variability in knockdown time was observed in insects when controlling for genotype, implicating the role of other mechanisms of resistance and warranting further study. This individual bottle bioassay method yielded a bimodal distribution of knockdown times. Insects with susceptible alleles did knockdown earlier, though some individuals with fully resistant genotypes exhibited knockdown times similar to those observed in insects with susceptible alleles. This indicates running bottle bioassays with the synergist PBO will provide control agencies with important information about the resistance profile of local *Ae. aegypti*. Pyrethrum without PBO (MERUS 3.O- Clarke) is likely to see continued use, as organic farming and some residences inhibit the use of PBO. Deltamethrin has recently been approved for use in California, and there is evidence of less deltamethrin resistance across the state. Deltamethrin correlative studies will also be needed. Further study into the resistance mechanisms at play in California *Ae. aegypti* populations will provide vital information to mosquito control districts across the state, as understanding the resistance mechanisms allows for careful planning of insecticide use, rotation, and spray cover.

The establishment of *Ae. aegypti* in California has raised concerns that they could facilitate local transmission of arboviruses such as dengue and Zika as seen in Florida, Texas and Puerto Rico(36–38). While pyrethroids are favored as a chemical method for their control, resistance is widespread in California and globally (7,8,32,39). Despite significant work on pyrethroid resistance in *Ae. aegypti*, questions remain concerning the identification and relative compounding roles that VGSC

mutations, metabolic detoxification mechanisms, other resistance mechanisms, and environmental factors have on conferring insecticide resistance. Additional resistance mechanisms such as reduced cuticular permeability and behavioral resistance have been shown to be of significant importance in insects (40,41).

The VGSC mutations V1016I, F1534C and V410L are prevalent in *Ae. aegypti* in the Americas and have been closely monitored by the California Department of Public Health (CDPH) (7,13,15,17). These surveillance efforts found that between 2015 and 2017 resistant alleles were largely fixed in the Central Valley population, and less abundant (>80% for F1534C and >61% for V1016I from 2015-2017) but increasing in prevalence in the Southern population of *Ae. aegypti (7)*. Studies in Mexico have identified similar trends (17). Our results were similar to the CDPH findings (Table 1). In our samples F1534C was nearly fixed, with only two heterozygous individuals (one from Clovis and one from Greater LA). The detection of a heterozygote from Clovis was surprising, given our relatively small sample size compared to the 1200 mosquitoes tested by Liebmen et al. between 2015 and 2017. Our sample mosquitoes were captured in late 2018, and follow the general trend found in Liebmen et al. 2019 (7). In California, *Ae. aegypti* appear to have dispersed along the major Interstate 5 route so it is possible that mosquitoes from the Southern Population and Central Valley population are routinely moved between regions (42). Recent whole genome sequencing data as well as SNP chip data support multiple introductions of *Ae. aegypti* into CA, and found evidence of distinct genetic clusters converging in the Central Valley (34,43).

Most of our samples had all 5 VGSC mutations (Fig 4-5). Resistance alleles were likely prevalent in the founder populations in California (Cornel et al. 2016). Previous research has found that the Central Valley populations appear genetically similar to *Ae. aegypti* from the South-Central US,

while the Greater LA mosquitoes are more similar to southwestern *Ae. aegypti* (2,34). Resistance is widespread in populations in the United States, though the F1534 allele is still found in some areas (44,45). Interestingly, bottle bioassay results reported by local control agencies had not always followed the patterns that would be expected given the relative proportions of resistance associated SNPs, which was also in line with our results using the individual bottle bioassay test (Fig 3-5).

The *Xenopus* oocyte expression system for *Ae. aegypti* sodium channels has facilitated investigation into the role of individual mutations assayed in pyrethroid resistance (13,31,46,47). The mutations V410L + F1534C and V1016I + F1534C have even been studied in combination (13,16,47). Little is known about the S723T mutation (48) though with the high frequency of this mutation in all populations tested (59% in Greater LA and >92% in all Central Valley populations, Table 1), future functional analyses would be important to understanding the role this mutation plays in resistance. These mutations act in combination with metabolic mechanisms of pyrethroid resistance mediated by the upregulation, overexpression or duplication of cytochrome P450 enzymes encoding genes (14,49–51). Bottle bioassays with cytochrome P450 inhibitor, piperonyl butoxide (PBO), have implicated that cytochrome P450s play a synergistic role in combination with VGSC mutations to confer resistance in mosquitoes from Clovis (32). The significant differences in knockdown time found in our samples with the resistant genotypes (Fig 5) supports the hypothesis that other mechanisms are important and variable within California populations and these other mechanisms should be explored in future studies. This indicates that running bottle bioassays that include the cytochrome P450 inhibitor PBO will provide local control agencies important information when evaluating resistance. Given the functional evidence in the literature, and our assay results (Fig 4-5), the increasing prevalence of the F1534C mutation, particularly in

combination with V410L and V1016I reliably indicates an elevated level of resistance to type I pyrethroids (7,13,31,47). However, the individual bottle assays employed here prompts us to question that, assuming good conditions, the presence of resistance alleles in the VGSC gene may not guarantee adulticide failure, and susceptible alleles may not ensure control success. For this reason, it is important to pursue detailed investigations in the biology underlying the resistance phenotype. Additional knowledge on this subject would facilitate identification of additional genetic and/or biochemical markers. The ideal goal would be to identify markers that provide quantitative measures of the resistance phenotype in field caught mosquitoes which would boost the predictive power of these assays. In addition, identification of new targets underlying the phenotype opens the door for development of alternative strategies and new synergists to augment existing insecticidal compounds.

Funding

AC acknowledges funding support from the Pacific Southwest Regional Center of Excellence for Vector-Borne Diseases funded by the U.S. Centers for Disease Control and Prevention (Cooperative Agreement 1U01CK000516) pacvec.us. The funding body played no role in the design of this experiment.

Authors' contributions

ETK, LKM, KB, AJC, YL, and GMA conceived and designed the study. ETK, LKM, KB, KVS, AZ, and TVS collected data. ETK, LKM, and GMA analyzed the data. ETK and LKM prepared the manuscript. ETK, LKM, AJC, YL, and GMA revised and edited the final manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank the California Department of Public Health, Vector-borne Disease Section for their consultation.

Figures:

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

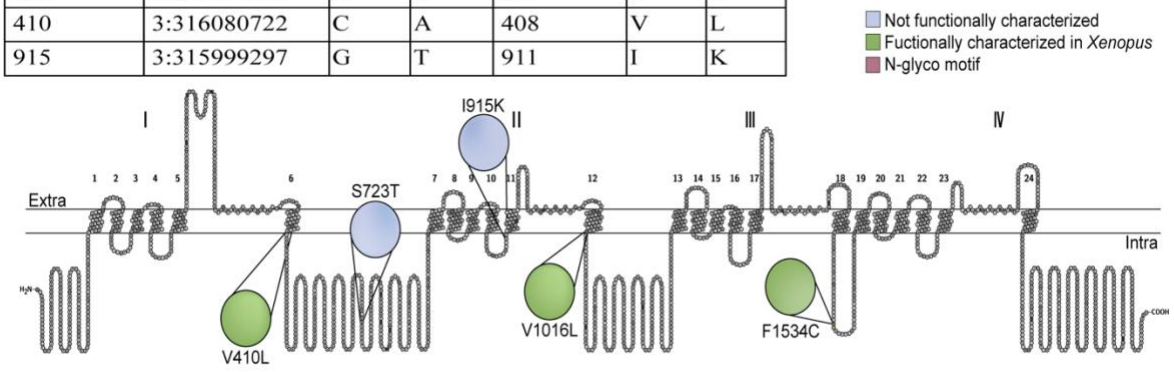


Fig 1. Topology of the mosquito sodium channel. The *Ae. aegypti* reference sequence was translated in CLC Main Workbench, Version 7 (52). The resulting amino acid sequence was aligned to the *Musca domestica* (ANW06229) and *Drosophila melanogaster* (AAB59195) reference protein sequences. SNP annotations were transferred to *Musca* to determine the *Musca* protein position, and structural annotations were transferred from *Drosophila* to *Aedes*. The topology of the sodium channel was illustrated using Protter version 1 (53).The sodium channel protein contains four homologous repeat domains (I–IV). Each repeat domain has six α-helical transmembrane segments (1–6, 7-12, 13-18, 19-24). Filled circles represent the five SNPs assayed in this study. Ref. =Reference. Alt. = alternate. aa = amino acid

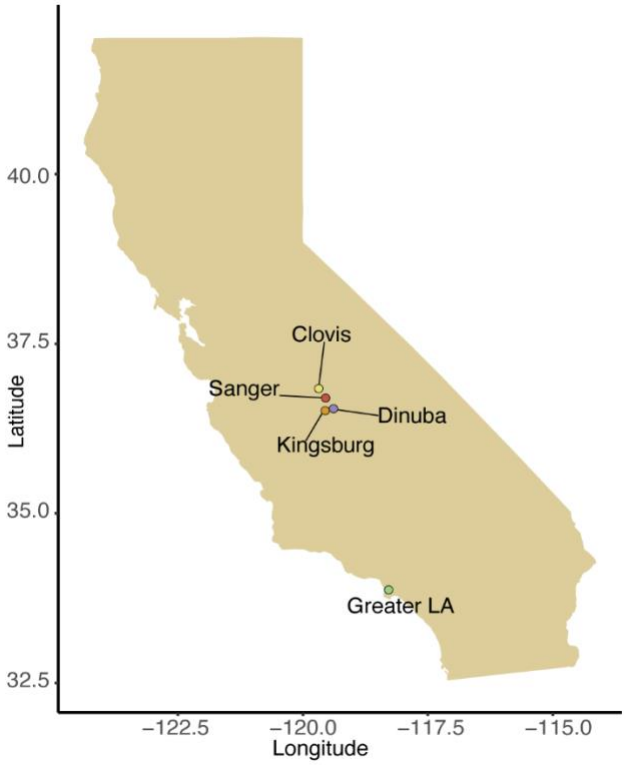


Fig 2. Locations from which *Ae. aegypti* were analyzed. Cities where founders for each lab strain were collected. Individuals were collected from the 5 cities we labeled on the map. Each lab strain was reared from individuals collected at various sites in these cities, and reared together to increase specimen numbers.

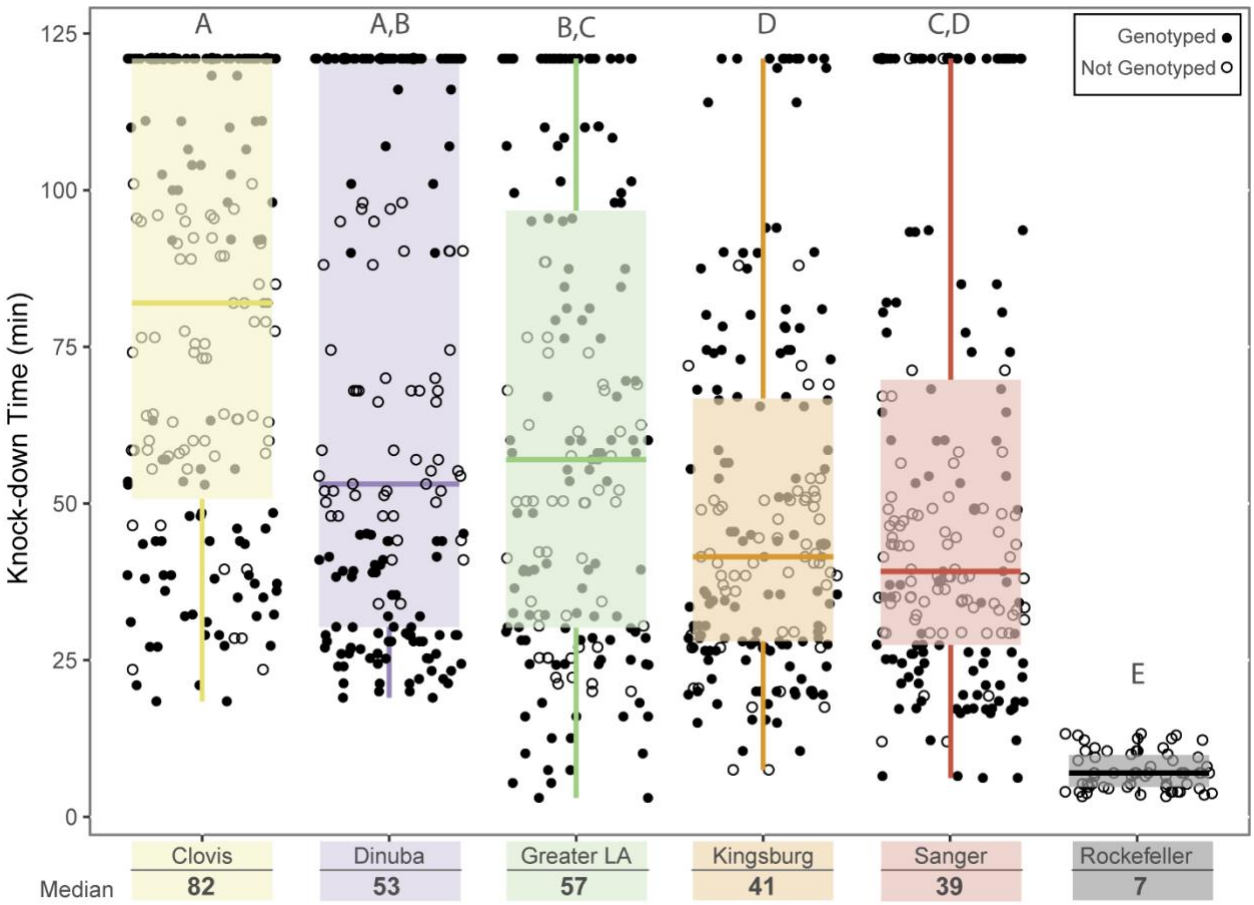
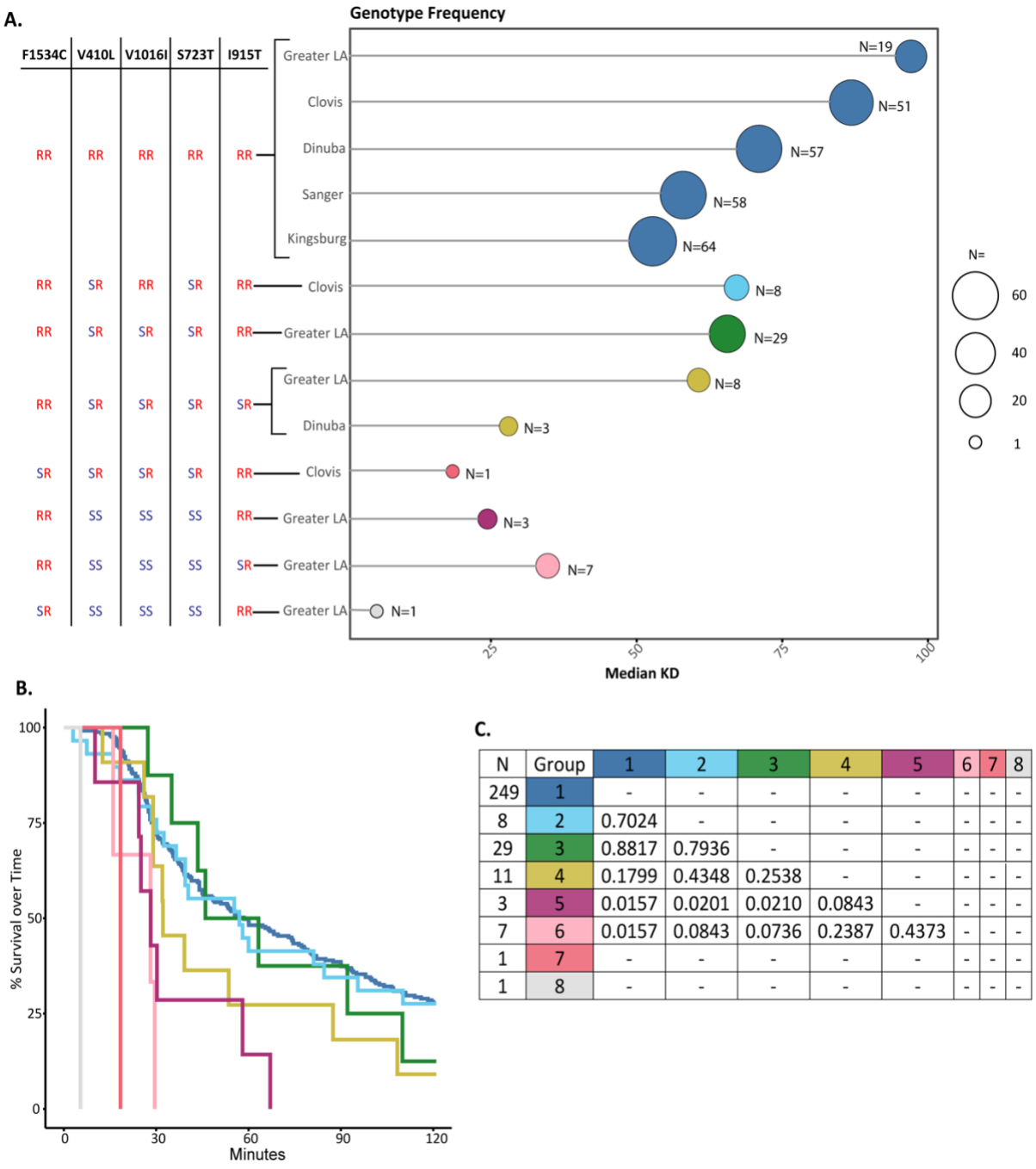


Fig 3. Distribution of knockdown times for each population. Each sample is represented by a circle. Filled circles indicate genotyped samples. Differences between group medians were determined by using the Mood's median test followed by fdr correction. Letters indicate statistically significant differences.

Table 1. Frequency of resistant alleles in each VGSC SNP in each population.

Genetic cluster	Population	N	F1534C				V1016I				V410L				S723T				I915K			
			SS	SR	RR	% R allele	SS	SR	RR	% R allele	SS	SR	RR	% R allele	SS	SR	RR	% R allele	SS	SR	RR	% R allele
Central Valley	Dinuba	60	0	0	60	100	0	3	57	97.50	0	3	57	97.50	0	3	57	97.50	0	3	57	97.50
	Clovis	60	0	1	59	99.17	0	1	59	99.17	0	9	51	92.50	0	9	51	92.50	0	0	60	100
	Kingsburg	64	0	0	64	100	0	0	64	100	0	0	64	100	0	0	64	100	0	0	64	100
	Sanger	58	0	0	58	100	0	0	58	100	0	0	58	100	0	0	58	100	0	0	58	100
Greater LA	Greater LA	67	0	1	66	99.25	11	37	19	59	11	37	19	59	11	37	19	59	0	15	52	88.80



N	Group	1	2	3	4	5	6	7	8
249	1	-	-	-	-	-	-	-	-
8	2	0.7024	-	-	-	-	-	-	-
29	3	0.8817	0.7936	-	-	-	-	-	-
11	4	0.1799	0.4348	0.2538	-	-	-	-	-
3	5	0.0157	0.0201	0.0210	0.0843	-	-	-	-
7	6	0.0157	0.0843	0.0736	0.2387	0.4373	-	-	-
1	7	-	-	-	-	-	-	-	-
1	8	-	-	-	-	-	-	-	-

Fig 4. Median knockdown time for each present genotype. A. Frequency of the 8 observed genotypes by population and their respective median knockdown times. B. Kaplan-Meier analysis of observed knockdown between the 8 genotypes within all populations. C. P-values for log-rank comparisons between each genotype.

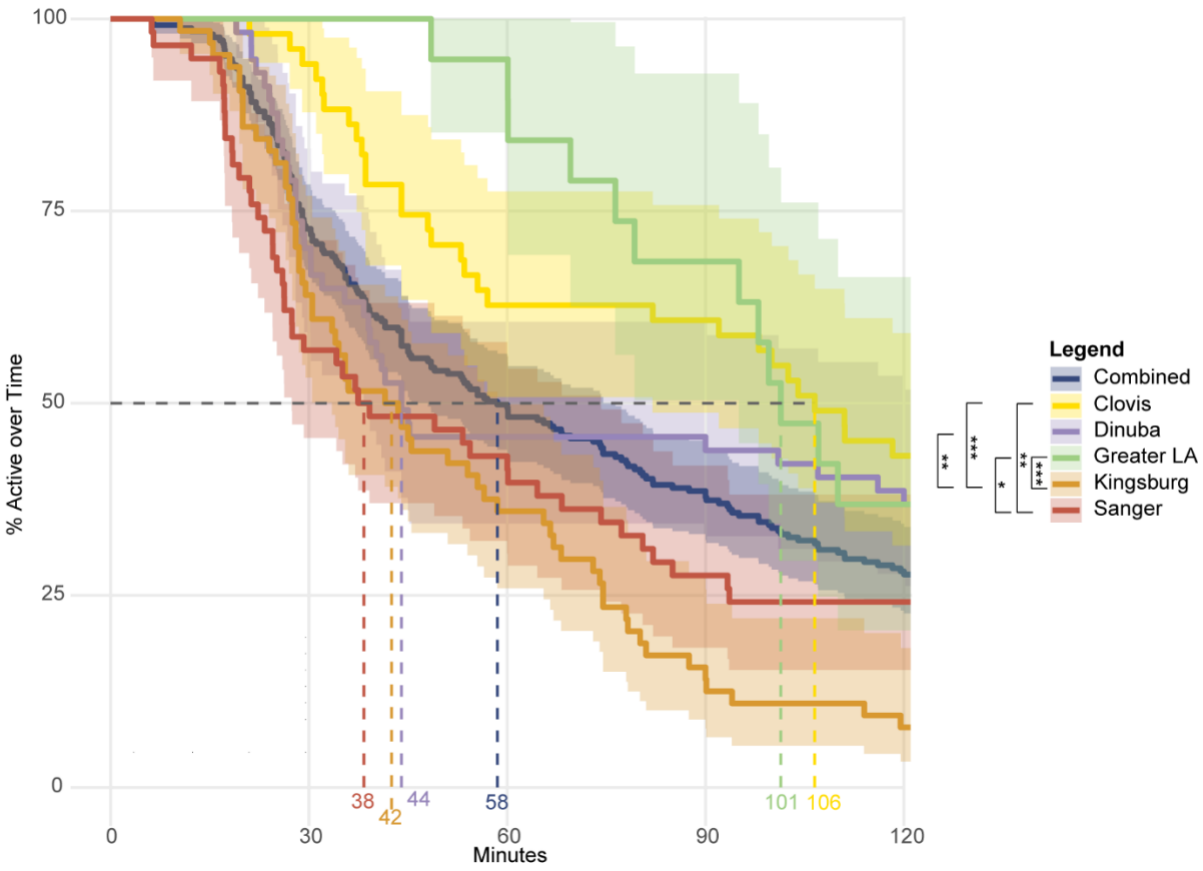


Fig 5. Kaplain-Meier survival curve analysis of the homozygous resistant genotype. A comparison of knockdown between the homozygous resistant phenotypes between all 5 populations tested, significant differences denoted by group.

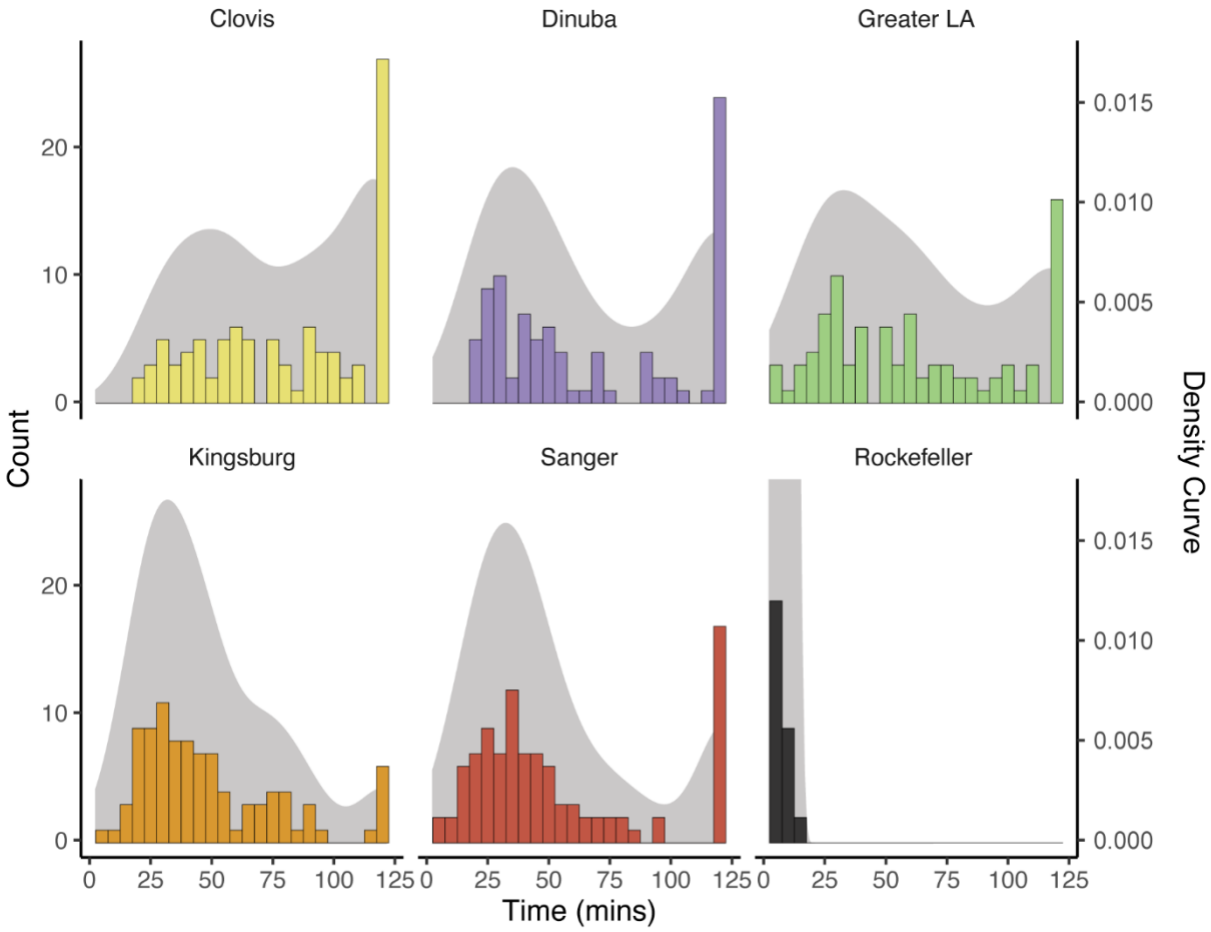


Fig S1. Histogram of knockdown times for assayed mosquitoes with kernel density plot. Knockdown time distribution was non-normal (Shapiro-Wilk test, $p < 0.00005$ for each population).

Supplemental Table 1. iPLEX MassARRAY primers for 8 SNPs used in this study.

Supplemental Table 2. Population name and generation for bottle assay testing.

Sample origin	Generation	Assayed/Collected
Greater Los Angeles (Los Angeles County)	F2	67/91
Sanger (Fresno County)	F1	58/103
Kingsburg (Fresno County)	F1	64/99
Dinuba (Tulare County)	F3	60/88
Clovis (Fresno County)	F2	60/95

References:

1. Metzger ME, Hardstone Yoshimizu M, Padgett KA, Hu R, Kramer VL. Detection and Establishment of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) Mosquitoes in California, 2011-2015. J Med Entomol. 2017 May 1;54(3):533–43.

2. Gloria-soria A, Brown JE, Kramer V, Yoshimizu MH, Powell JR. Origin of the Dengue Fever Mosquito , Aedes aegypti , in California. 2014;8(7). Available from: http://dx.doi.org/10.1371/journal.pntd.0003029

3. Invasive Aedes in California [Internet]. https://cdphdata.maps.arcgis.com. [cited 2020 May 27]. Available from: https://cdphdata.maps.arcgis.com/apps/webappviewer/index.html?id=57367199287a4d18a2cecf107854255b

4. Aedes aegypti and Aedes albopictus mosquitoes [Internet]. CDPH. [cited 2020 Jun 16]. Available from: https://www.cdph.ca.gov/Programs/CID/DCDC/Pages/Aedes-aegypti-and-Aedes-albopictus-mosquitoes.aspx

5. Housset P, Dickmann R, Others. A promise fulfilled--pyrethroid development and the benefits for agriculture and human health. In: Pyrethroid Scientific Forum 2009. researchgate.net; 2009. p. 135.

6. Cornel AJ, Holeman J, Nieman CC, Lee Y, Smith C, Amorino M, et al. Surveillance, insecticide resistance and control of an invasive. Genesis [Internet]. 2016; Available from: https://pdfs.semanticscholar.org/4a13/7685a38530874dadc19514f4115221447773.pdf

7. Liebman KA, Billeter SA, Yoshimizu MH, Yang F, Metzger ME, Schildhauer S, et al. Identification of Molecular Determinants of Resistance to Pyrethroid Insecticides in Aedes aegypti (Diptera: Culicidae) Populations in California, USA. J Med Entomol. 2019 Sep 3;56(5):1353–8.

8. Chen M, Du Y, Nomura Y, Zhorov BS, Dong K. Chronology of sodium channel mutations associated with pyrethroid resistance in Aedes aegypti. Arch Insect Biochem Physiol. 2020 Jun;104(2):e21686.

9. Vijverberg HPM, vanden Bercken J. Neurotoxicological effects and the mode of action of pyrethroid insecticides. Crit Rev Toxicol. 1990;21(2):105–26.

10. Du Y, Nomura Y, Zhorov BS, Dong K. Sodium Channel Mutations and Pyrethroid Resistance in Aedes aegypti. Insects [Internet]. 2016 Oct 31;7(4). Available from: http://dx.doi.org/10.3390/insects7040060

11. Williamson MS, Martinez-Torres D, Hick CA. Identification of mutations in the houseflypara-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Molecular and General [Internet]. 1996; Available from: https://idp.springer.com/authorize/casa?redirect_uri=https://link.springer.com/article/10.1007/BF02173204&casa_token=uPnQ-862O_kAAAAA:0OzdIVHsLtV3E6ir84K_5k8NhZEKD_-bdeUAoRnriJjMl7_6WSPsaE_p1xWQFU9Nvx9wbzRJM2gQpl0

12. Sun H, Tong KP, Kasai S, Scott JG. Overcoming super-knock down resistance (super-kdr) mediated resistance: multi-halogenated benzyl pyrethroids are more toxic to super-kdr than kdr house flies. Insect Mol Biol. 2016 Apr;25(2):126–37.

13. Haddi K, Tomé HVV, Du Y, Valbon WR, Nomura Y, Martins GF, et al. Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of Aedes aegypti: a potential challenge for mosquito control. Sci Rep. 2017 Apr 19;7:46549.

14. Saavedra-Rodriguez K, Campbell CL, Lenhart A, Penilla P, Lozano-Fuentes S, Black WC 4th. Exome-wide association of deltamethrin resistance in Aedes aegypti from Mexico. Insect Mol Biol. 2019 Oct;28(5):591–604.

15. Vera-Maloof FZ, Saavedra-Rodriguez K, Elizondo-Quiroga AE, Lozano-Fuentes S, Black WC IV. Coevolution of the Ile1,016 and Cys1,534 Mutations in the Voltage Gated Sodium Channel Gene of Aedes aegypti in Mexico. PLoS Negl Trop Dis. 2015;9(12):1–22.

16. Chen M, Du Y, Wu S, Nomura Y, Zhu G, Zhorov BS, et al. Molecular evidence of sequential evolution of DDT- and pyrethroid-resistant sodium channel in Aedes aegypti. PLoS Negl Trop Dis. 2019 Jun;13(6):e0007432.

17. Saavedra-Rodriguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P, et al. Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant Aedes aegypti from Mexico. Sci Rep. 2018 Apr 30;8(1):6747.

18. Brogdon WG, Chan A. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. CDC Methods. 2012;1–28.

19. Clemons A, Mori A, Haugen M, Severson DW, Duman-Scheel M. Culturing and egg collection of Aedes aegypti. Cold Spring Harb Protoc. 2010;5(10):1–6.

20. CDC - Parasites - Education and Training - CDC Bottle Bioassay [Internet]. 2019 [cited 2020 May 8]. Available from: https://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html

21. Brogdon WG, McAllister JC. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J Am Mosq Control Assoc. 1998 Jun;14(2):159–64.

22. Martins AJ, Lins RMM de A, Linss JGB, Peixoto AA, Valle D. Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant Aedes aegypti from Brazil. Am J Trop Med Hyg. 2009 Jul;81(1):108–15.

23. Kawada H, Higa Y, Komagata O, Kasai S, Tomita T, Thi Yen N, et al. Widespread distribution of a newly found point mutation in voltage-gated sodium channel in pyrethroid-resistant Aedes aegypti populations in Vietnam. PLoS Negl Trop Dis. 2009 Oct 6;3(10):e527.

24. Lee Y, Weakley AM, Nieman CC, Malvick J, Lanzaro GC. A multi-detection assay for malaria transmitting mosquitoes. J Vis Exp. 2015 Feb 28;(96):e52385.

25. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. [cited 2020 Jun 3]. Available from: https://www.R-project.org

26. RStudio Team. RStudio: Integrated Development for R [Internet]. 2015 [cited 2020 Jun 3]. Available from: http://www.rstudio.com/.

27. Terry M. Therneau PMG. A Package for Survival Analysis in R [Internet]. 2020. Available from: https://CRAN.R-project.org/package=survival

28. Hervé M. RVAideMemoire: testing and plotting procedures for biostatistics. R Package Version 09-69 Available online at: https [Internet]. 2018; Available from:

https://scholar.google.ca/scholar?cluster=11480313434998080824,5512342904207808814,1742940659148127598 2,5991997385139527444,10679832498456940388&hl=en&as_sdt=0,5&sciodt=0,5

29. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. Springer-Verlag New York; 2016 [cited 2020 Jun 24]. Available from: https://ggplot2.tidyverse.org

30. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Series B Stat Methodol. 1995;57(1):289–300.

31. Hu Z, Du Y, Nomura Y, Dong K. A sodium channel mutation identified in Aedes aegypti selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. Insect Biochem Mol Biol. 2011 Jan;41(1):9–13.

32. Cornel AJ, Holeman J, Nieman CC, Lee Y, Smith C, Amorino M, et al. Surveillance , insecticide resistance and control of an invasive Aedes aegypti (Diptera : Culicidae) population in California. 2016;(0):1–16.

33. Lee Y, Schmidt H, Collier TC, Conner WR, Hanemaaijer MJ, Slatkin M, et al. Genome-wide divergence among invasive populations of Aedes aegypti in California. 2019;1–10.

34. Pless E, Gloria-Soria A, Evans BR, Kramer V, Bolling BG, Tabachnick WJ, et al. Multiple introductions of the dengue vector, Aedes aegypti, into California. PLoS Negl Trop Dis. 2017 Aug;11(8):e0005718.

35. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. J Am Stat Assoc. 1958 Jun 1;53(282):457–81.

36. Grubaugh ND, Ladner JT, Kraemer MUG, Dudas G, Tan AL, Gangavarapu K, et al. Genomic epidemiology reveals multiple introductions of Zika virus into the United States. Nature. 2017 Jun 15;546(7658):401–5.

37. Teets FD, Ramgopal MN, Sweeney KD, Graham AS, Michael SF, Isern S. Origin of the dengue virus outbreak in Martin County, Florida, USA 2013. Virol Rep. 2014 Mar;1-2:2–8.

38. Murray KO, Rodriguez LF, Herrington E, Kharat V, Vasilakis N, Walker C, et al. Identification of dengue fever cases in Houston, Texas, with evidence of autochthonous transmission between 2003 and 2005. Vector Borne Zoonotic Dis. 2013 Dec;13(12):835–45.

39. Smith LB, Kasai S, Scott JG. Pyrethroid resistance in Aedes aegypti and Aedes albopictus: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016 Oct;133:1–12.

40. Zalucki MP, Furlong MJ. Behavior as a mechanism of insecticide resistance: evaluation of the evidence. Curr Opin Insect Sci. 2017 Jun;21:19–25.

41. Balabanidou V, Grigoraki L, Vontas J. Insect cuticle: a critical determinant of insecticide resistance. Current Opinion in Insect Science. 2018 Jun;27:68–74.

42. VectorSurv - California Vectorborne Disease Surveillance System [Internet]. [cited 2020 Jun 18]. Available from: https://vectorsurv.org/

43. Lee Y, Schmidt H, Collier TC, Conner WR, Hanemaaijer MJ, Slatkin M, et al. Genome-wide divergence among invasive populations of Aedes aegypti in California. BMC Genomics. 2019 Mar 12;20(1):204.

44. Kandel Y, Vulcan J, Rodriguez SD, Moore E, Chung H-N, Mitra S, et al. Widespread insecticide resistance in Aedes aegypti L. from New Mexico, USA. PLoS One. 2019;14(2):e0212693.

45. Mundis SJ, Estep AS, Waits CM, Ryan SJ. Spatial variation in the frequency of knockdown resistance genotypes in Florida Aedes aegypti populations. Parasit Vectors. 2020 May 11;13(1):241.

46. Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY, et al. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Proc Natl Acad Sci U S A. 2013 Jul 16;110(29):11785–90.

47. Fan Y, Scott JG. The F1534C voltage-sensitive sodium channel mutation confers 7- to 16-fold resistance to pyrethroid insecticides in Aedes aegypti. Pest Manag Sci. 2020 Jun;76(6):2251–9.

48. Itokawa K, Sekizuka T, Maekawa Y, Yatsu K, Komagata O, Sugiura M, et al. High-throughput genotyping of a full voltagegated sodium channel gene via genomic DNA using target capture sequencing and analytical pipeline MoNaS to discover novel insecticide resistance mutations. PLoS Negl Trop Dis. 2019;13(11):1–17.

49. Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H. Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, Aedes aegypti. PLoS Negl Trop Dis. 2012 Jun 12;6(6):e1692.

50. Smith LB, Tyagi R, Kasai S, Scott JG. CYP-mediated permethrin resistance in Aedes aegypti and evidence for trans-regulation. Foster GM, editor. PLoS Negl Trop Dis. 2018 Nov;12(11):e0006933.

51. Smith LB, Sears C, Sun H, Mertz RW, Kasai S, Scott JG. CYP-mediated resistance and cross-resistance to pyrethroids and organophosphates in Aedes aegypti in the presence and absence of kdr. Pestic Biochem Physiol. 2019;160(May):119–26.

52. CLC Genomics Workbench 7 [Internet]. [cited 2020 Jun 3]. Available from: https://digitalinsights.qiagen.com

53. Omasits U, Ahrens CH, Müller S, Wollscheid B. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics. 2014 Mar 15;30(6):884–6.

Time-series analysis of transcriptomic changes due to permethrin exposure: *Aedes aegypti* undergoes detoxification metabolism over 24 hours

Lindsey K. Mack[1] and Geoffrey M. Attardo[1]

1. Department of Entomology and Nematology, University of California, Davis, Davis, CA

Abstract:

Insecticide resistance is a multifaceted response and an issue across taxa. *Aedes aegypti,* the mosquito that vectors Zika, dengue, chikungunya, and yellow fever, demonstrates high levels of pyrethroid resistance across the globe, presenting a challenge to public health officials. To examine the transcriptomic shifts across time after exposure to permethrin, a 3'tag-seq analysis was employed on samples 6, 10, and 24 hours after exposure along with controls. Differential expression analysis revealed significant shifts in detoxifying enzymes and various energy-producing metabolic processes. Among the upregulated detoxifying enzymes are cytochrome P450s, glutathione s-transferases and peroxidases, and ATP-binding cassette transporters. Additionally, eight heat shock genes or genes with heat shock domains exhibit the highest degree of fold change across time. Twenty-four hours after exposure, samples indicate a global downregulation of these processes, though principal component analysis suggests lasting signatures of the response. Understanding the recovery response to insecticide exposure provides information on possible new genetic and synergist targets to explore.

Introduction:

Global insecticide intolerance is an issue across taxa. Insects overcome this stressor via a combination of genetic resistance, metabolic resistance, and behavioral resistance[1]. Due to the multifaceted nature of this response, achieving long-lasting population control with current insecticides is difficult, if not impossible[2]. Further exploration into this response could yield important results for the development of new insecticides with alternate modes of action or synergists to enhance efficacy of existing insecticides.

A particularly important model for exploring this complex response is the yellow fever mosquito, *Aedes aegypti*, due to ease of collection and rearing, as well as real world implications of this insect

in public health. In addition, the availability of an array of genetic tools for this species facilitate in depth investigation and manipulation for research and control purposes. *Ae. aegypti* threatens over half the world's population with one or more of the viral pathogens it carries (Zika, dengue, chikungunya, yellow fever, etc), and presents a significant burden on the global health system. This mosquito is found on all continents aside from Antarctica due to physiological characteristics that improve its invasion abilities. *Ae. aegypti* lay eggs which are desiccation resistant, surviving for months after oviposition. Typically, eggs are laid in man-made containers and humans are the preferred host of these mosquitoes. In 2013, *Ae. aegypti* first established a population California's Central Valley, but has now spread as far south as the Mexico border and as far north to Redding, CA[3]. Unfortunately, *Ae. aegypti* poses a significant challenge to public health officials due to widespread insecticide resistance.

Pyrethroids are a commonly used class of insecticides in public health applications as they show significant toxicity in arthropods, but relatively little toxicity to mammals due to differences in the target site binding affinity and metabolic rate[4]. These chrysanthemum derived xenobiotics bind to open voltage gated sodium channels, causing these channels to remain open leading to constant depolarization of the membrane leading to death in insects[5].

In *Ae. aegypti,* several single nucleotide polymorphisms (SNPs) in the voltage gated sodium channel gene (VGSC) are recognized as conferring resistance, though the prevalence of these SNPs is location dependent. In California, the primary SNPs are V1016I, F1534C, and V410L, though the frequency of these differs across the state[6]. Additionally, overexpression or overactivation of detoxifying genes are associated with resistance, though in *Ae. aegypti* there are over 150 genes that code for these types of enzymes and the overexpression and activation of these genes in resistant mosquitoes is highly variable geographically[7].

Gene expression responses are not instantaneous but rather complex and time intensive. In xenobiotic response, there are multiple stages of detoxification. Stage I is dominated by cytochrome p450s (CYPs), initially neutralizing xenobiotics and tagging them for further breakdown. Stage II is dominated by glutathione-s-transferases (GSTs) which breakdown these molecules further into compounds that can be excreted. Stage III is characterized by various transporter proteins moving these compounds across the membrane into the gut or rectum for excretion[8].

While many genes have been identified as conferring resistance in *Ae. aegypti*, the temporal response of the transcriptome to exposure to these compounds is relatively unknown, especially in adults. Researchers studied the response to pyrethroid exposure in *Anopheles funestus* larvae across time, but application of pyrethroids typically target adult mosquitoes rather than the aquatic larvae[9]. Defining this response over time is important for a nuanced understanding of the progression and complexities of the response to pyrethroid exposure across multiple physiological systems. Additionally, a deeper understanding of this response could highlight genes or pathways that may be ideal targets for novel monitoring and/or control methods, both chemical and genetic. To study this response in adult *Aedes aegypti*, a high-throughput analysis of gene expression over time was performed using 3'Tag-seq. Here, we characterize the response of a field strain of *Aedes aegypti* to a sub-lethal pyrethroid challenge over the course of 24 hours. The results reveal the identity and timing of insecticide responsive genes and pathways involved in the xenobiotic response of this strain of *Ae. aegypti.*

Results and Discussion

Principal Component Analysis Highlights Major Divergence in 24-Hour Post-Permethrin Treatment Expression Profile:

To assess transcriptomic changes across the first 24 hours after exposure to permethrin, 35 libraries were created from samples collected before exposure, and 6, 10, and 24 hours after exposure (summarized in Fig. 1a). These time points were chosen based on a microarray study in *Anopheles gambiae* that showed significant changes in response at these intervals[10]. All 35 libraries produced high quality 3' Tag-Seq[11] single end reads, with an average library size of 4,092,249 reads (min:3,493,811—unexposed replicate 2, max: 4,640,468—24 hrs post permethrin exposure replicate 3). Across samples, 96.7% of reads mapped to the reference genome at least once on average. Gene annotations were extracted from Vectorbase and genes with an "unspecified product" annotation were searched with BlastP against a custom database of the Culicidae and Drosophila databases to search for orthologs of unannotated genes. Of the 19,804 genes annotated in the *Aedes aegypti* genome, 11,155 (56.3%) were expressed in this dataset.

To assess how samples grouped based on overall expression profile, a principal component analysis was performed to ensure grouping was consistent with treatment conditions. PC1 explained 35.4% of the variation, while PC2 explained 16.4% of the variation (Fig. 1b). Because only 51.8% of the variation in the dataset was explained by PC1 and PC2, further clustering analyses were performed.

To further define the differences and similarities between groups, a kmeans cluster analysis was performed and the gap statistic was calculated to determine the optimal number of clusters within the data set[12]. Through this analysis, it was determined that the data could be categorized into 4 clusters as follows: baseline and 24 hours post-acetone treatment, 6- and 10-hours post-acetone treatment, 6- and 10-hours post permethrin treatment, and 24 hours post permethrin treatment (Fig.

1B). Based on the clustering, the acetone exposed insects appear to return to baseline 24 hours after exposure, indicating that the use of this reagent as a control in insecticide resistance testing experiments is adequate.

Because replicate samples from the 24 hours post-permethrin treatment demonstrate the greatest variance relative to the other treatment groups, we further investigated the genes driving this separation using a biplot. Biplot analysis (Supp. Fig. 1), reveals that the gene most strongly influencing this treatment group is catalase (AAEL013407), which codes for a protein that mediates oxidative stress by catalyzing the breakdown of hydrogen peroxide into two water molecules. Catalase has undergone thorough investigation in mosquitoes and is associated with multiple physiological processes incluing insecticide resistance, fecundity, pathogen infection, diapause, and maintenance of homeostasis after blood feeding[13–16]. All of these processes result in high levels of reactive oxygen species (ROS) production, driving increased catalase expression to neutralize these molecules and mediate oxidative stress. Some studies in *Anopheles* species have found that insecticide resistant individuals live longer than their susceptible counterparts, likely due to an increase in catalase, though this phenotype seems to be strain specific[17–20]. Further study on the role of catalase in the strain specific longevity trends observed in resistant populations would improve our understanding of life-history tradeoffs due to resistance. For this reason, exploring chemical agents that exploit ROS production or knock down catalase activity could be viable for mosquito control.

Redox Homeostasis Processes Exhibit Significant Expression Changes Over Time After Insecticide Exposure:

We next characterized the time course expression response to pyrethroid exposure to holistically assess differential expression. This analysis provides information on the relationship between time

points and expression values, and assess which genes are experiencing significant changes in expression across time. To accomplish this, we used a cubic regression spline curve with 3 degrees of freedom to assess expression trends across the time course. The analysis revealed significant responses over time after insecticide exposure with 383 genes upregulated and 200 downregulated. We performed a gene ontology (GO) analysis on the upregulated and downregulated genes to identify enriched functions within these groups. Of the 383 upregulated genes, we found enrichment of 5 primary GO categories. Among these are acetyl-CoA biosynthesis, glucose metabolism, lipid metabolism, redox homeostasis processes, and ATP production (Fig. 2).

Redox homeostasis processes are the primary response component to many stressors, including insecticides. Pyrethroid exposure causes oxidative stress, resulting from an imbalance of reactive oxygen species (ROS) and enzymes capable of neutralizing them[21–24]. Free radicals are formed by many cellular processes including the electron transport chain, fatty acid beta oxidation, and many other processes that require the breaking of molecular bonds such as the breakdown of xenobiotics. Gene families involved in the neutralization of free radicals to maintain redox homeostasis are also involved in the breakdown of xenobiotics, these include CYPs and GSTs[22,25]. Expression of these genes is often induced by increases in ROS[26]. ROS can cause conformational changes that result in the release of a cofactor from a transcription factor allowing for nuclear localization of said transcription factor. One example is the release of the KEAP-1 protein from cap n collar transcription factor[27].

In *Ae. aegypti,* the ortholog of the *Drosophila melanogaster cap n' collar* gene is *AAEL019563*. The cap n'collar C isoform (CncC) is the invertebrate equivalent of the vertebrate nuclear factor erythroid 2-related factor (Nrf2), which is known to bind antioxidant response elements[28]. CncC binding to these promoters leads to the expression of the previously mentioned gene families under

insecticide stress[29]. Importantly, CncC has been found to be constitutively active in resistant populations of *Drosophila* and *Anopheles gambiae*[30,31]. Studies have also found that this transcription factor is responsible for metabolic shifts in response to stress, which are illustrated by this data set as well[32,33]. Interestingly, in this dataset, *AAEL019653* does not display a significant change until 24 hours after exposure, when it is downregulated (logFC=-1.04, FDR=0.00019). It is unsurprising that this gene does not experience any upregulation throughout the time course as it is a constitutively expressed transcription factor which is released from its repressor upon increases in ROS within the cell.

Both lipid and carbohydrate metabolism were upregulated, along with acetyl-CoA biosynthesis. This is consistent with results in many other studies on the response to insecticide exposure[10,23,34]. Additional evidence supports the role of CncC in increased lipid and carbohydrate metabolism, as constitutive activation of this protein leads to a decrease in lipid stores in *Drosophila* and mouse cells showed increased glucose uptake in the same condition[33,35]. Acetyl-CoA is derived from the catabolism of proteins, lipids, and carbohydrates. Acetyl-CoA is a primary monitor of the metabolic state of an organism. When acetyl-CoA is plentiful, it is shuttled into lipid synthesis and while in a depleted state, it is transported into the mitochondria for ATP synthesis[36]. Other processes involved in ATP production include genes involved in NAD binding and FAD processes. Both of these molecules are high energy electron acceptors in the electron transport chain. This paired with ATP transmembrane transport overrepresentation clarifies the energetic stress the insect is under after exposure to a xenobiotic.

Heat Shock Proteins Exhibit the Largest Fold Changes Among Upregulated Genes Across Time

Eight of the top 20 genes with the largest fold change belong to the heat shock protein (HSP) family (Fig. 3). Each of the HSPs displays a similar patterns of transcript abundance over time,

with an increase through 10 hours followed by a gradual decline. Acetone treated samples show a response in these gene as well, though expression levels return to baseline at 24 hours while permethrin treated samples still have elevated expression. HSPs are associated with response to environmental stressors and have been associated with insecticide exposure response in many insects[37–42]. HSPs have a wide array of functions, aiding in all aspects of protein processing while not acting as a part of the mature protein[43]. One of these functions is as chaperones to aid in initial protein folding or to repair damage following stress. The role these molecules play in insecticide response may be due to protein damage due to ROS. One study found protein oxidation products increase following inoculation with the larvicidal bacteria *Bacillus thuringiensis kurstaki,* however, direct evidence of these products has not been studied in adult mosquitoes following insecticide exposure[44].

In a warming climate, the interactions between heat stress and insecticide resistance have the potential to pose major issues for vector control professionals[45]. There is even evidence that heat tolerance can protect from insecticide exposure and vice versa[37,42]. Continued research on the development of cross-resistance between heat and pyrethroids will be important for future vector control efforts.

Time Point Specific Pairwise Comparisons Identify 7 Detoxifying Genes Upregulated At All Time Points

To investigate time point specific detoxification signatures, we employed pairwise comparisons at each time point. 1245 (11.16%) were differentially expressed in at least 1 time point, 645 upregulated and 599 downregulated, using an FDR cut off of 0.05. Thirty-eight of the 645 upregulated genes were upregulated at all 3 time points (5.9%) while only 1 gene (a putative

oxidase/peroxidase) was downregulated at all 3 time points (AAEL019639, FDR=7.97x10^{-9}) (Fig. 4B).

Gene ontology analysis was used to explore functional characteristics of genes upregulated at all time points. Due to the small number of genes, the classic fisher algorithm was used which tests GO terms independently and is therefore less conservative than the TopGO default algorithm. Genes shared across time points fall into four primary categories: lipid metabolism, detoxifying enzymes, purine breakdown, and FAD binding (Fig. 4A). Among the detoxifying enzymes are CYP6M11, CYP4D38, esterase B1, CYP4C38, GPHX1, a microsomal GST and an ABC transporter. These are part of different stages of detoxification. It could be speculated that these detoxifying enzymes exhibit a more generalized function, aiding in the breakdown and excretion of various byproducts. Alternatively, the process of insecticide detoxification in insects might be so slow that they continue to sequester insecticides and break down entire molecules, even 24 hours after exposure. This could be an important consideration for vector control professionals who routinely perform insecticide resistance testing, as the time at which they are observing knockdown may not be an accurate depiction of resistance state.

Fatty Acid Beta Oxidation and ATP Production are Predominant Processes at 6-hours Post-Exposure

Six hours post exposure, 95 genes were upregulated while 15 genes were downregulated. Processes overrepresented at this time point are related to fatty acid beta oxidation and ATP production and transport. The most significantly upregulated gene is that coding for the protein carnitine O-palmitoyl transferase (whd) (*AAEL005458*, FDR=2.36 x10^{-5}) which is involved in fatty acid beta oxidation[46]. Of the 95 upregulated genes, 34 (35.7%) are only upregulated at 6 hours, one of which is the transcription factor, Hnf4 (*AAEL011323*, FDR=0.028). This factor is associated with a

metabolic switch to oxidative phosphorylation in *Drosophila* and fatty acid beta oxidation in *Ae. aegypti*[47,48]. Additionally, it is likely that the *withered* gene (*whd*) is activated by Hnf4, as researchers found that the expression of *whd* significantly decreased upon RNAi knockdown of *Hnf4*[48].

Downregulated genes were more specific to the 6-hour time point, with 12 of the 15 only showing reduced abundance at 6 hours. However, there was no significant overrepresentation of function among this group based on a gene ontology analysis. An interesting gene within this group is a cytochrome P450, CYP9J22 (*AAEL014619*, FDR=0.017), as the CYP9J family is implicated in insecticide detoxification. Because it is downregulated so early in the time course, it may be one of the first CYPs to respond to permethrin followed by a drastic downregulation, or it may just be an example of strain specific detoxifying gene responses[7]. The 6-hour time point shared 15 upregulated genes with the 10-hour time point, though this group did not contain any significantly overrepresented GO terms. However, among these genes is *whd,* indicating a continuation of increased fatty acid beta oxidation into 10 hours post exposure.

Carbohydrate Metabolism Elevates Ten Hours Post-Exposure and Sustains Through 24 Hours:

Gene expression changes at 10-hours post-exposure represent a switch in energy source as well as the peak of expression for genes displaying differential upregulation at all time points. At ten hours post exposure, 219 genes were upregulated, while 77 genes were downregulated. GO term overrepresentation among upregulated genes shows an increase in carbohydrate metabolism, with a continuation of fatty acid beta oxidation as well. This may indicate that mosquitoes have burned through much of their lipid stores by 10 hours post exposure, leaving them to function off of carbohydrates derived from sugar feeding. The most significant upregulated gene is that coding for glycine N-methyltransferase (GNMT) (*AAEL012764*, FDR=6.31×10^{-7}). This gene has not been

previously associated with insecticide response to our knowledge, however the role of the mouse ortholog in stress response has been investigated. Knockout of *GNMT* in mice resulted in a reduction in expression of detoxifying genes including CYPs, GSTs, catalase, and superoxide dismutase, as well as an increase in lipid peroxidation products[49]. Further study of the role of this gene in insecticide response may provide additional information on resistance mechanisms.

Eighty-one (37.0%) upregulated genes are only upregulated at 10 hours, though no significant GO term overrepresentation was found. Among these are 4 detoxifying genes previously associated with insecticide exposure: *GSTD6*, *CYP9J9*, *CYP304B2*, and *CYP304C1*, and 1 gene previously studied in response to insecticide exposure with negative results: *GSTT1*[29,50–53]. Differing results in our study may be due to observation of expression changes over a longer span of time or merely strain specific differences. Forty-five of the 77 (58.4%) downregulated genes were only downregulated at 10 hours. *GSTT4*, a detoxifying gene previously linked to pyrethroid exposure, is among this group. Although it was found to be overexpressed in a study where GSTT1 was deemed insignificant[53]. This suggests that the role of individual GSTs in the detoxification response may vary between strains in their response to pyrethroid exposure.

Ten hours post exposure shares 85 upregulated genes with 24 hours post exposure, many of which are involved in oxidoreductase activity (among which are *catalase*, *CYP9J6*, *CYP6BB2*, and a CYP with no family assigned), carbohydrate metabolism, and organic acid metabolism. *CYP9J6* and *CYP6BB2* are well associated with pyrethroid response[50,54,55]. In some strains of *Ae. aegypti, CYP6BB2* is increased in copy number, and has been specifically shown to aid in metabolism of permethrin[56,57]. Additionally, 9 HSP genes or genes with HSP-like binding regions are upregulated, consistent with results from the time course analysis.

Downregulated Genes at 24 Hours Post-Exposure are Tied to Energy Consumption:

Expression signatures 24-hours post-exposure were investigated to further assess broad differences creating the clustering divide observed in Figure 1b. Twenty-four hours post exposure, 371 genes are upregulated while 476 genes are downregulated. The most significant upregulated gene was a cytochrome P450, possibly part of the CYP6B family, and experienced a 2-fold change (*AAEL009018*, FDR=1.42×10^{-7})[58]. This gene has been associated with both pyrethroid and bendiocarb resistance[58,59]. The specificity of its differential expression solely at 24 hours after exposure to permethrin in this strain is unknown.

The upregulated genes at this time point largely mirror the upregulated genes throughout the time course, however the downregulated genes are unique to 24 hours, with 445 of the 476 downregulated genes only downregulated at 24 hours. The GO hits fall within 6 categories: ATP binding, GTPase activity, protein processing, signal transduction, spermidine biosynthesis, and transcription (Fig. 5). Spermidine is associated with protection against the toxic effects of pyrethroids in zebrafish, though the downregulation of genes involved in its synthesis may indicate that the insect is no longer experiencing toxic effects at this time[60]. Rather, the insect is primarily breaking down byproducts of earlier metabolism of the pyrethroid molecules. Decreases in ATP binding, signal transduction, protein processing, GTPase activity, and transcription indicate an overall decrease in the molecular responses to pyrethroid exposure. This combination of activity suggests that the insect is ramping down their overall response to return to a basal physiological state.

Thirty-eight detoxifying enzymes exhibit an association with pyrethroid response

To further classify those xenobiotic response-associated genes that may not have exhibited a statistically significant change in expression, we conducted a weighted gene correlation network analysis utilizing the R package WGCNA .[61] This analysis groups genes based on expression

patterns over time as well as by their correlation with treatment. Thirty modules were identified with one highly correlated with treatment (Supp. Fig. 3). This module contains 931 genes, 305 of which are differentially expressed using an FDR cutoff of 0.05 from the time course analysis (245 upregulated, 60 downregulated). Interestingly, no correlation with time was observed in this module. This suggests that the expression changes witnessed over this time course are attributed to the xenobiotic response, rather than merely to time-associated expression alterations. Forty-two genes within the oxidoreductase GO term are significant based on the time course analysis within this group (Fig. 6A). Oxidoreductase activity is mediated by many enzymes associated with detoxification functions.

Within the module most related to treatment are 25 cytochrome P450s (without filtering based on FDR). Eleven of these are significant when considering expression over time while 17 are significant in at least 1 time point (Fig. 6B). Expression patterns of these genes are inconsistent, but cluster into 5 groups based on these patterns. One CYP is significantly downregulated at 10- and 24-hours post exposure, *CYP307A1*. This gene is included in the Halloween gene cluster and is the ortholog of the *Drosophila* gene *Spook*. Spook is part of ecdysone synthesis, so this downregulation may have implications for the role of ecdysone metabolism in the insecticide stress response[62,63].

We also found members of the CYP9J family is which is associated with pyrethroid resistance[7,64–67]. Four genes from this family are present in the treatment associated module, 3 of which cluster together based on expression pattern (*CYP9J6, CYP9J9*, and *CYP9J31*). In prior work, researchers performed an extensive micro-array study on rhythmic gene expression in female *Ae. aegypti* heads with a special focus on the CYP9J family and found two groups of co-oscillating members

of this family[68]. However, none of the genes explored in that study were differentially expressed in this study.

Nine GSTs/GPXs are present in the treatment-associated module, 5 of which are significant. Expression trends are more consistent within this group, with expression peaking at 10 hours post exposure (Fig. 6B). GSTX2 is associated with DDT resistance in South America, however researchers in Thailand performed in-vitro experiments with this protein derived from a Thai population and found no evidence of direct metabolism of DDT by this enzyme[69,70]. GPXH1 appears to be induced by xenobiotic exposure, however, overexpression of this gene is not associated with resistance[71].

Four ABC transporters are in this group, 3 of which are upregulated. ABC transporters are responsible for transporting insecticide and byproducts of insecticide metabolism out of the cell to avoid accumulation of these toxic compounds[72]. Transcription of these are also initiated by CncC, and their role in xenobiotic detoxification has been validated by RNAi experiments in crop pests[73].

Conclusion:

Exposure to permethrin results in not only an immediate physiological response, but a response that continues through at least 24 hours after exposure. This response is characterized by shifts in metabolism and energy production along with redox balancing and detoxifying genes. Understanding the recovery response to insecticide exposure provides information on possible new genetic and synergist targets to explore. Additionally, observing these genetic trends provides evidence that two phase treatments of insecticides with different modes of action may be effective, though the logistics of such an application would be difficult and likely cost prohibitive. This study also highlights strain specific differences in the response of detoxifying enzymes. Further

investigation into the evolutionary mechanisms behind these differences may give improved understanding of the evolution of insecticide resistance.

Methods:

Mosquitoes:

Mosquitoes were collected from Reedley, CA (36.5809032,-119.4553858,16.51) and reared according to existing protocols for 2 generations in the lab[74]. Briefly, larvae were reared in trays of 200 in 1 L of dechlorinated tap water and fed Fluval fish pellets. Upon pupation, pupae were removed from larval trays and placed in BugDorm #6 cages and fed 10 % sucrose *ad libitum* until the 5th day post-eclosion. To determine the resistance phenotype of this strain to permethrin, a CDC bottle bioassay was performed, using the diagnostic dose of 43ug/mL[75]. Based on the diagnostic time of 10 minutes, the strain was determined to be resistant to permethrin (Supp. Fig. 4).

Permethrin Exposure:

In groups of 15-25, females 5 days-post eclosion were placed in 250 mL Wheaton bottles coated in permethrin at a concentration of 15 ug/mL in acetone or 1 mL acetone as a control for 1 hour. Mosquitoes were then removed from bottles and placed in BugDorm #6 cages. Five replicates of 5 mosquitoes were collected 6, 10, and 24 hours after cessation of exposure and homogenized in the lysis buffer supplied with the Zymo Quick RNA Mini-Prep kit (R1054). Additionally, 5 replicates of 5 mosquitoes were collected prior to permethrin/acetone exposure to act as a baseline. RNA was extracted and samples were assessed for contamination with a NanoDrop One^{c}, then analyzed via Bioanalyzer for RIN score and concentration by the UCDGC.

Library Prep and Sequencing:

RNA was submitted to the UC Davis Genome Center for library prep and 3' Tag-seq analysis. Gene expression profiling was carried out using a 3'-Tag-RNA-Seq protocol. Barcoded sequencing libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the recommendations of the manufacturer using both the UDI-adapter and UMI Second-Strand Synthesis modules (Lexogen). The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a LabChip GX system (PerkinElmer, Waltham, MA). The libraries were quantified by fluorometry on a Qubit fluorometer (Life Technologies, Carlsbad, CA), and pooled in equimolar ratios. The library pool was quantified via qPCR with a Kapa Library Quant kit (Kapa Biosystems / Roche, Basel, Switzerland) on a QuantStudio 5 system (Applied Biosystems, Foster City, CA). The libraries were sequenced on a HiSeq 4000 sequencer (Illumina, San Diego, CA) with single-end 100 bp reads. 3'Tag-seq is a quick and efficient form of sequencing in which single-end sequencing is performed on the 3' end, creating only an initial read of 80 or 90 base pairs.[11] This creates low noise data that can easily be aligned to the existing, well annotated genome.

Reads were checked for quality using FastQC v0.11.9, then trimmed using bbduk, a function within bbmap (v37-50) [76,77]. Resulting reads were aligned to the *Aedes aegypti* LVP_AGWG-50 genome, indexed with an –sjdbOverhang 99 using STAR v2.7.2a[78,79]. Read files were then indexed using samtools v1.3.1[80]. Raw read data is available via the NCBI SRA database under the accession number PRJNA988225.

Gene Expression Analysis:

All statistical analyses were carried out using R Statistical Software (v.4.2.1)[81]. To observe similarities and groupings among samples, a principal component analysis and kmeans clustering analysis were performed.

Differential gene expression analysis was performed using edgeR[82]. To begin filtering the data set, genes with low expression (less than or equal to 1 CPM in more than 2 samples) were ignored. For a gene to be considered differentially expressed, a false discovery rate (FDR) of < 0.05 was used as a threshold. To characterize differential expression while considering time, a cubic regression spline curve with 3 degrees of freedom was used to assess expression trends across the time course with regard to treatment.

Co-expression network analysis was performed using the WGCNA package in R[61]. This analysis groups genes based on expression trends over time as well as their association with treatment.

Gene ontology enrichment analysis was performed using the R package TopGO[83]. All genes detectably expressed within this dataset were used as the background for this analysis. The TopGO fisher test was used to determine significance, using a p-value cutoff of 0.05.

References

1. George-Rafael Samantsidis *et al.* 'What I cannot create, I do not understand': functionally validated synergism of metabolic and target site insecticide resistance. *Proceedings of The Royal Society B: Biological Sciences* (2020) doi:10.1098/rspb.2020.0838.
2. Macoris, M. L., Martins, A. J., Andrighetti, M. T. M., Lima, J. B. P. & Valle, D. Pyrethroid resistance persists after ten years without usage against Aedes aegypti in governmental campaigns: Lessons from Sao Paulo State, Brazil. *PLoS Negl. Trop. Dis.* **12**, e0006390 (2018).
3. VectorSurv Development Team. VectorSurv - Vectorborne Disease Surveillance System. (2023).
4. Bradbury, S. P. & Coats, J. R. Comparative toxicology of the pyrethroid insecticides. *Rev. Environ. Contam. Toxicol.* **108**, 133–177 (1989).
5. Coats, J. R. Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environ. Health Perspect.* **87**, 255–262 (1990).
6. Mack, L. K. *et al.* Frequency of sodium channel genotypes and association with pyrethrum knockdown time in populations of Californian Aedes aegypti. *Parasit. Vectors* **14**, 141 (2021).
7. Bariami, V., Jones, C. M., Poupardin, R., Vontas, J. & Ranson, H. Gene amplification, abc transporters and cytochrome p450s: Unraveling the molecular basis of pyrethroid resistance in the dengue vector, aedes aegypti. *PLoS Negl. Trop. Dis.* **6**, (2012).
8. Amezian, D., Nauen, R. & Le Goff, G. Transcriptional regulation of xenobiotic detoxification genes in insects - An overview. *Pestic. Biochem. Physiol.* **174**, 104822 (2021).

9. De Marco, L. *et al.* The choreography of the chemical defensome response to insecticide stress: insights into the Anopheles stephensi transcriptome using RNA-Seq. *Sci. Rep.* **7**, 41312 (2017).

10. Vontas, J. *et al.* Gene expression in insecticide resistant and susceptible Anopheles gambiae strains constitutively or after insecticide exposure. *Insect Mol. Biol.* **14**, 509–521 (2005).

11. When do you recommend 3′-Tag RNA-seq? https://dnatech.genomecenter.ucdavis.edu/faqs/when-do-you-recommend-3-tag-rna-seq/.

12. Tibshirani, R., Walther, G. & Hastie, T. Estimating the Number of Clusters in a Data Set via the Gap Statistic. *J. R. Stat. Soc. Series B Stat. Methodol.* **63**, 411–423 (2001).

13. DeJong, R. J. *et al.* Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito Anopheles gambiae. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2121–2126 (2007).

14. Sim, C. & Denlinger, D. L. Catalase and superoxide dismutase-2 enhance survival and protect ovaries during overwintering diapause in the mosquito Culex pipiens. *J. Insect Physiol.* **57**, 628–634 (2011).

15. Magalhaes, T., Brackney, D. E., Beier, J. C. & Foy, B. D. Silencing an Anopheles gambiae catalase and sulfhydryl oxidase increases mosquito mortality after a blood meal. *Arch. Insect Biochem. Physiol.* **68**, 134–143 (2008).

16. Oliver, S. V. & Brooke, B. D. The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors Anopheles arabiensis and Anopheles funestus. *PLoS One* **11**, 1–19 (2016).

17. Tchouakui, M. *et al.* Fitness Costs of the Glutathione S-Transferase Epsilon 2 (L119F-GSTe2) Mediated Metabolic Resistance to Insecticides in the Major African Malaria Vector Anopheles Funestus. *Genes* **9**, (2018).
18. Osoro, J. K. *et al.* Insecticide resistant Anopheles gambiae have enhanced longevity but reduced reproductive fitness and a longer first gonotrophic cycle. *Sci. Rep.* **12**, 8646 (2022).
19. Smith, L. B., Silva, J. J., Chen, C., Harrington, L. C. & Scott, J. G. Fitness costs of individual and combined pyrethroid resistance mechanisms, kdr and CYP-mediated detoxification, in Aedes aegypti. *PLoS Negl. Trop. Dis.* **15**, e0009271 (2021).
20. Martins, A. J. *et al.* Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected aedes aegypti populations. *PLoS One* **7**, 1–9 (2012).
21. Smd, A., Hc, S., Sk, J. & Sreeramulu, K. Effect of pyrethroids, permethrin and fenvalarate, on the oxidative stress of. http://oar.icrisat.org/5934/1/WJST_2_1_01-05_2012.pdf (2012).
22. Terhzaz, S. *et al.* A novel role of Drosophila cytochrome P450-4e3 in permethrin insecticide tolerance. *Insect Biochem. Mol. Biol.* **67**, 38–46 (2015).
23. Brinzer, R. A. *et al.* Metabolomic profiling of permethrin-treated {Drosophila} melanogaster identifies a role for tryptophan catabolism in insecticide survival. *Insect Biochem. Mol. Biol.* **67**, 74–86 (2015).
24. Touaylia, S., Ali, M., Abdellhafidh, K. & Bejaoui, M. Permethrin induced oxidative stress and neurotoxicity on the freshwater beetle Laccophilus minutus. *CHEMISTRY AND ECOLOGY* **35**, 459–471 (2019).

25. Sun, L. *et al.* Differential transcription of cytochrome P450s and glutathione S transferases in DDT-susceptible and -resistant Drosophila melanogaster strains in response to DDT and oxidative stress. *Pestic. Biochem. Physiol.* **100**, 7–15 (2011).

26. Lu, K. *et al.* Activation of CncC pathway by ROS burst regulates cytochrome P450 CYP6AB12 responsible for λ-cyhalothrin tolerance in Spodoptera litura. *J. Hazard. Mater.* **387**, 121698 (2020).

27. Pitoniak, A. & Bohmann, D. Mechanisms and functions of Nrf2 signaling in Drosophila. *Free Radical Biology and Medicine* **88**, 302–313 (2015).

28. Wilding, C. S. Regulating resistance: CncC:Maf, antioxidant response elements and the overexpression of detoxification genes in insecticide resistance. *Curr Opin Insect Sci* **27**, 89–96 (2018).

29. Liu, Y. *et al.* Exploring the correlation between deltamethrin stress and Keap1-Nrf2-ARE pathway from Drosophila melanogaster RNASeq data. *Genomics* **112**, 1300–1308 (2020).

30. Misra, J. R., Lam, G. & Thummel, C. S. Constitutive activation of the Nrf2/Keap1 pathway in insecticide-resistant strains of Drosophila. *Insect Biochem. Mol. Biol.* **43**, 1116–1124 (2013).

31. Ingham, V. A., Pignatelli, P., Moore, J. D., Wagstaff, S. & Ranson, H. The transcription factor Maf-S regulates metabolic resistance to insecticides in the malaria vector Anopheles gambiae. *BMC Genomics* **18**, 669 (2017).

32. Hayes, J. D. & Dinkova-Kostova, A. T. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* **39**, 199–218 (2014).

33. Heiss, E. H., Schachner, D., Zimmermann, K. & Dirsch, V. M. Glucose availability is a decisive factor for Nrf2-mediated gene expression. *Redox Biol* **1**, 359–365 (2013).

34. Araújo, R. A., Guedes, R. N. C., Oliveira, M. G. A. & Ferreira, G. H. Enhanced activity of carbohydrate- and lipid-metabolizing enzymes in insecticide-resistant populations of the maize weevil, Sitophilus zeamais. *Bull. Entomol. Res.* **98**, 417–424 (2008).

35. Karim, M. R., Taniguchi, H. & Kobayashi, A. Constitutive activation of Drosophila CncC transcription factor reduces lipid formation in the fat body. *Biochem. Biophys. Res. Commun.* **463**, 693–698 (2015).

36. Shi, L. & Tu, B. P. Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. *Curr. Opin. Cell Biol.* **33**, 125–131 (2015).

37. Oliver, S. V. & Brooke, B. D. The effect of elevated temperatures on the life history and insecticide resistance phenotype of the major malaria vector Anopheles arabiensis (Diptera: Culicidae). *Malar. J.* **16**, 73 (2017).

38. Dong, B. *et al.* A heat shock protein protects against oxidative stress induced by lambda-cyhalothrin in the green peach aphid Myzus persicae. *Pestic. Biochem. Physiol.* **181**, 104995 (2022).

39. Si, F.-L. *et al.* HSP superfamily of genes in the malaria vector Anopheles sinensis: diversity, phylogenetics and association with pyrethroid resistance. *Malar. J.* **18**, 132 (2019).

40. Traverso, L. *et al.* Transcriptomic modulation in response to an intoxication with deltamethrin in a population of Triatoma infestans with low resistance to pyrethroids. *PLoS Negl. Trop. Dis.* **16**, e0010060 (2022).

41. Zhang, L. J. *et al.* Thermotolerance, oxidative stress, apoptosis, heat-shock proteins and damages to reproductive cells of insecticide-susceptible and-resistant strains of the diamondback moth Plutella xylostella. *Bull. Entomol. Res.* **107**, 513–526 (2017).

42. Patil, N. S., Lole, K. S. & Deobagkar, D. N. Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes Anopheles stephensi and Aedes aegypti. *Med. Vet. Entomol.* **10**, 277–282 (1996).

43. Rosenzweig, R., Nillegoda, N. B., Mayer, M. P. & Bukau, B. The Hsp70 chaperone network. *Nat. Rev. Mol. Cell Biol.* **20**, 665–680 (2019).

44. Ahmed, A. M. Lipid peroxidation and oxidative protein products as biomarkers of oxidative stress in the autogenous mosquito, Aedes caspius, upon infection with the mosquitocidal bacterium, Bacillus thuringiensis kurstaki. *Pak. J. Zool.* **44**, 525–536 (2012).

45. Chen, B., Feder, M. E. & Kang, L. Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Mol. Ecol.* **27**, 3040–3054 (2018).

46. Hoppel, C. L. Carnitine and carnitine palmitoyltransferase in fatty acid oxidation and ketosis. *Fed. Proc.* **41**, 2853–2857 (1982).

47. Barry, W. E. & Thummel, C. S. The Drosophila HNF4 nuclear receptor promotes glucose-stimulated insulin secretion and mitochondrial function in adults. *Elife* **5**, (2016).

48. Wang, X. *et al.* Hormone and receptor interplay in the regulation of mosquito lipid metabolism. *Proceedings of the National Academy of Sciences* **114**, E2709–E2718 (2017).

49. Liao, Y.-J. *et al.* Deficiency of glycine N-methyltransferase results in deterioration of cellular defense to stress in mouse liver. *Proteomics Clin. Appl.* **4**, 394–406 (2010).

50. Marcombe, S. *et al.* Exploring the molecular basis of insecticide resistance in the dengue vector Aedes aegypti: a case study in Martinique Island (French West Indies). *BMC Genomics* **10**, 494 (2009).

51. Fonseca-González, I., Quiñones, M. L., Lenhart, A. & Brogdon, W. G. Insecticide resistance status of Aedes aegypti (L.) from Colombia. *Pest Manag. Sci.* **67**, 430–437 (2011).

52. Estep, A. S., Sanscrainte, N. D., Waits, C. M., Louton, J. E. & Becnel, J. J. Resistance Status and Resistance Mechanisms in a Strain of Aedes aegypti (Diptera: Culicidae) From Puerto Rico. *J. Med. Entomol.* **54**, 1643–1648 (2017).

53. Durant, A. C., Grieco Guardian, E., Kolosov, D. & Donini, A. The transcriptome of anal papillae of Aedes aegypti reveals their importance in xenobiotic detoxification and adds significant knowledge on ion, water and ammonia transport mechanisms. *J. Insect Physiol.* **132**, 104269 (2021).

54. Ishak, I. H. *et al.* The Cytochrome P450 gene CYP6P12 confers pyrethroid resistance in kdr-free Malaysian populations of the dengue vector Aedes albopictus. *Sci. Rep.* **6**, 24707 (2016).

55. Epelboin, Y. *et al.* CYP450 core involvement in multiple resistance strains of Aedes aegypti from French Guiana highlighted by proteomics, molecular and biochemical studies. *PLoS One* **16**, e0243992 (2021).

56. Marcombe, S. *et al.* Distribution of insecticide resistance and mechanisms involved in the arbovirus vector Aedes aegypti in Laos and implication for vector control. *PLoS Negl. Trop. Dis.* **13**, e0007852 (2019).

57. Kasai, S. *et al.* Mechanisms of Pyrethroid Resistance in the Dengue Mosquito Vector, Aedes aegypti: Target Site Insensitivity, Penetration, and Metabolism. *PLoS Negl. Trop. Dis.* **8**, (2014).

58. Faucon, F. *et al.* In the hunt for genomic markers of metabolic resistance to pyrethroids in the mosquito Aedes aegypti: An integrated next-generation sequencing approach. *PLoS Negl. Trop. Dis.* **11**, 1–20 (2017).

59. Cattel, J. *et al.* Combining genetic crosses and pool targeted DNA-seq for untangling genomic variations associated with resistance to multiple insecticides in the mosquito Aedes aegypti. *Evol. Appl.* 303–317 (2019).

60. Liu, X. *et al.* Protective Effects of Spermidine and Melatonin on Deltamethrin-Induced Cardiotoxicity and Neurotoxicity in Zebrafish. *Cardiovasc. Toxicol.* **21**, 29–41 (2021).

61. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559 (2008).

62. Namiki, T. *et al.* Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. *Biochem. Biophys. Res. Commun.* **337**, 367–374 (2005).

63. Pondeville, E. *et al.* Microarray and RNAi analysis of P450s in Anopheles gambiae male and female steroidogenic tissues: CYP307A1 is required for ecdysteroid synthesis. *PLoS One* **8**, e79861 (2013).

64. Gong, Y., Li, T., Zhang, L., Gao, X. & Liu, N. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, Culex quinquefasciatus. *Int. J. Biol. Sci.* **9**, 863–871 (2013).

65. Liu, N., Li, T., Reid, W. R., Yang, T. & Zhang, L. Multiple Cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, Culex quinquefasciatus. *PLoS One* **6**, e23403 (2011).

66. Al Nazawi, A. M., Aqili, J., Alzahrani, M., McCall, P. J. & Weetman, D. Combined target site (kdr) mutations play a primary role in highly pyrethroid resistant phenotypes of Aedes aegypti from Saudi Arabia. *Parasites and Vectors* **10**, (2017).

67. Rault, L. C., O'Neal, S. T., Johnson, E. J. & Anderson, T. D. Association of age, sex, and pyrethroid resistance status on survival and cytochrome P450 gene expression in Aedes aegypti (L.). *Pestic. Biochem. Physiol.* **156**, 96–104 (2019).

68. Ptitsyn, A. A. *et al.* Rhythms and synchronization patterns in gene expression in the Aedes aegypti mosquito. *BMC Genomics* **12**, 153 (2011).

69. Lumjuan, N. *et al.* The Aedes aegypti glutathione transferase family. *Insect Biochem. Mol. Biol.* **37**, 1026–1035 (2007).

70. Grant, D. F. & Hammock, B. D. Genetic and molecular evidence for a trans-acting regulatory locus controlling glutathione S-transferase-2 expression in Aedes aegypti. *Mol. Gen. Genet.* **234**, 169–176 (1992).

71. Otali, D. *et al.* Increased production of mitochondrial reactive oxygen species and reduced adult life span in an insecticide-resistant strain of Anopheles gambiae. *Bull. Entomol. Res.* **104**, 323–333 (2014).

72. Wu, C., Chakrabarty, S., Jin, M., Liu, K. & Xiao, Y. Insect ATP-Binding Cassette (ABC) Transporters: Roles in Xenobiotic Detoxification and Bt Insecticidal Activity. *Int. J. Mol. Sci.* **20**, (2019).

73. Kalsi, M. & Palli, S. R. Cap n collar transcription factor regulates multiple genes coding for proteins involved in insecticide detoxification in the red flour beetle, Tribolium castaneum. *Insect Biochem. Mol. Biol.* **90**, 43–52 (2017).

74. Clemons, A., Mori, A., Haugen, M., Severson, D. W. & Duman-Scheel, M. Culturing and egg collection of Aedes aegypti. *Cold Spring Harb. Protoc.* **5**, 1–6 (2010).

75. Brogdon, W. G. & Chan, A. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. *CDC Methods* 1–28 (2012).

76. Andrews, S. *et al.* FastQC. Preprint at (2012).
77. Bushnell, B. *BBMap: A fast, accurate, splice-aware aligner*. https://www.osti.gov/biblio/1241166 (2014).
78. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
79. Matthews, B. J. *et al.* Improved reference genome of Aedes aegypti informs arbovirus vector control. *Nature* **563**, 501–507 (2018).
80. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
81. R Core Team. R: A Language and Environment for Statistical Computing. Preprint at https://www.R-project.org/ (2023).
82. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression dataB. *Bioinformatics* **26**, 139–140 (2009).
83. Alexa, A. & Rahnenführer, J. Gene set enrichment analysis with topGO. *Bioconductor Improv* **27**, 1–26 (2009).

Acknowledgments

The authors thank Anton Cornel and Katherine Brisco for providing the mosquito eggs for this experiment. This work funded by the Bill Hazeltine Student Research Scholarship. Funding for the lab is received partially from Agricultural Experiment Station Grant (NIFA) - CA-D-ENM-2477-H. This study was supported by cooperative agreement U01CK000649 funded by the U.S. Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and

Prevention or the Department of Health and Human Services. The library preparations and sequencing were carried out at the UC Davis Genome Center DNA Technologies and Expression Analysis Core, supported by NIH Shared Instrumentation Grant 1S10OD010786-01.

Author Contribution Statement

LKM and GMA developed the question and methods for this experiment, as well as reviewed the manuscript. LKM completed all sample acquisition steps, analysis, and manuscript preparation.

Data Availability Statement

Raw data for this project can be found in the Sequence Read Archive with accession number: PRJNA988225. Reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA988225?reviewer=31a7vt72pisnn88mt80v4i28a5

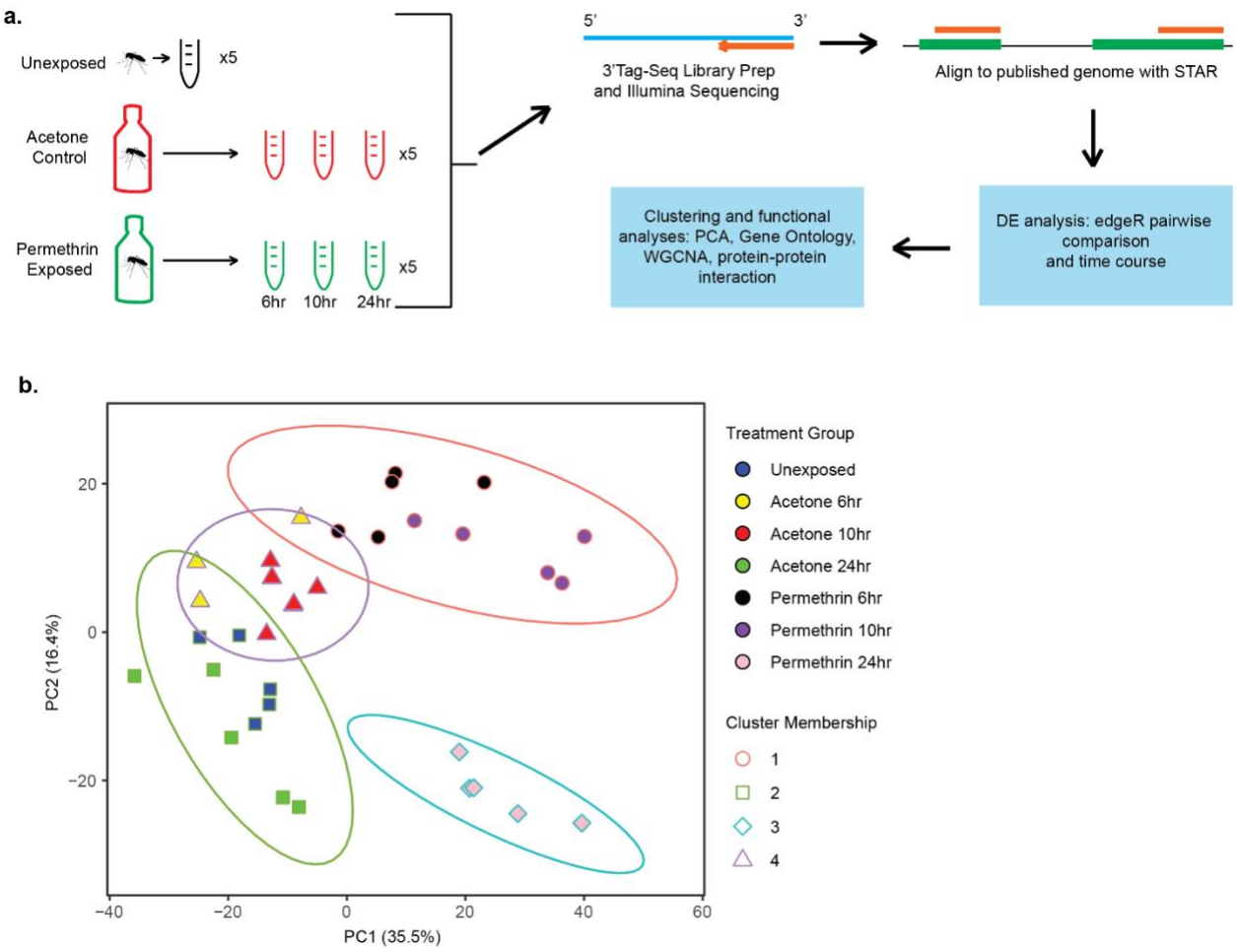


Figure 1. Summary of experimental design and cluster analysis of expression profiles. a) Depiction of experimental design and b) Principal component analysis of all samples using log transformed gene counts. Kmeans clustering analysis indicated 4 clusters to be ideal for the amount of variation in this dataset. Biplot available in Supp. Fig 1.

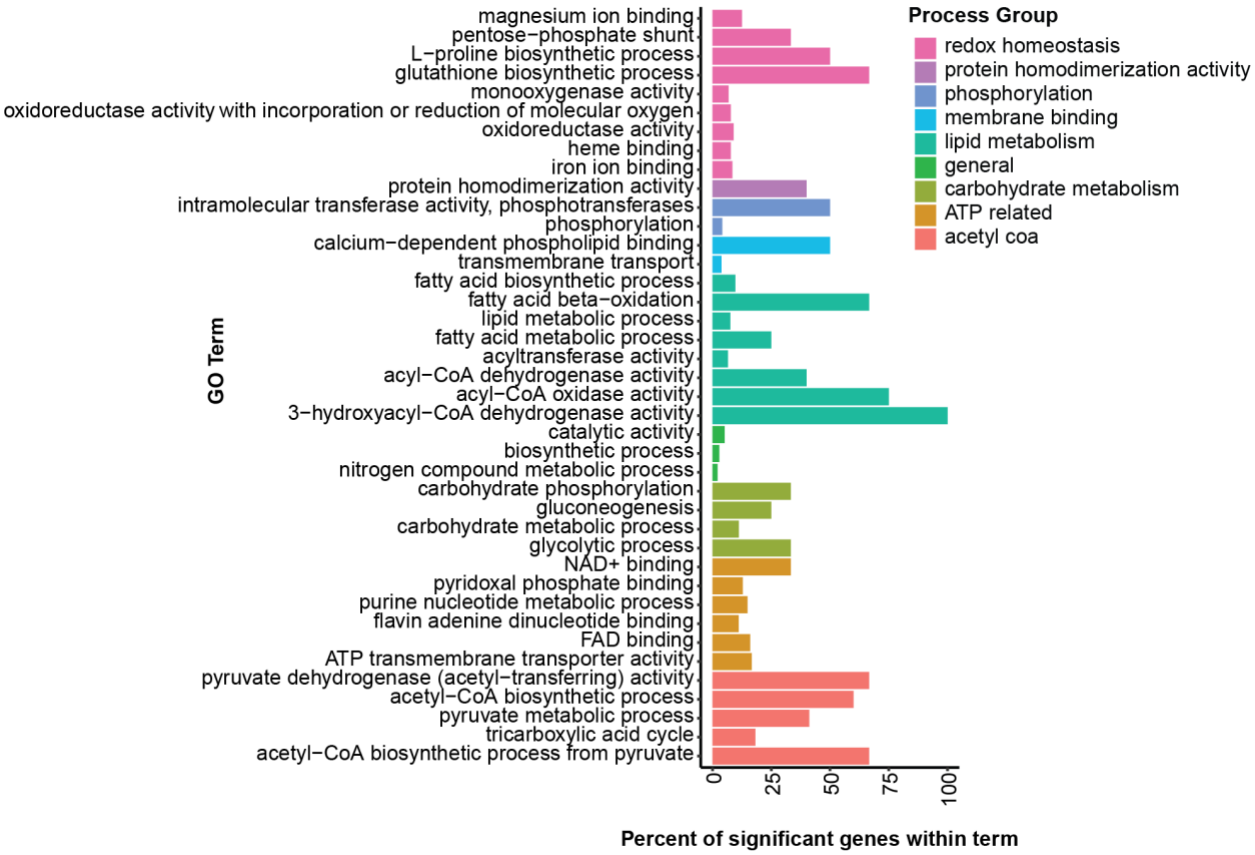


Figure 2. Gene ontology analysis of genes upregulated throughout the time course. Time course modeled using cubic regression spline curve and pairwise comparison between treatments. A high percent of significant genes within a term indicates a large proportion of genes annotated with a specific term were found significantly upregulated in this analysis.

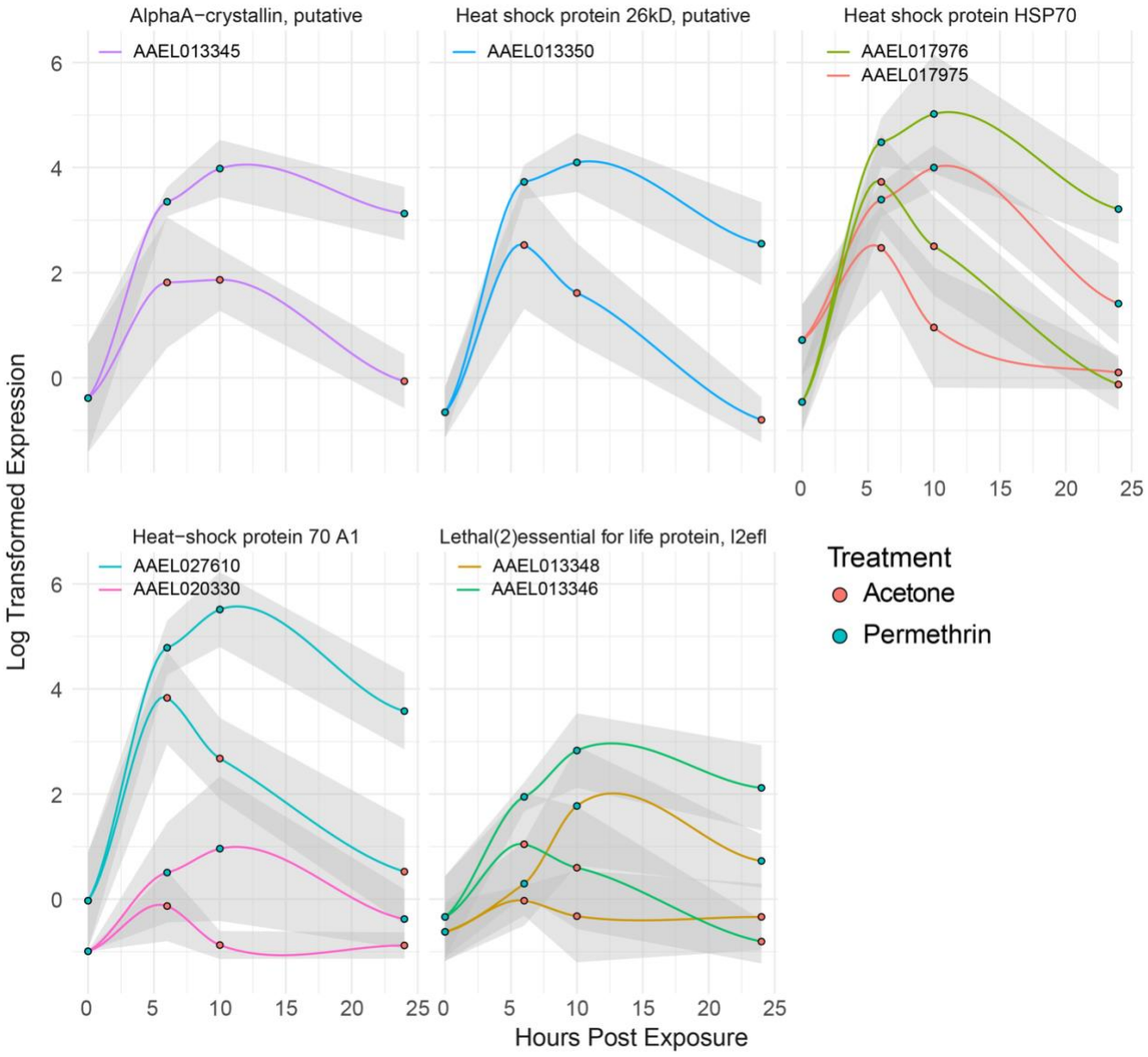


Figure 3. Heat shock proteins experience large fold changes over time after exposure to permethrin. Genes are faceted by product descriptions found in Vectorbase, and 95% CI shown with grey shading. *AlphaA-crystallin* is a small HSP (Jakob et al 1993). *l2efl* is a member of the HSP20 family (Runtuwene et al 2020)

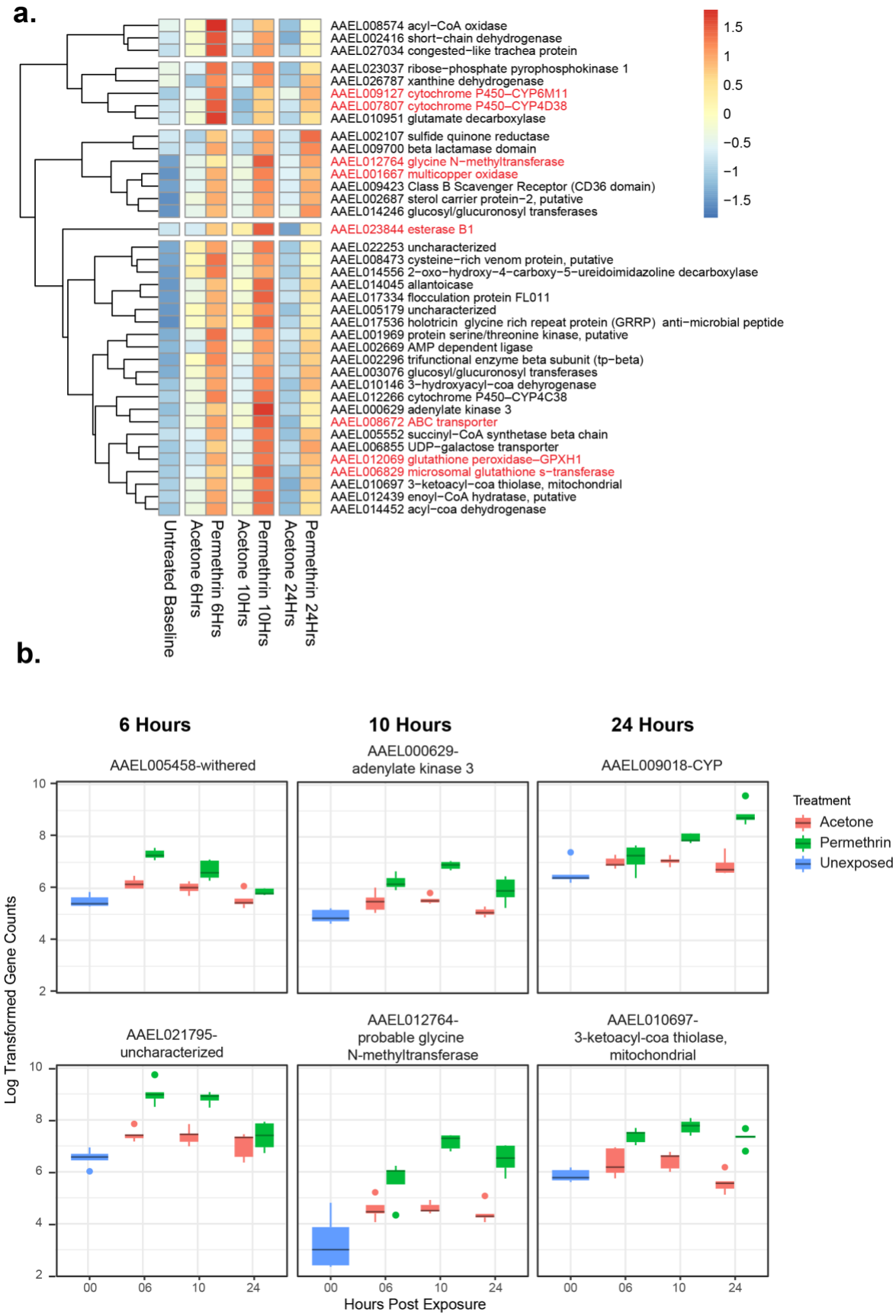

a.
AAEL008574 acyl−CoA oxidase
AAEL002416 short−chain dehydrogenase
AAEL027034 congested−like trachea protein
AAEL023037 ribose−phosphate pyrophosphokinase 1
AAEL026787 xanthine dehydrogenase
AAEL009127 cytochrome P450−CYP6M11
AAEL007807 cytochrome P450−CYP4D38
AAEL010951 glutamate decarboxylase
AAEL002107 sulfide quinone reductase
AAEL009700 beta lactamase domain
AAEL012764 glycine N−methyltransferase
AAEL001667 multicopper oxidase
AAEL009423 Class B Scavenger Receptor (CD36 domain)
AAEL002687 sterol carrier protein−2, putative
AAEL014246 glucosyl/glucuronosyl transferases
AAEL023844 esterase B1
AAEL022253 uncharacterized
AAEL008473 cysteine−rich venom protein, putative
AAEL014556 2−oxo−hydroxy−4−carboxy−5−ureidoimidazoline decarboxylase
AAEL014045 allantoicase
AAEL017334 flocculation protein FL011
AAEL005179 uncharacterized
AAEL017536 holotricin glycine rich repeat protein (GRRP) anti−microbial peptide
AAEL001969 protein serine/threonine kinase, putative
AAEL002669 AMP dependent ligase
AAEL002296 trifunctional enzyme beta subunit (tp−beta)
AAEL003076 glucosyl/glucuronosyl transferases
AAEL010146 3−hydroxyacyl−coa dehyrogenase
AAEL012266 cytochrome P450−CYP4C38
AAEL000629 adenylate kinase 3
AAEL008672 ABC transporter
AAEL005552 succinyl−CoA synthetase beta chain
AAEL006855 UDP−galactose transporter
AAEL012069 glutathione peroxidase−GPXH1
AAEL006829 microsomal glutathione s−transferase
AAEL010697 3−ketoacyl−coa thiolase, mitochondrial
AAEL012439 enoyl−CoA hydratase, putative
AAEL014452 acyl−coa dehydrogenase
1.5
1
0.5
0
−0.5
−1
−1.5
Untreated Baseline
Acetone 6Hrs
Permethrin 6Hrs
Acetone 10Hrs
Permethrin 10Hrs
Acetone 24Hrs
Permethrin 24Hrs
b.
6 Hours
10 Hours
24 Hours
AAEL005458-withered
AAEL000629-
adenylate kinase 3
AAEL009018-CYP
AAEL021795-
uncharacterized
AAEL012764-
probable glycine
N-methyltransferase
AAEL010697-
3-ketoacyl-coa thiolase,
mitochondrial
Log Transformed Gene Counts
Hours Post Exposure
00
06
10
24
Treatment
Acetone
Permethrin
Unexposed

Figure 4. Summary of pairwise comparisons. A. Heat map of genes upregulated at all time points (FDR < 0.05), red text indicates detoxifying genes. B. Top 2 upregulated genes with smallest FDR at each time point.

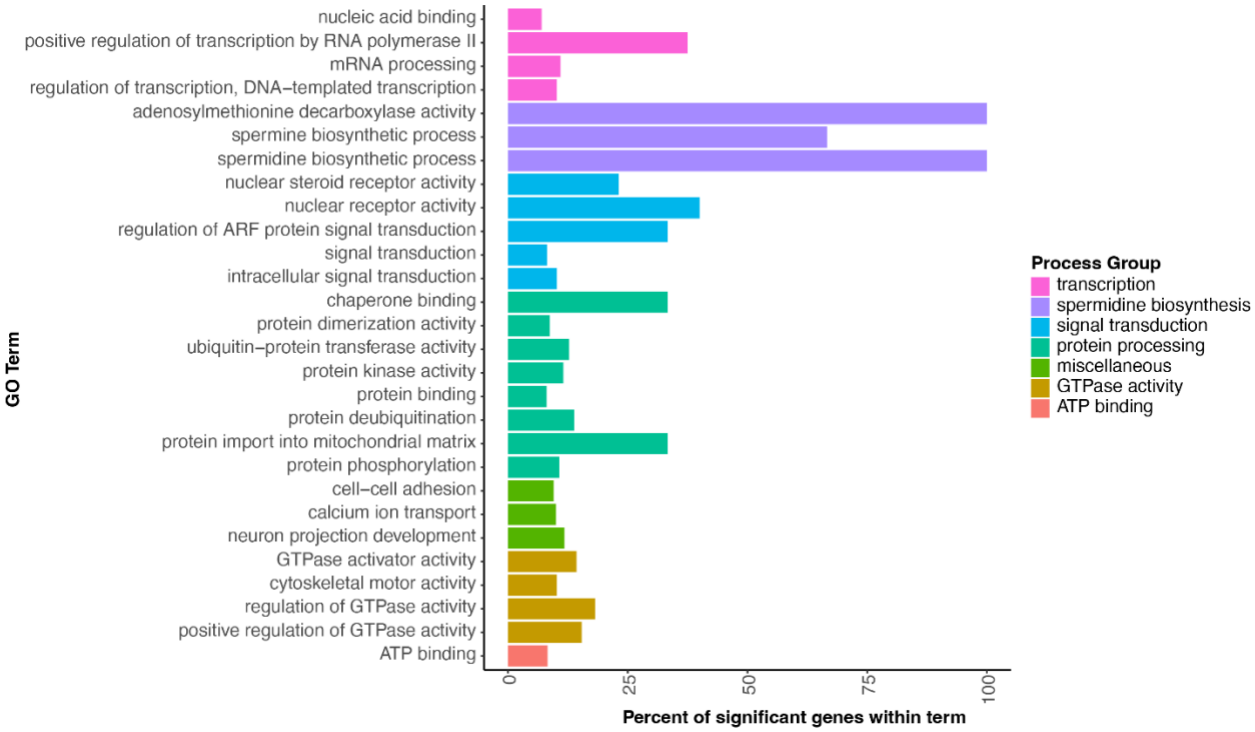


Figure 5. Gene Ontology analysis of downregulated genes at 24 hours post exposure. Genes with FDR < 0.05 and logFC < 0 included in analysis.

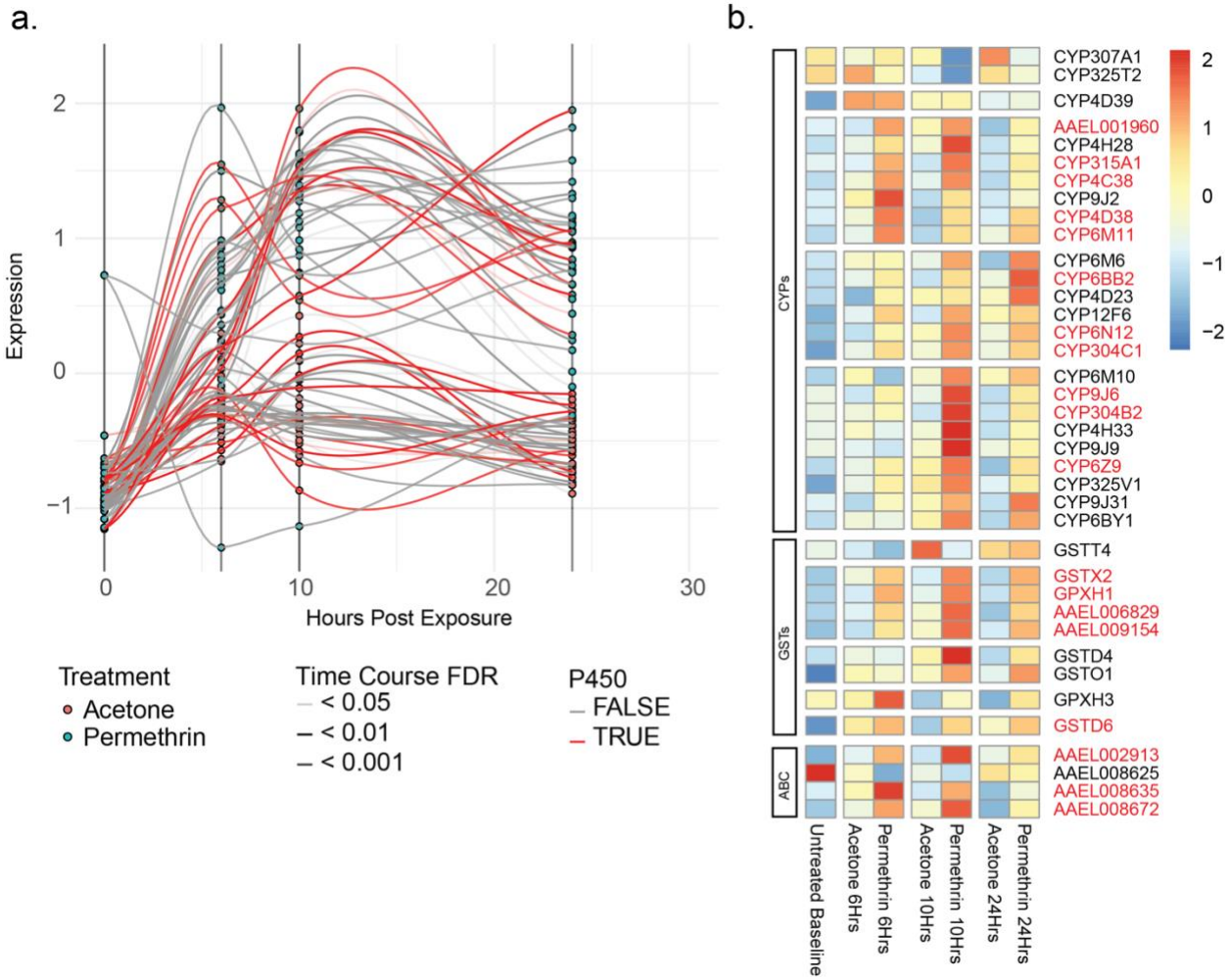


Figure 6. Summary of oxidoreductase activity genes in the WGCNA module with the highest correlation to treatment. A. Line plot of oxidoreductase activity gene expression over time highlighting the domination of CYPs within this group. B. Heat map of CYPs, GSTs, and ABC transporters within this module, regardless of FDR. Red text indicates an FDR < 0.05.

Long read RNA-seq reveals upregulation of detoxifying enzymes and downregulation of growth rate related genes after permethrin exposure in developed ovaries of Aedes aegypti

Lindsey K. Mack[1], Dula Parkinson[2], Matthew Pinch[3], Erin Taylor Kelly[1], Immo Hansen[3], and Geoffrey M. Attardo[1]

1. Department of Entomology and Nematology, University of California, Davis, Davis, CA
2. Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA
3. Department of Biology, New Mexico State University, Las Cruces, NM

Abstract:

Stress responses are energetically costly, but they can also prime an insect to encounter future stressors. Climate change is driving dramatic environmental perturbations and stressors are increasing in intensity and frequency, particularly for the mosquito, *Aedes aegypti.* While some insects will be diminished due to stress, it appears *Ae. aegypti* is thriving. Once restricted to tropical environments, this mosquito has now spread to much drier, hotter locations. This is unfortunate, as *Ae. aegypti* transmits many arboviruses, including dengue, chikungunya, and yellow fever. Additionally, this insect displays high levels of pyrethroid resistance globally. Pyrethroids are commonly used pesticides across industries, and resistance to these chemicals has increased across taxa. For *Ae. aegypti* this means that the chance of encountering a sublethal dose is high. Sublethal exposures can improve survival to subsequent exposures, and these effects can be experienced transgenerationally, without mutations occurring. To detect and explore potential transgenerational effects of insecticide exposure, we employed Nanopore sequencing of developed *Ae. aegypti* ovaries after maternal permethrin exposure. Through differential expression analysis, we discovered 11 stress response genes upregulated in permethrin treated ovaries, along with an upregulation of genes involved in DNA mismatch repair. Additionally, mitochondrial and ribosomal component genes were downregulated, in which suggests that the development rate of resulting offspring will be lower. Further studies will need to be performed to identify the functional effects of these results, but significant changes to offspring physiology appear to occur as a result of maternal permethrin exposure. This has implications for vector control and should be explored to determine the effectiveness of pyrethroid use over time.

Introduction:

Stress events have immediate and long-term effects on organisms. All stress responses require allocation of resources to carry out said response, resulting in tradeoffs with other fitness-enhancing processes. Additionally, stressors can cause damage to molecular and cellular structures in the body, leading to decrease in physiological condition. However, stress events can prime organisms for future encounters with stressors. For example, experiencing multiple stressors in quick succession can protect an individual from deleterious effects of the stressor, though extending the time between events reduces the protective nature (1).

Some stressors induce hormesis, a response in which low doses are beneficial to the organism while very high doses are toxic (2). In the context of insecticide exposure, insects can experience improved tolerance and increased reproduction after sublethal doses (3–6). These benefits can be experienced transgenerationally as well through epigenetic mechanisms (7–10).

This phenomenon is of growing concern, as global insecticide resistance is an issue across taxa. Prevalence of pyrethroid resistance is particularly high as pyrethroids are used in agricultural, public health, and structural pest treatments. Resistance is developed through mutations at the target site, increased expression or overactivation of detoxifying proteins, or thickening of the cuticle to reduce penetration of the chemical (11). This multifaceted defense leads to reduced efficacy of common pest control methodologies.

A pest of particular concern are mosquitoes, the deadliest animal in the world, as the diseases they transmit account for about 700,000 deaths per year according to the WHO (12). They carry the pathogens that cause a wide range of diseases, like malaria, dengue, chikungunya, and yellow fever. In the field of mosquito biology, *Aedes aegypti* accounts for a vast majority of the basic

research on these organisms, due to the ease with which they are reared in a lab environment. Part of this ease is due to their unique, desiccation-resistant eggs, which remain viable for months after deposition. They are also a highly anthropophilic species, preferring to feed on and live near humans. This biology allows them to easily invade new environments, making them a particularly frustrating pest. Native to sub-Saharan Africa, *Ae. aegypti* is now found on all continents aside from Antarctica (13).

In the last decade, *Ae. aegypti* has invaded, established, and spread throughout California (14,15). Population genetics show evidence of 3 separate introductions into the state from differing genetic backgrounds, and all 3 of these genetic clusters indicate high levels of pyrethroid resistance (14,16). Mosquitoes undergo rapid evolution due to their short lifespans and large egg clutch size, though *Ae. aegypti* that invaded California arrived in the state resistant to pyrethroids (17). Similar dynamics exist in other locations and the environmental changes associated with climate change facilitate continuing invasion by *Ae. aegypti*.

California's landscape, particularly in the Central Valley, seems especially conducive to the persistence of pyrethroid resistance. According to the US Geological Service, the Central Valley produces 25% of the country's food while occupying less than 1% of the total agricultural land in the US. Epigenetic evidence of "ambient" pyrethroid exposure was found in humans in communities near agricultural land in the Central Valley (18). We can assume *Ae. aegypti* experience similar sublethal exposure to pyrethroids in these communities, maintaining resistance even when public health professionals are not using pyrethroids to target them. As previously stated, sublethal insecticide doses can trigger a hormetic response resulting in improved tolerance and increase reproduction in insects. Exploring these dynamics in mosquitoes could be helpful to improved understanding of sublethal pyrethroid effects on these insects.

Anautogenous mosquitoes like *Ae. aegypti* are an excellent model system for exploring generational changes and reproductive physiology. Their reproductive cycles are only initiated by a bloodmeal, synchronizing oogenesis, and making it easy to collect enough tissue for high-throughput applications. In just 72 hours, a mother develops a full clutch of eggs ready to be oviposited (Fig. 1). Along with the potential benefits of sublethal insecticide exposure, exposure can also lead to tradeoffs leading to decreased fitness. How are mothers balancing their limited resources with providing their offspring with the best chance of survival?

To further explore the effects of a sublethal insecticide exposure on oogenesis and the ovarian transcriptome, we performed high-throughput gene expression analysis on developed oocytes after maternal exposure. We hypothesize that changes to the transcriptome of ovaries after exposure to permethrin will indicate significant fitness improvements for resulting offspring. Improving our understanding of the sublethal effects of insecticides in mosquitoes can assist in vector control decisions and the production of novel control mechanisms.

Results and Discussion:

Differential expression analysis reveals transcriptomic downregulation to a higher degree than upregulation:

To explore differentially expressed genes in oocytes after maternal exposure to permethrin, we performed a differential expression analysis comparing permethrin exposed and acetone control groups (Fig. 2). Of the 9065 genes expressed in this dataset, 3545 genes had an adjusted p value of less than 0.05, 1639 with a log2 fold change greater than 0 and 1906 with a log2 fold change of less than 0. Further filtering these genes by degree of fold change, 1532 genes had a log2 fold change greater than 1, 81 upregulated and 1450 downregulated. We did not hypothesize this degree of difference in upregulation versus downregulation, it seems that oocytes are depleted more than

they are provisioned. To validate the homogeneity of expression profiles in samples from identical treatments, a principal component analysis was conducted. Samples clustered well by treatment with principal component 1 explaining 73.38% of the variation and principal component 2 with 8.56% (supp. Fig. 1). Overall, it appears that permethrin treatment results in significant differences in ovarian gene expression.

Insecticide response related genes are upregulated in permethrin treated ovaries while the *vgsc* gene is downregulated:

To assess the association between stress response gene expression in the ovaries and permethrin exposure, we specifically explored upregulated differential expression results for genes involved in stress response. Eleven stress response genes were upregulated in the permethrin treated group. These include heat shock proteins, ATP-binding cassette (ABC) transporters, cytochrome P450s (CYPs), glutathione synthetase, and catalase (Fig. 3). Three of these were upregulated in response to permethrin in mothers as observed in chapter 2. CYP4C38 was upregulated across time points in chapter 2, and was found to respond to permethrin in *Culex quinquefasciatus* (19). CYPs are tasked with the first step of detoxification. These enzymes are well associated with insecticide resistance (11). Glutathione synthetase is part of the glutathione biosynthesis pathway and acts as an antioxidant, and glutathione-s-transferases are involved in the second step of detoxification (20). ABC transporters are involved in expulsion of insecticide or modified insecticide out of the cell. This is the final stage of detoxification and necessary for excretion of byproducts from the body (21). Provisioning oocytes with these genes could increase insecticide tolerance.

Maternal deposition of mRNAs related to environmental stressors has been described in *Drosophila* in the context of heat shock protein genes. When a small heat shock protein gene was maternally loaded into oocytes, *Drosophila* larvae were more tolerant of heat stress (10).

Heat shock proteins are molecular chaperones whose role is to help fold proteins or repair misfolded proteins. Reactive oxygen species produced by stress can cause damage to proteins, so heat shock proteins are necessary stress response genes. Three heat shock proteins were upregulated in the permethrin treated group, though only at a fold change of around 0.5 (AAEL017315, p=0.0025; AAEL025697, p=0.0044; AAEL000301, p=0.0143) (Fig. 3). Cross tolerance between heat and pyrethroids was demonstrated in mosquito larvae. Exposure to heat shock prior to a pyrethroid proved to increase pyrethroid tolerance and vice versa to a lesser extent (22). Priming of offspring following a pyrethroid exposure may lead to increased thermal tolerance, which is an important consideration in a changing climate.

Catalase is responsible for the breakdown of hydrogen peroxide, a reactive oxygen species, into 2 water molecules. It is associated with many physiological processes, including fecundity and insecticide tolerance (23). Expression of catalase increases in response to a blood meal, and is necessary for high fecundity in mosquitoes (24). Oxidative stress following insecticide exposure causes increases in catalase even up to and possibly beyond 24 hours post exposure as observed in chapter 2. Increases in this enzyme in the permethrin treated group may indicate investment by the mother to improve her fecundity.

Finally, pyrethroids target voltage gated sodium channels, binding to open channels resulting in constant depolarization of the membrane, paralysis, and death (25). Interestingly, the permethrin treated group experienced a 1.7-fold decrease in expression of the *vgsc* gene when compared to the control (AAEL013277, p=0.0375). This gene is required for survival, as it propagates action potentials across axons, a process necessary for movement and nervous system function in general. In *Drosophila,* a mutation in *maleless,* reduces expression of these channels in homozygous individuals. These individuals experience reduced eclosion and increased susceptibility to low and

high temperatures (26). Additional experiments are necessary to determine if similar dynamics exist in offspring of permethrin exposed mothers, or if the increase in heat shock protein expression could protect them from heat shock.

Maternal permethrin treatment results in downregulation of protein production machinery:

To explore functional groups among the downregulated genes, a gene ontology analysis and pathway analysis were performed. Among the most overrepresented groups and pathways were genes involved in various components of protein production, primarily ribosomes (Supp. Tables 1&4). Every ribosomal protein gene identified within this dataset (31 60S subunits and 23 40S subunits) is downregulated around 2-fold (Fig. 4B). The implications of this are vast. In a new study using a mouse model, researchers determined that oocyte ribosome occupancy determined protein synthesis in the zygote using an individual zygote (27). The reduction in ribosomal protein genes may indicate slower embryonic development due to decreased protein availability, which could be advantageous in a stressful environment. Essentially, it could allow the offspring to "wait out" the stressor, keeping them safe within their protective egg until a more suitable environment is present. However, this could also be indicative of a resource tradeoff.

In *Drosophila*, eggs from mothers that experienced larval nutritional stress, ribosomal protein genes and ribosome biogenesis were downregulated (28). Additional studies have shown a connection between the juvenile hormone receptor, Methoprene tolerant (Met), expression and ribosomal protein L32 (RpL32) expression (29). Met expression is tightly regulated by nutritional condition. Met is a juvenile hormone (JH) receptor; which acts a transcription factor regulating vitellogenic gene expression. Juvenile hormone primes the mosquito body for vitellogenesis, providing signals on the nutritional condition of the mother. Methoprene is an analog of JH, and topical treatment of mosquitoes with Methoprene mimics the effects of a 20% sucrose diet (30).

As evidenced in chapter 2, mothers treated with permethrin experience a massive upregulation in genes related to the metabolism of stored energy products. This may indicate that the mothers were nutritionally depleted when they were bloodfed for this experiment, which would result in a reduction in expression of ribosomal protein genes.

The proteasome complex is necessary for maintaining protein homeostasis in the cell, breaking down damaged proteins (31). Proteolysis plays important roles in insect development. Among the downregulated functional categories and pathways are proteosome complex processes (Supp Tables 1&4). In the brown planthopper (*Nilaparvata lugens),* researchers discovered that proteasome activity is necessary for the transcription of lipase, vitellogenin, and some Halloween genes (32). These genes are all required for the process of oogenesis. Lipase (AAEL007055, p=0.031) and vitellogenin (AAEL006966, pvalue=0.00059) are downregulated in the ovaries after permethrin exposure, but none of the Halloween genes experience a significant change. Paradoxically, upregulation of proteasomes have been identified to illicit resistance to deltamethrin, a pyrethroid, in mosquito cells and that the addition of a proteasome inhibitor increased sensitivity to the insecticide (33). It is unclear what effect downregulation of the proteasome complex may have on offspring of permethrin treated mothers.

<u>Downregulation of mitochondrial components and upregulation of mitophagy pathway indicate mitochondrial damage resulting from permethrin treatment:</u>

Damaged mitochondria can cause oxidative stress due to production/escape of reactive oxygen species from mitochondrial processes. To combat these damaging molecules, cells perform mitophagy, autophagy directed mitochondrial death. Based on a pathway analysis of the upregulated genes in this dataset, the mitophagy pathway was significantly overrepresented (p=0.0327). Upregulated genes within this pathway include those coding for PINK1

(AAEL011594, log2FC=0.743, FDR=0.0004) and TBK1 (AAEL020848, log2FC=0.773, FDR=0.005) (Fig 5A). PINK1 is responsible for ubiquitin directed mitophagy, it is activated by various damage signals (34). PINK1 recruits parkin to the damaged mitochondria which ubiquitinates Miro and Mfn1 repressing them. Miro is involved in movement of mitochondria while Mfn1 is involved in mitochondrial fusion. Repressing these two genes keeps damaged mitochondria from causing widespread damage. Interestingly, both genes are upregulated in the permethrin treated group (Miro, AAEL001853, log2FC=0.962, FDR=$6.4x10^{-7}$) (Mfn1, AAEL004471, log2FC=0.847, FDR=$9.8x10^{-6}$), which may be indicative of mitochondrial stress because the oocyte is attempting to make use of a limited number of mitochondria. TBK1 phosphorylates autophagy receptors, which initiate autophagy of damaged mitochondria (35). TBK1 is also upregulated (AAEL020848, log2FC=0.773, FDR=0.004).

Gene ontology analysis of the downregulated genes revealed additional evidence of decreased mitochondrial function in the permethrin treated group. Genes with reduced transcript abundance represent biological processes involved in mitochondrial function including: NADH dehydrogenase activity, cytochrome c oxidase activity, molecular functions involved in general electron transport chain activity, and cellular components that make up the mitochondria including mitochondrial ribosomes and the inner membrane (fig. 5B). These processes take place in the mitochondria and produce energy for the cell. Additionally, pathway analysis found oxidative phosphorylation and the pentose phosphate pathway overrepresented in the downregulated genes (Supp Table 1). Other studies have found a decrease in the oxidative phosphorylation pathway as a result of sublethal insecticide exposure as well (36). This all may be related to mitophagy as a way to mediate oxidative stress, as cap n' collar, a well-known mediator of oxidative stress, was shown to activate mitophagy in *Drosophila* (37). Cap n' collar is activated by increases in reactive

oxygen species and is a central player in the xenobiotic response, associated with insecticide exposure (38).

Evidence of genotoxic effects of permethrin in ovaries:

Exposure to pyrethroids causes a significant production of reactive oxygen species (ROS), from increases in metabolic processes as well as the increased functioning of CYPs, as observed in chapter 2. ROS are molecules containing unpaired electrons which are highly reactive, stealing electrons from other molecules. An imbalance between ROS and antioxidants can lead to oxidative stress in cells, resulting in damage to DNA, proteins, and lipids.

Mosquito ovaries are likely highly susceptible to DNA damage due to the continuous production of oocytes throughout the life of the mosquito, as well as the impressive increase in mitotic activity in follicular cells after a bloodmeal (i.e. development of ovaries) (Fig. 1) (39). Clear signs of DNA damage are present in this dataset, primarily in the upregulation of mismatch repair and translesion synthesis related genes (Fig. 6B, supp. fig 2). Studies in *Drosophila* have shown that organism condition affects the particular repair mechanism employed by individuals, as some are more energetically taxing than others or can repress each other (40–43). For example, mismatch repair represses homologous recombination when there are too many mismatches in a strand, a dynamic that appears to exist in permethrin treated ovaries (fig. 6) (43). Cellular stress can increase the rate of mutation, which can be explained by DNA damage (44). Interestingly, the physiological condition of the mother may increase her likelihood of passing mutations to her offspring as well (45). Further evidence that mothers in our experiment are passing more mutations to their offspring is the upregulation of translesion synthesis machinery which incorporates unrepaired mutations into replicated DNA. Studies in vertebrates have shown many pyrethroids to have genotoxic effects due to increases in ROS in the cell, however these effects have not been studied extensively

in insects, likely because these compounds are meant to be detrimental to insects (46–49). However, exploring genotoxic effects in insects could give more context and understanding to how they are able to evolve resistance so rapidly to insecticides.

DNA damage can also cause downregulation of histone protein genes, as seen in this dataset (Fig. 7) (50). This can have developmental consequences, like those observed in *C. elegans* in mutants with H3.3 mutations. Interestingly, depletion of H3.3 assembly factors leads to an adult-onset lethal effect (51). Histones are also important for regulating gene expression, as histone modifications are primary drivers of epigenetic inheritance. In permethrin treated ovaries, we see an upregulation of histone lysine-N-methyltransferases (Fig. 7B).

A differential transcript usage analysis was also performed to help us understand the differences in expression of gene isoforms caused by alternative splicing or promoter usage among the different groups. Sixty-two genes displayed differential transcript usage, though no common functional features were found within these genes by gene ontology analysis (supp. table 3). One of the most interesting among these genes (*AAEL010826-RA -RB*), has two splice isoforms that encode different biochemical functions. The *AAEL010826-RA* isoform contains a domain coding for a histone-N-methyltransferase and shows increased transcript abundance in the permethrin treated group, while the isoform dominant in the control codes for a eukaryotic transcription initiation factor (eIF2) (Fig. 8). Based on the structure of these two transcripts, only two small exons on the 5' end of the gene is shared (Fig. 8). Interestingly, the fusion of these two transcripts appears to be a common feature in insects, dating back 400 million years (52). The orthologs of these isoforms in *Drosophila* are well studied and function in H3-K9 methylation, which is associated with heterochromatin formation leading to gene silencing, as well as GTP and methionine binding to initiate translation (53,54). Based on function, these isoforms are active in

the nucleus and cytoplasm, respectively, presenting further questions for consideration. This gene and its function in pyrethroid mediated developmental changes should be investigated in *Ae. aegypti* to expand our understanding of epigenetic mechanisms of pyrethroid resistance.

Conclusion:

Permethrin exposure greatly affects the transcriptome of developed ovaries in *Ae. aegypti,* with evidence of maternal priming for stress tolerance, nutritional trade-offs, and DNA damage. Provisioning of stress tolerance genes and DNA damage have significant implications for the evolution of insecticide resistance and should be investigated further for a deeper understanding of the mechanisms behind the development of insecticide resistance. More specific epigenetic analysis, like quantification and specificity of methylation, of mosquitoes after exposure to pyrethroids would be helpful in further characterizing heritable effects of pyrethroid exposure. This is only the first study of insecticide effects on developed ovaries of *Ae. aegypti* but raises many questions and new avenues of investigation into the evolution of pyrethroid resistance.

Methods:

Mosquitoes:

Mosquitoes for this project were collected from Grace Ranch (38.42508778383484, -122.87800484630372) in the Central Valley of California. These were reared according to existing protocols in the lab for 4 generations prior to use for experiments (55).

Five-day old adult female mosquitoes were exposed to 43 ug/mL permethrin in acetone via coated 250 mL Wheaton bottles for 30 minutes. Control samples were placed in bottles coated with 1 mL acetone for 30 minutes. After 30 minutes, treated adults were returned to a clean cage to recover. Two days after exposure, mosquitoes were offered a blood meal, and all mosquitoes exhibiting the

signature red belly indicating a successful blood meal were removed from the cage and placed into another clean cage.

MicroCT imaging:

The Rockefeller lab strain of *Ae. aegypti* were reared according to existing protocols (55). Five days post eclosion, unfed female mosquitoes were placed in Bouin's solution for fixation, followed by alcohol dehydration, while the rest were bloodfed. Seventy-two hours later, bloodfed mosquitoes were collected in the same way. Fixed samples were placed in 600mL microcentrifuge tubes filled with hand sanitizer and sent to the Lawerence Berkeley National Laboratory Advanced Light Source to be scanned on beamline 8.3.2.

Scans were assembled and segmented using Dragonfly v 2022.1 (Object Research Systems).

RNA sample collection:

Three days later, ovaries were dissected out of 15 individuals per replicate, with 4 replicates for the permethrin treated group and 3 replicates for the acetone group. Ovaries were collected directly into the lysis buffer provided with the Zymo Quick RNA Miniprep kit (R1054) and crushed using a plastic pestle. RNA was then extracted from all samples following the kit procedure. RNA quality was checked by Nanodrop One as well as Agilent Bioanalyzer for accurate quantification.

RNA samples were submitted to the UC Davis Genome Center (UCDGC) (1 ug per replicate) for cDNA library prep and Oxford Nanopore sequencing via Promethion. cDNA preparation was performed using the NEBNext Single Cell/Low Input cDNA synthesis kit with VNP and SSP primers from the DCS109 kit from Oxford Nanopore and barcoded with the PBC096 kit from Oxford Nanopore. Samples were then sequenced on a PromethION sequencer.

Mapping:

Reads ranged from 4.24 million reads (permethrin sample 3) to 7 million reads per sample (acetone sample 1). Data files were concatenated and filtered by length and quality using fastcat (56). To orient reads and trim adapters, we used pychopper (57). Rescued reads were analyzed with pychopper a second time in the reverse orientation to collect all quality reads. All pychopper passing reads were concatenated for mapping. Once prepared, reads were mapped to the *Aedes aegypti* genome (AaegyptiLVP-AGWG-62) from Vectorbase using minimap2, with an average of 99% mapping to the genome (58). Counts were obtained through the summarizeOverlaps function from the GenomicFeatures package in R (59).

Differential analyses:

Differential expression analysis was performed via DESeq2 and differential transcript usage analysis was performed using DRIMSeq (60,61). DRIMSeq p-values were adjusted using StageR (62).

Functional analyses:

Further analyses were performed on differential results. Gene ontology analysis was performed using topGO, relying on the default algorithm to determine overrepresentation (63). Additionally, pathway analysis was performed using KEGGREST to acquire the genes assigned to pathways followed by a wilcox test to compare the number of genes annotated within the pathway with those not in the pathway (64).

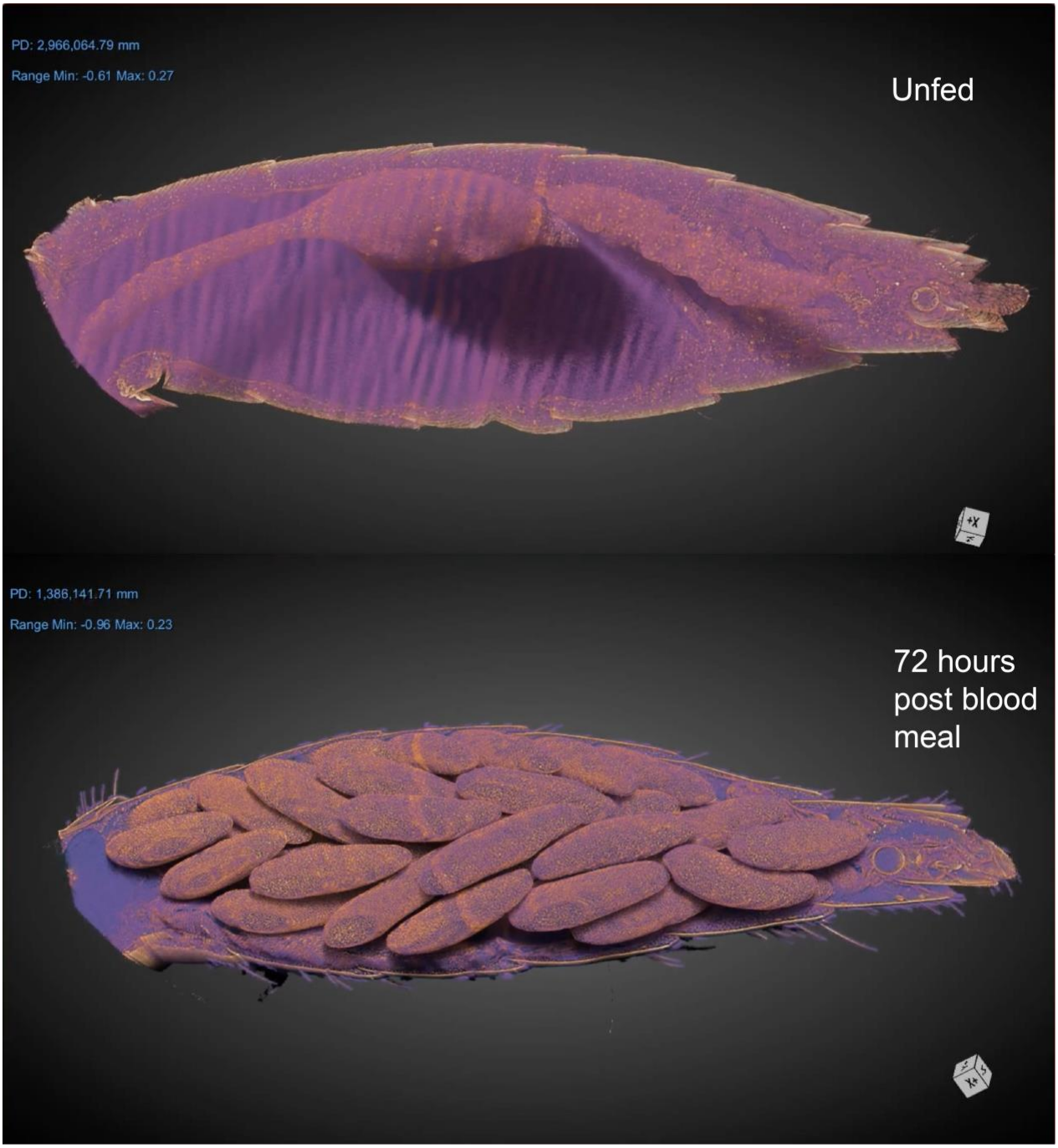


Figure 1. MicroCT images depicting anatomical changes after blood feeding. Mosquitoes undergo immense physiological and anatomical changes after blood feeding, with mature oocytes growing to occupy over 50% of the abdominal volume.

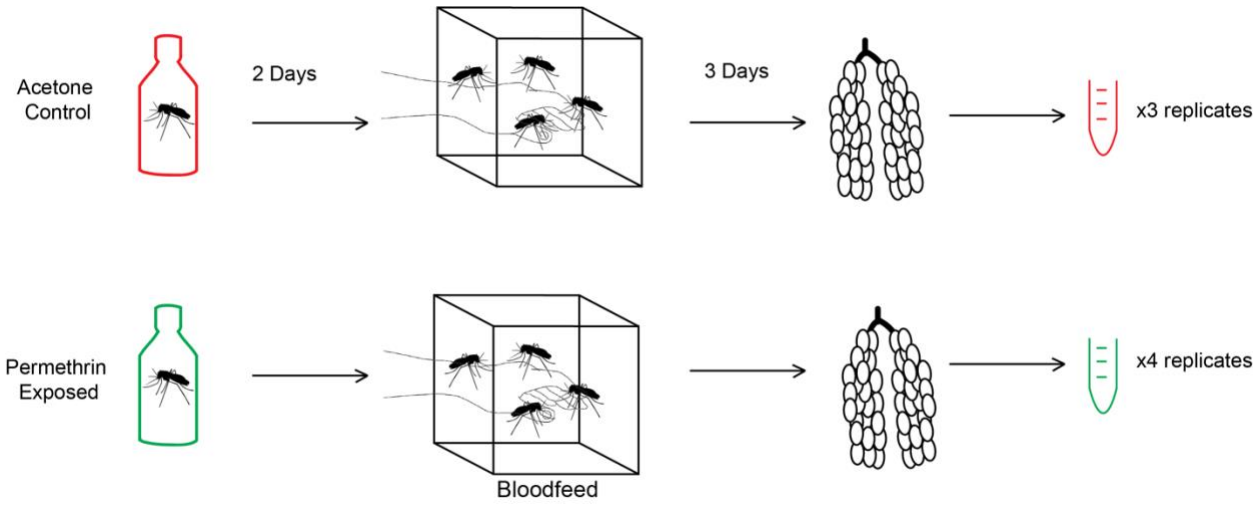


Figure 2. Diagram of the experimental design. Five-day-old female mosquitoes were exposed to permethrin or acetone as a control, blood fed 2 days later, and ovaries dissected 3 days after blood feeding.

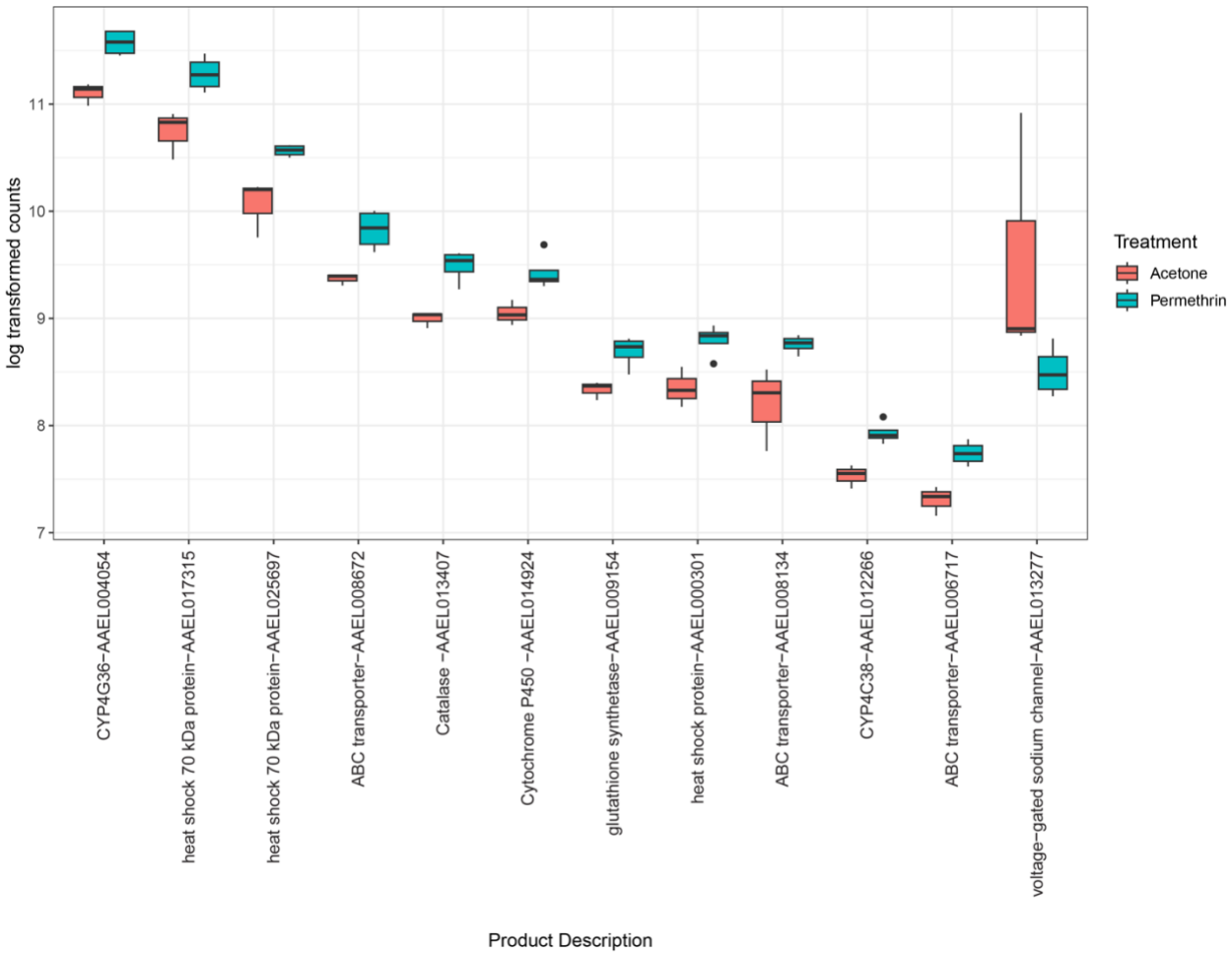
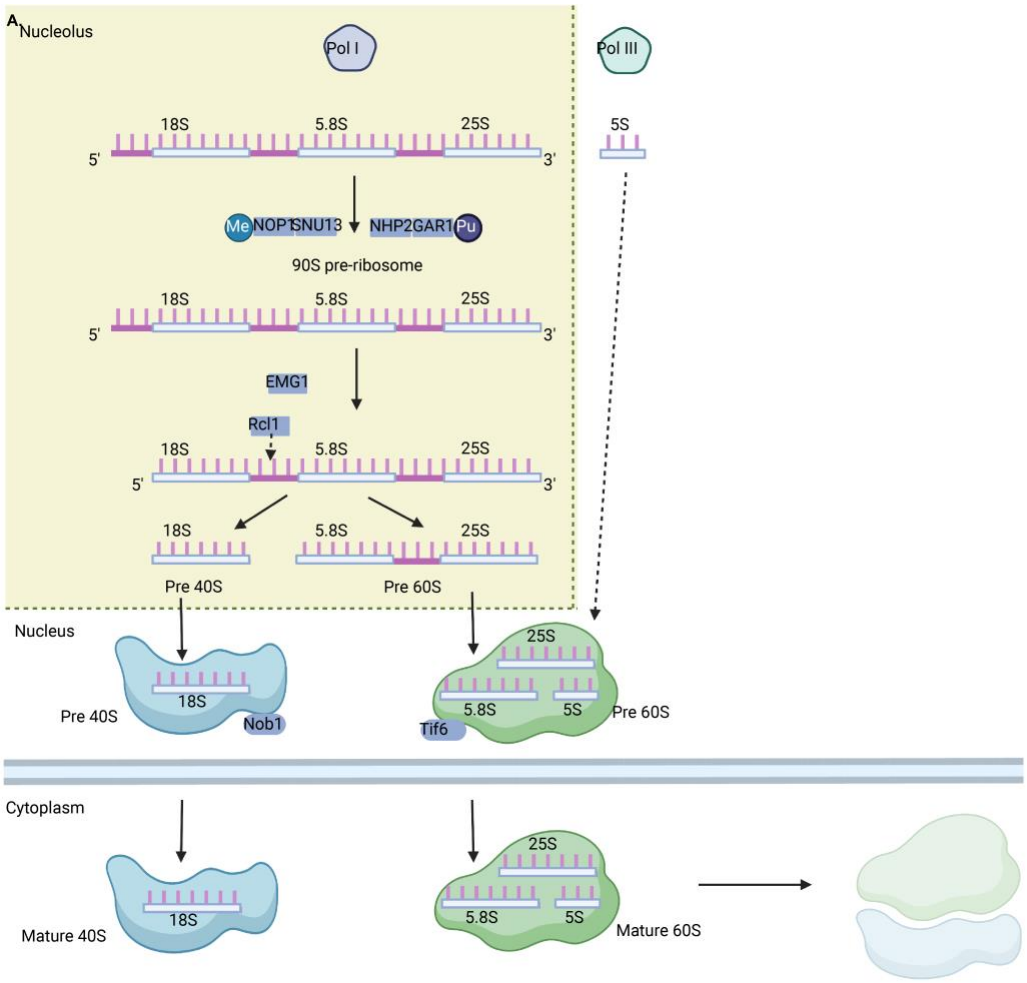
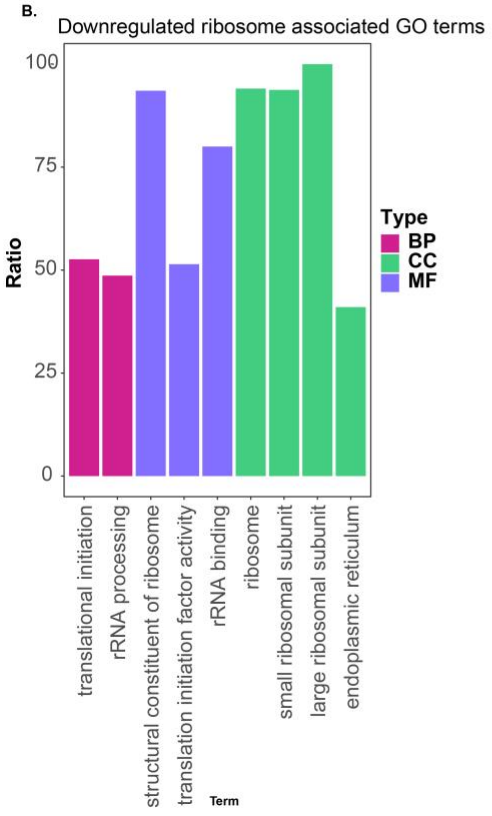


Figure 3. Known insecticide response related genes upregulated in ovaries of permethrin treated mosquitoes. Significance based on an adjusted p-value cutoff of 0.05.


A
Nucleolus
Pol I
Pol III
18S
5.8S
25S
5'
3'
5S
Me
NOP1
SNU13
NHP2
GAR1
Pu
90S pre-ribosome
EMG1
Rcl1
Pre 40S
Pre 60S
Nucleus
Nob1
Tif6
Cytoplasm
Mature 40S
Mature 60S


B.
Downregulated ribosome associated GO terms
Ratio
Type
BP
CC
MF
translational initiation
rRNA processing
structural constituent of ribosome
translation initiation factor activity
rRNA binding
ribosome
small ribosomal subunit
large ribosomal subunit
endoplasmic reticulum
Term

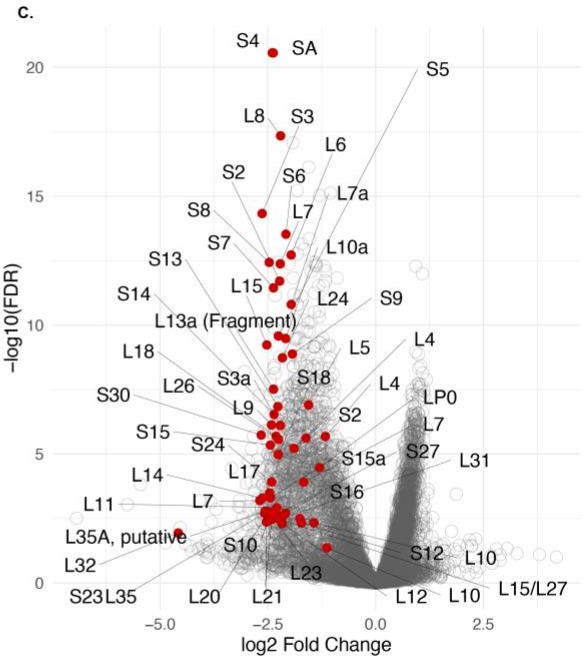

C.
−log10(FDR)
log2 Fold Change

Figure 4. Oocytes of permethrin treated mosquitoes experience a downregulation of ribosomal protein genes. A. Based on a gene ontology analysis and pathway analysis, ribosome biogenesis is overrepresented among the downregulated genes. All genes shown in the diagram are downregulated. B. Multiple gene ontology categories of ribosome related processes are overrepresented in gene ontology. Ratio refers to the percent of genes within an ontology category that were found in this dataset. C. All ribosomal protein genes in this data set are downregulated more than 2-fold. Created with Biorender.

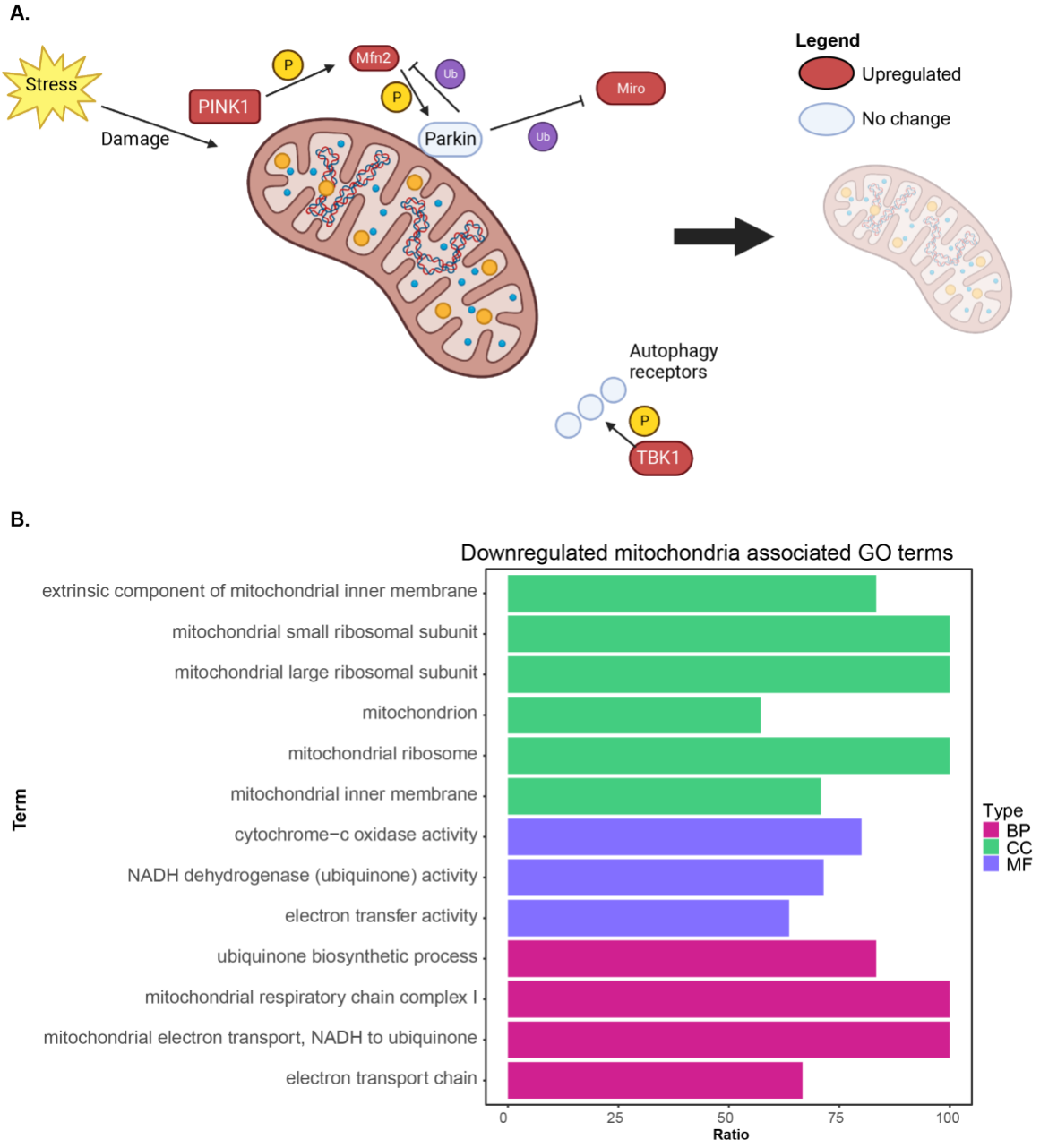


Figure 5. Oocytes of permethrin-treated mosquitoes show transcriptional indications of mitochondrial depletion. A. Genes in the mitophagy pathway are overrepresented among the upregulated genes. B. Mitochondria-related gene ontology categories are overrepresented among downregulated genes. Together, these results suggest that permethrin treatment causes impressive damage to mitochondria within the oocytes. Created with Biorender.

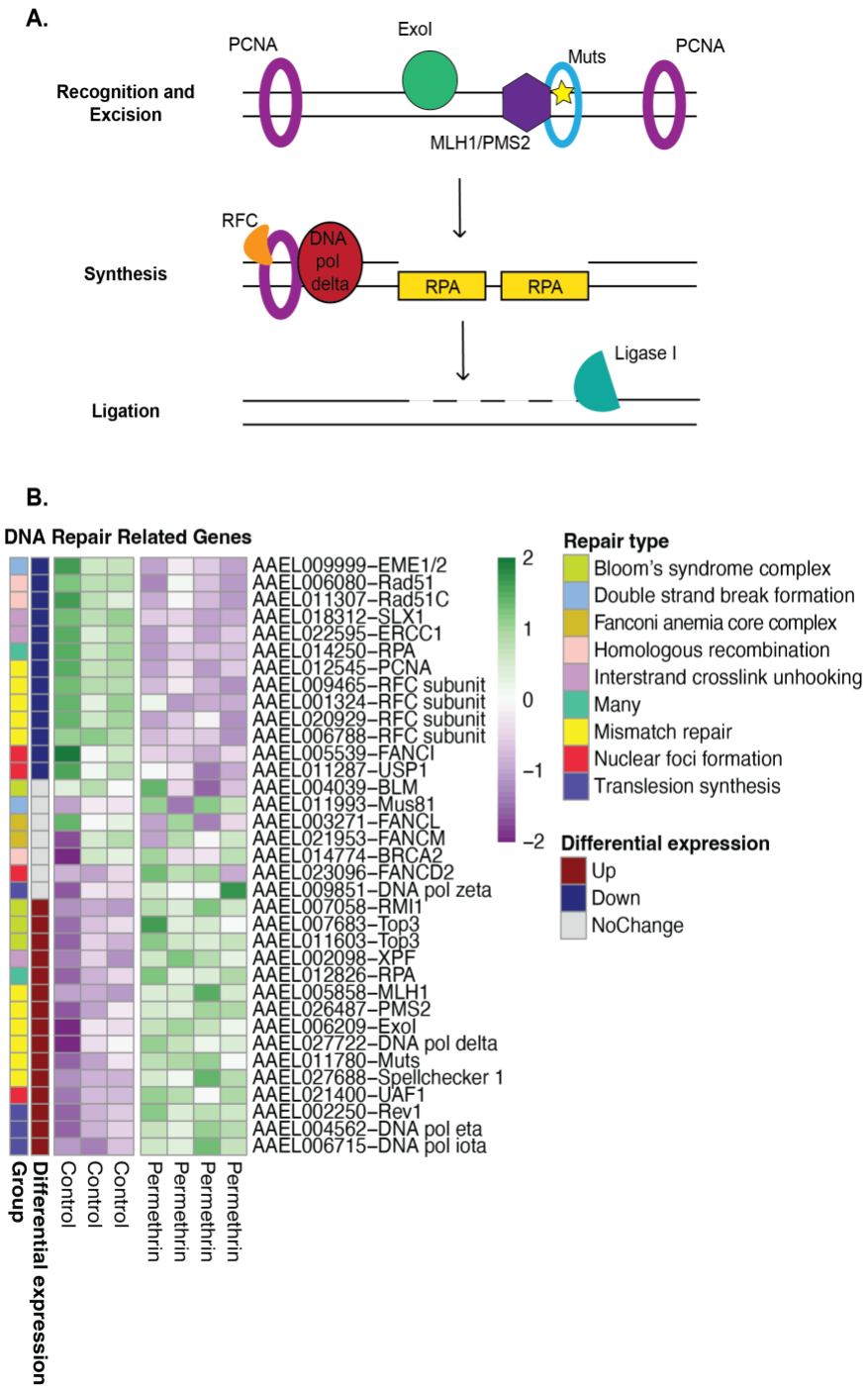


Figure 6. Exposure to permethrin induces DNA damage response gene expression in the ovaries. A. Schematic of mismatch repair pathway. B. Heatmap of DNA repair related genes, values are z-scaled counts. Through a pathway analysis, the Fanconi Anemia pathway was overrepresented among upregulated genes, a pathway involved in DNA repair.

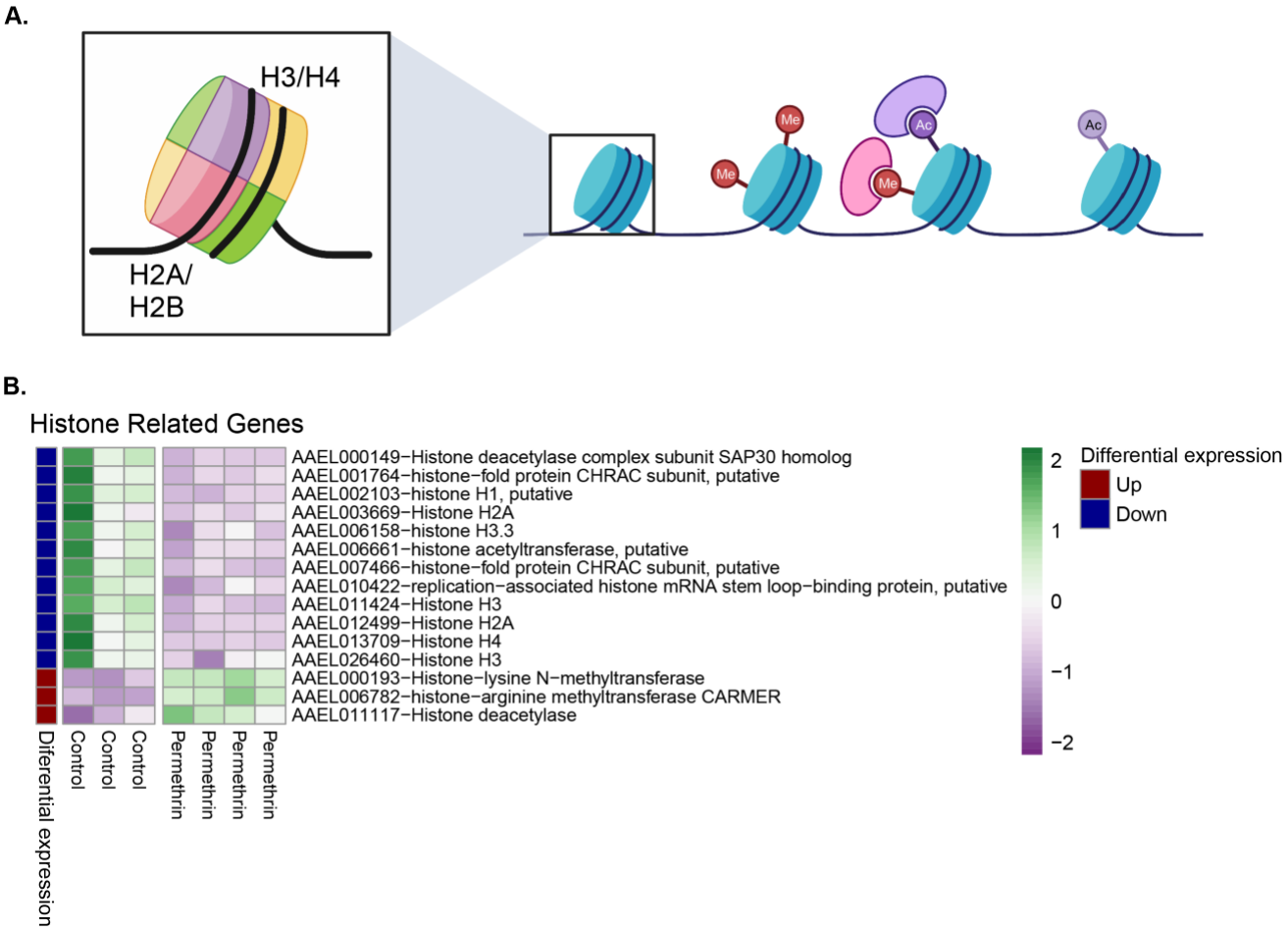


Figure 7. Exposure to permethrin causes downregulation of histone related genes in ovaries. A. Schematic of the nucleosome and histone modifications. B. Heatmap of histone related genes. Histone genes are downregulated, though 2 histone methyltransferases and 1 histone deacetylase are upregulated. Created with Biorender.

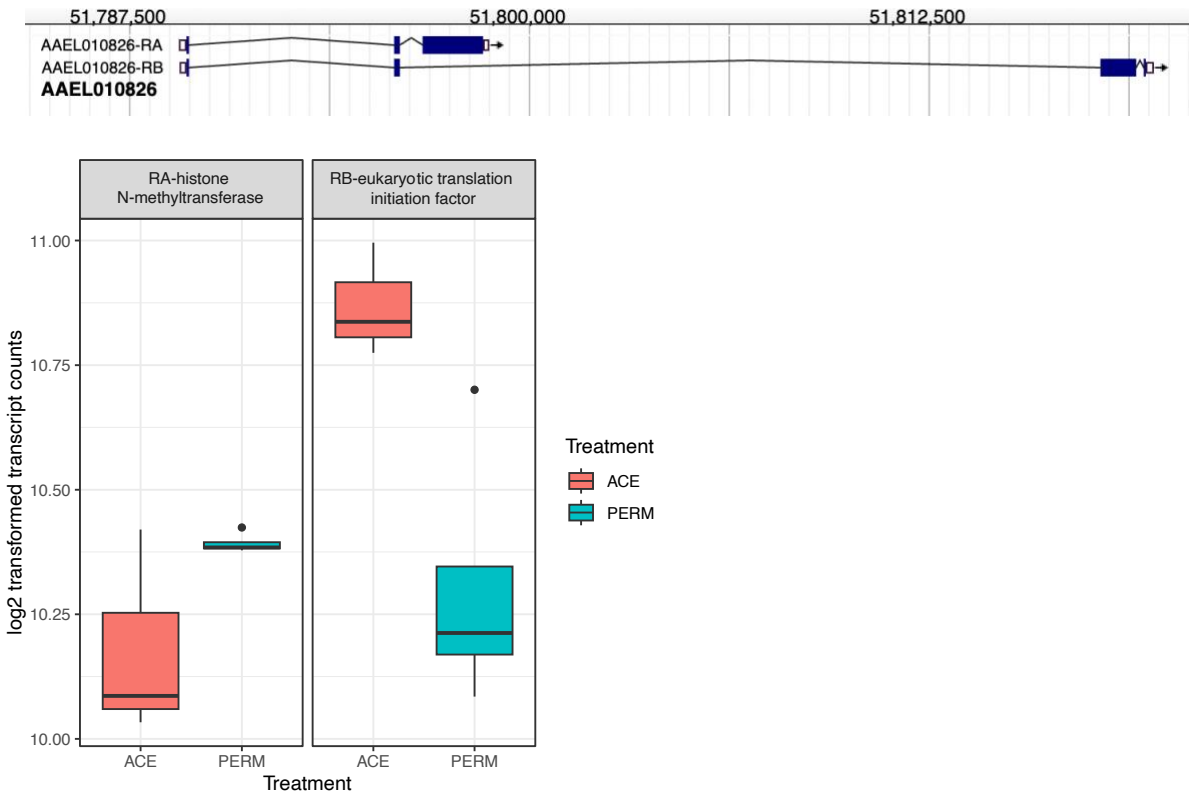
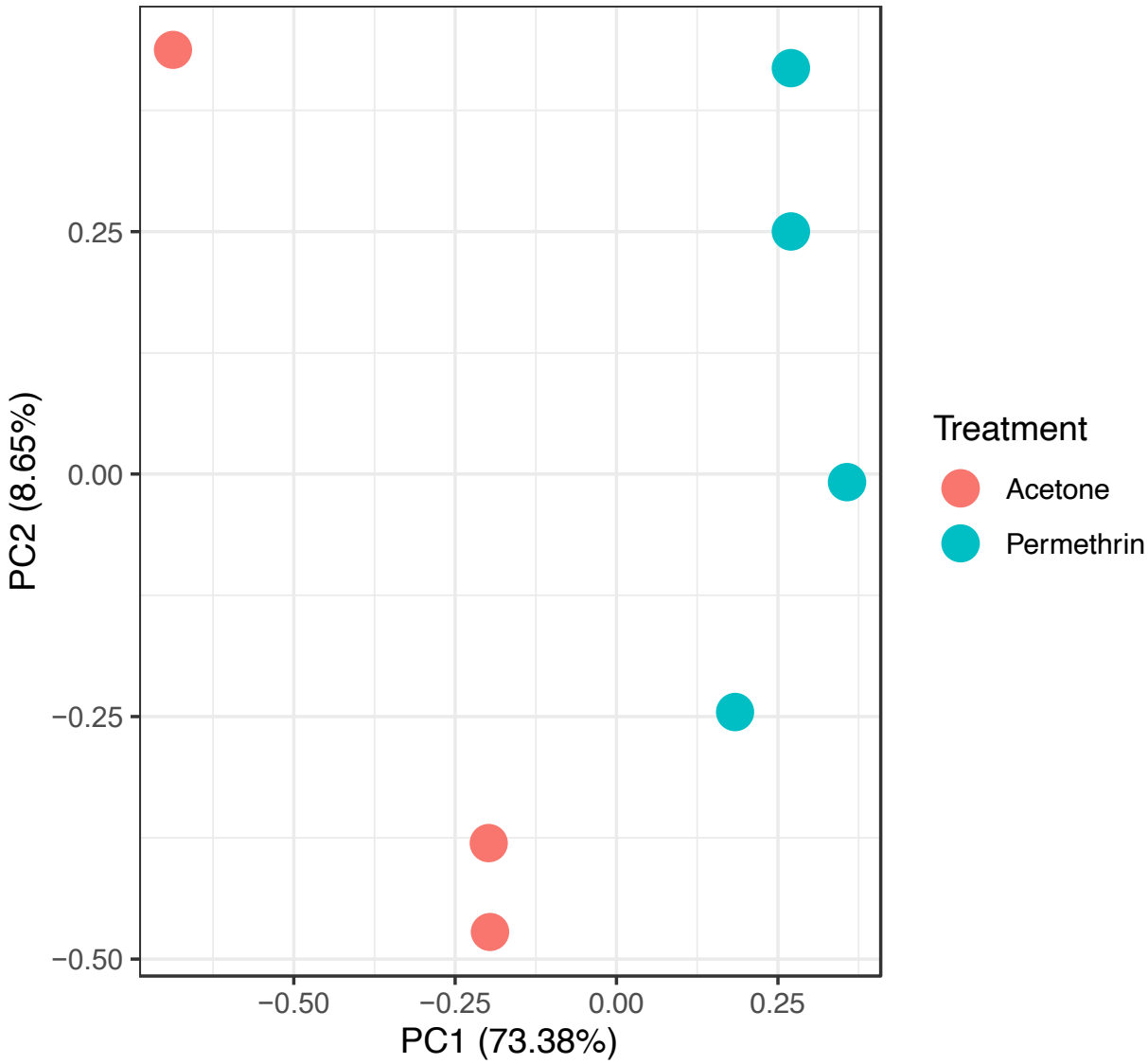
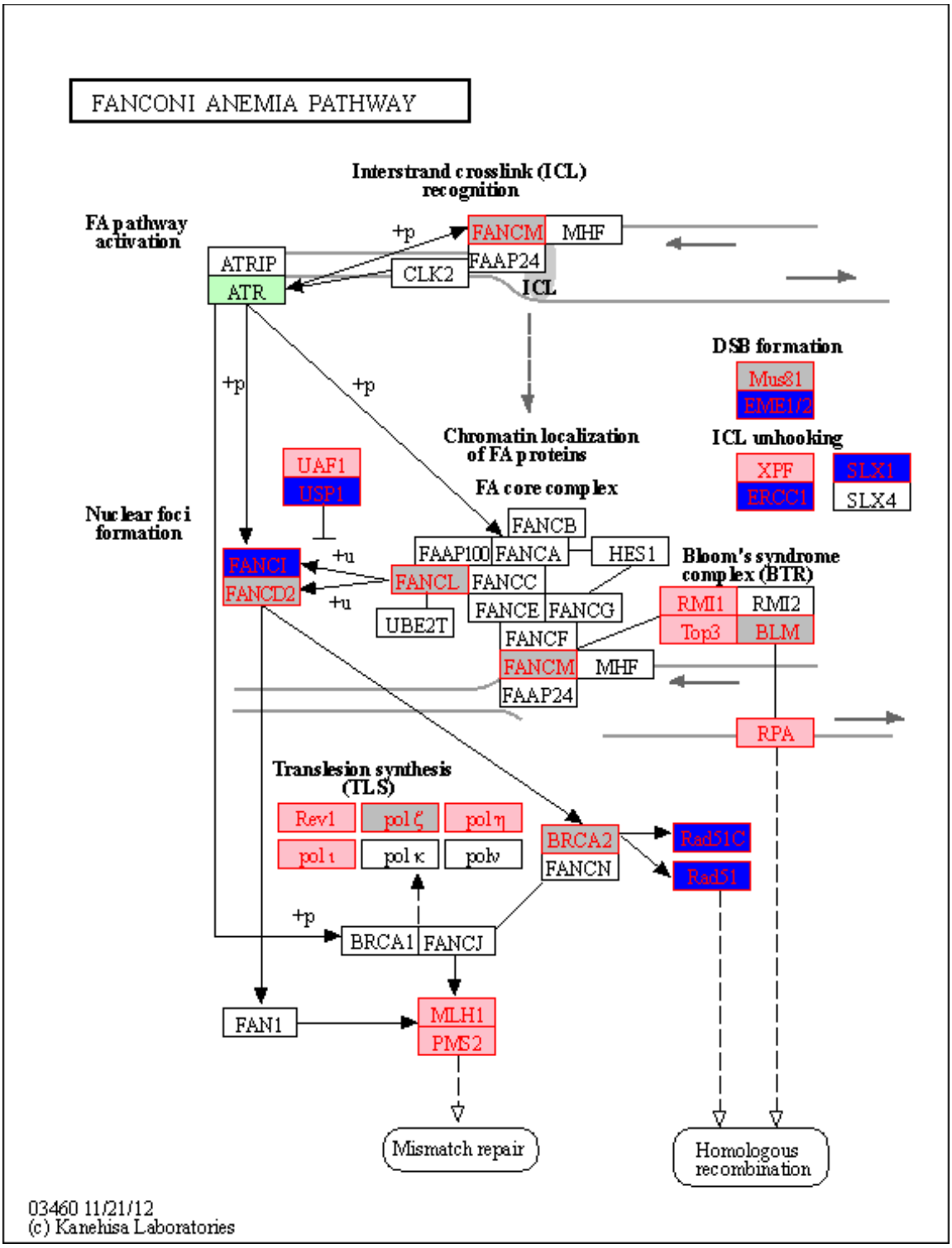


Figure 8. Differential transcript usage analysis reveals switch to histone methylation from translation initiation factor after permethrin exposure. The two isoforms contain much different coding regions of the gene.



Supplementary Figure 1. Principal component analysis of samples.



Supplementary Figure 2. Full pathway of Fanconi Anemia Pathway from KEGG. Red boxes indicated upregulated genes and blue indicated downregulated genes.

Supplementary Table 3. Upregulated GO Terms

GO.ID	Term	Annotated	Significant	Expected	classicFisher	elimFisher	topgoFisher	parentchildFisher	GOtype
GO:0006886	intracellular protein transport	98	47	20.1	6.80E-10	2.10E-08	1.40E-08	0.00012	BP
GO:0006270	DNA replication initiation	10	7	2.05	0.001	0.001	0.001	0.00544	BP
GO:0006891	intra-Golgi vesicle-mediated transport	4	4	0.82	0.0018	0.0018	0.0018	0.00531	BP
GO:0007179	transforming growth factor beta receptor...	4	4	0.82	0.0018	0.0018	0.0018	0.04545	BP
GO:0006635	fatty acid beta-oxidation	6	5	1.23	0.0018	0.0018	0.0018	1	BP
GO:0006904	vesicle docking involved in exocytosis	6	5	1.23	0.0018	0.0018	0.0018	1	BP
GO:0007020	microtubule nucleation	6	5	1.23	0.0018	0.0018	0.0018	0.02557	BP
GO:0006606	protein import into nucleus	7	5	1.44	0.0052	0.0052	0.0052	0.1855	BP
GO:0006298	mismatch repair	5	4	1.03	0.0074	0.0074	0.0074	0.01321	BP
GO:0006402	mRNA catabolic process	24	9	4.92	0.0414	0.0414	0.0085	0.09796	BP
GO:1904668	positive regulation of ubiquitin protein...	3	3	0.62	0.0086	0.0086	0.0086	1	BP
GO:0042555	MCM complex	6	6	1.13	4.30E-05	4.30E-05	4.30E-05	7.60E-05	CC
GO:0000145	exocyst	8	7	1.5	5.40E-05	5.40E-05	5.40E-05	0.13282	CC
GO:0031461	cullin-RING ubiquitin ligase complex	15	9	2.82	0.00047	0.00047	0.00023	0.18073	CC

GO:0017119	Golgi transport complex	5	5	0.94	0.00023	0.00023	0.00023	0.00578	CC
GO:0000922	spindle pole	8	6	1.5	0.00086	0.00086	0.00086	0.00082	CC
GO:0005634	nucleus	1002	214	188.5	0.00857	0.00857	0.00177	0.632	CC
GO:0005667	transcription regulator complex	41	12	7.71	0.0692	0.0692	0.00267	0.12416	CC
GO:0005815	microtubule organizing center	20	10	3.76	0.00155	0.00155	0.00342	0.00144	CC
GO:0032300	mismatch repair complex	3	3	0.56	0.00663	0.00663	0.00663	0.00891	CC
GO:0005524	ATP binding	609	172	122.77	2.60E-07	2.60E-07	2.60E-07	0.0071	MF
GO:0003676	nucleic acid binding	1400	301	282.23	0.08155	0.11018	0.00031	0.6308	MF
GO:0031625	ubiquitin protein ligase binding	7	6	1.41	0.00038	0.00038	0.00038	1	MF
GO:0022857	transmembrane transporter activity	358	81	72.17	0.12922	0.12922	0.0008	0.3135	MF
GO:0005515	protein binding	1459	342	294.12	0.0002	0.00086	0.00114	0.0117	MF
GO:0035091	phosphatidylinositol binding	24	14	4.84	4.30E-05	0.001	0.00151	0.0133	MF
GO:0016746	acyltransferase activity	105	26	21.17	0.14418	0.14418	0.00316	0.5182	MF
GO:0003678	DNA helicase activity	20	10	4.03	0.00271	0.00271	0.00439	0.127	MF
GO:0008324	monoatomic cation transmembrane transpor...	125	21	25.2	0.85604	0.85604	0.00497	0.5274	MF

GO:0008270	zinc ion binding	565	138	113.9	0.00524	0.00524	0.00524	3.60E-06	MF
GO:0030983	mismatched DNA binding	5	4	1.01	0.0069	0.0069	0.0069	0.1833	MF
GO:0043015	gamma-tubulin binding	5	4	1.01	0.0069	0.0069	0.0069	0.0131	MF
GO:0042578	phosphoric ester hydrolase activity	105	29	21.17	0.03953	0.03953	0.00806	0.0485	MF
GO:0010997	anaphase-promoting complex binding	3	3	0.6	0.00818	0.00818	0.00818	0.0369	MF
GO:0051377	mannose-ethanolamine phosphotransferase ...	3	3	0.6	0.00818	0.00818	0.00818	0.0833	MF
GO:0061608	nuclear import signal receptor activity	3	3	0.6	0.00818	0.00818	0.00818	0.1	MF
GO:0097027	ubiquitin-protein transferase activator ...	3	3	0.6	0.00818	0.00818	0.00818	0.0454	MF

Supplementary Table 4. Downregulated GO Terms

GO.ID	Term	Annotated	Significant	Expected	classicFisher	elimFisher	topgoFisher	parentchildFisher	GOtype
GO:0006412	translation	206	116	42.73	< 1e-30	8.80E-27	< 1e-30	5.60E-26	BP
GO:0022900	electron transport chain	36	24	7.47	2.90E-09	2.60E-06	1.90E-06	0.00078	BP
GO:0006413	translational initiation	38	20	7.88	1.30E-05	1.30E-05	6.40E-06	0.00045	BP
GO:0006120	mitochondrial electron transport, NADH t...	6	6	1.24	7.80E-05	7.80E-05	7.80E-05	1	BP
GO:0006364	rRNA processing	37	18	7.67	0.00013	0.00013	0.00021	0.32987	BP

GO:0032981	mitochondrial respiratory chain complex ...	5	5	1.04	0.00038	0.00038	0.00038	0.28571	BP
GO:0000413	protein peptidyl-prolyl isomerization	20	11	4.15	0.00076	0.00076	0.00076	0.47619	BP
GO:0008380	RNA splicing	50	25	10.37	3.60E-06	0.00145	0.00177	0.03416	BP
GO:0051603	proteolysis involved in protein cataboli...	79	25	16.39	0.01434	0.01434	0.00183	7.00E-05	BP
GO:0006465	signal peptide processing	4	4	0.83	0.00184	0.00184	0.00184	0.0865	BP
GO:0046940	nucleoside monophosphate phosphorylation	4	4	0.83	0.00184	0.00184	0.00184	0.0033	BP
GO:0006383	transcription by RNA polymerase III	10	7	2.07	0.00107	0.00107	0.00188	0.0009	BP
GO:0006744	ubiquinone biosynthetic process	6	5	1.24	0.00189	0.00189	0.00189	1	BP
GO:0000398	mRNA splicing, via spliceosome	37	17	7.67	0.00048	0.00782	0.00507	0.14913	BP
GO:0090502	RNA phosphodiester bond hydrolysis, endo...	15	8	3.11	0.00528	0.00528	0.00528	0.64238	BP
GO:0006366	transcription by RNA polymerase II	75	25	15.56	0.00704	0.00704	0.00529	0.00284	BP
GO:0016226	iron-sulfur cluster assembly	7	5	1.45	0.00548	0.00548	0.00548	0.00479	BP
GO:0006457	protein folding	38	15	7.88	0.00625	0.00625	0.00625	0.01071	BP
GO:0000387	spliceosomal snRNP assembly	5	4	1.04	0.00768	0.00768	0.00768	0.10937	BP
GO:0006826	iron ion transport	3	3	0.62	0.0089	0.0089	0.0089	0.17857	BP

GO:0030150	protein import into mitochondrial matrix	3	3	0.62	0.0089	0.0089	0.0089	0.14706	BP
GO:0032367	intracellular cholesterol transport	3	3	0.62	0.0089	0.0089	0.0089	1	BP
GO:0005840	ribosome	101	95	23.74	< 1e-30	< 1e-30	< 1e-30	< 1e-30	CC
GO:0015935	small ribosomal subunit	16	15	3.76	4.20E-09	1.00E-06	2.00E-06	1	CC
GO:0015934	large ribosomal subunit	13	13	3.06	6.20E-09	2.00E-06	3.70E-05	0.55172	CC
GO:0000786	nucleosome	22	14	5.17	6.60E-05	6.60E-05	6.60E-05	0.00023	CC
GO:0005839	proteasome core complex	13	13	3.06	6.20E-09	3.70E-05	0.00016	5.30E-06	CC
GO:0016272	prefoldin complex	6	6	1.41	0.00017	0.00017	0.00017	0.00353	CC
GO:0019773	proteasome core complex, alpha-subunit c...	6	6	1.41	0.00017	0.00017	0.00017	0.00087	CC
GO:0016592	mediator complex	21	13	4.94	0.00018	0.00018	0.00018	0.02204	CC
GO:0005730	nucleolus	35	19	8.23	7.50E-05	7.50E-05	0.0004	0.1278	CC
GO:0005681	spliceosomal complex	14	10	3.29	0.00019	0.00019	0.00059	0.04165	CC
GO:0005743	mitochondrial inner membrane	48	34	11.28	3.50E-12	0.00025	0.00062	0.09925	CC
GO:0005761	mitochondrial ribosome	13	13	3.06	6.20E-09	0.00068	0.00068	0.00522	CC
GO:0005666	RNA polymerase III complex	5	5	1.18	0.00071	0.00071	0.00071	0.01204	CC

GO:0005739	mitochondrion	159	91	37.37	7.00E-21	1.70E-05	0.00092	2.30E-15	CC
GO:0005747	mitochondrial respiratory chain complex ...	4	4	0.94	0.00303	0.00303	0.00303	0.75	CC
GO:0005762	mitochondrial large ribosomal subunit	4	4	0.94	0.00303	0.00303	0.00303	0.66972	CC
GO:0005763	mitochondrial small ribosomal subunit	4	4	0.94	0.00303	0.00303	0.00303	0.66972	CC
GO:0000178	exosome (RNase complex)	6	5	1.41	0.00343	0.00343	0.00343	0.01303	CC
GO:0031314	extrinsic component of mitochondrial inn...	6	5	1.41	0.00343	0.00343	0.00343	0.42705	CC
GO:0005783	endoplasmic reticulum	78	32	18.33	0.00038	0.01611	0.00505	0.01228	CC
GO:0003735	structural constituent of ribosome	93	87	16.56	< 1e-30	< 1e-30	< 1e-30	4.70E-26	MF
GO:0004298	threonine-type endopeptidase activity	13	13	2.31	1.70E-10	1.70E-10	1.70E-10	3.60E-12	MF
GO:0046982	protein heterodimerization activity	35	22	6.23	3.70E-09	3.70E-09	3.70E-09	8.10E-06	MF
GO:0003723	RNA binding	298	97	53.06	1.80E-10	1.40E-08	1.50E-07	1.80E-08	MF
GO:0003743	translation initiation factor activity	35	18	6.23	6.00E-06	6.00E-06	6.00E-06	0.1713	MF
GO:0019843	rRNA binding	10	8	1.78	3.20E-05	3.20E-05	0.00014	0.0025	MF
GO:0003899	DNA-directed 5'-3' RNA polymerase activi...	23	13	4.1	3.30E-05	3.30E-05	0.00019	1	MF
GO:0003755	peptidyl-prolyl cis-trans isomerase acti...	20	11	3.56	0.00019	0.00019	0.00019	1.50E-05	MF

GO:0003677	DNA binding	421	90	74.96	0.02913	0.02913	0.00045	0.3207	MF
GO:0009055	electron transfer activity	33	21	5.88	6.10E-09	5.40E-05	0.00056	8.70E-08	MF
GO:0004322	ferroxidase activity	4	4	0.71	0.001	0.001	0.001	1	MF
GO:0008199	ferric iron binding	4	4	0.71	0.001	0.001	0.001	2.60E-05	MF
GO:0008080	N-acetyltransferase activity	26	11	4.63	0.00307	0.00307	0.00107	0.0599	MF
GO:0008137	NADH dehydrogenase (ubiquinone) activity	7	5	1.25	0.00271	0.00271	0.00271	0.494	MF
GO:0004129	cytochrome-c oxidase activity	5	4	0.89	0.00429	0.00429	0.00429	0.0629	MF
GO:0051920	peroxiredoxin activity	5	4	0.89	0.00429	0.00429	0.00429	0.0276	MF
GO:0003712	transcription coregulator activity	38	17	6.77	0.00011	0.00011	0.00431	4.00E-08	MF
GO:0008168	methyltransferase activity	88	32	15.67	2.30E-05	0.00002	0.00445	0.3093	MF
GO:0003839	gamma-glutamylcyclotransferase activity	3	3	0.53	0.00563	0.00563	0.00563	0.1	MF
GO:0051537	2 iron, 2 sulfur cluster binding	14	7	2.49	0.00596	0.00596	0.00596	0.0494	MF

References:

1. Schunck F, Liess M. Time between Sequential Exposures to Multiple Stress Turns Antagonism into Synergism. Environ Sci Technol. 2022 Oct 18;56(20):14660–7.
2. Calabrese EJ, Mattson MP. Hormesis provides a generalized quantitative estimate of biological plasticity. J Cell Commun Signal. 2011 Mar;5(1):25–38.
3. Rix RR, Ayyanath MM, Christopher Cutler G. Sublethal concentrations of imidacloprid increase reproduction, alter expression of detoxification genes, and prime Myzus persicae for subsequent stress. J Pest Sci . 2016 Jun;89(2):581–9.
4. Ayyanath M-M, Cutler GC, Scott-Dupree CD, Prithiviraj B, Kandasamy S, Prithiviraj K. Gene expression during imidacloprid-induced hormesis in green peach aphid. Dose Response. 2014 Jul;12(3):480–97.
5. Ayyanath M-M, Cutler GC, Scott-Dupree CD, Sibley PK. Transgenerational shifts in reproduction hormesis in green peach aphid exposed to low concentrations of imidacloprid. PLoS One. 2013 Sep 6;8(9):e74532.
6. Poupardin R, Reynaud S, Strode C, Ranson H, Vontas J, David J-P. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito Aedes aegypti: impact on larval tolerance to chemical insecticides. Insect Biochem Mol Biol. 2008 May;38(5):540–51.
7. Gong Y, Cheng S, Desneux N, Gao X, Xiu X, Wang F, et al. Transgenerational hormesis effects of nitenpyram on fitness and insecticide tolerance/resistance of Nilaparvata lugens. J Pest Sci . 2023 Jan 1;96(1):161–80.

8. Castano-Sanz V, Gomez-Mestre I, Garcia-Gonzalez F. Evolutionary consequences of pesticide exposure include transgenerational plasticity and potential terminal investment transgenerational effects. Evolution . 2022 Nov 1;76(11):2649–68.
9. Agathokleous E, Guedes RNC, Calabrese EJ, Fotopoulos V, Azevedo RA. Transgenerational hormesis: What do parents sacrifice for their offspring? Current Opinion in Environmental Science & Health. 2022 Oct 1;29:100380.
10. Lockwood BL, Julick CR, Montooth KL. Maternal loading of a small heat shock protein increases embryo thermal tolerance in Drosophila melanogaster. J Exp Biol. 2017 Dec 1;220(Pt 23):4492–501.
11. Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H. Gene amplification, abc transporters and cytochrome p450s: Unraveling the molecular basis of pyrethroid resistance in the dengue vector, aedes aegypti. PLoS Negl Trop Dis [Internet]. 2012;6(6). Available from: http://dx.doi.org/10.1371/journal.pntd.0001692
12. Vector-borne diseases [Internet]. [cited 2023 May 14]. Available from: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases
13. Kraemer MUG, Sinka ME, Duda KA, Mylne A, Shearer FM, Barker CM, et al. the global distribution of the arbovirus vectors aedes aegypti and ae albopictus. Elife [Internet]. 2015; Available from: https://pubmed.ncbi.nlm.nih.gov/26126267
14. Lee Y, Schmidt H, Collier TC, Conner WR, Hanemaaijer MJ, Slatkin M, et al. Genome-wide divergence among invasive populations of Aedes aegypti in California. BMC Genomics. 2019 Mar 12;20(1):204.

15. Kelly ET, Mack LK, Campos M, Grippin C, Chen T-Y, Romero-Weaver AL, et al. Evidence of Local Extinction and Reintroduction of Aedes aegypti in Exeter, California. Frontiers in Tropical Diseases. 2021;2:8.
16. Mack LK, Kelly ET, Lee Y, Brisco KK, Shen KV, Zahid A, et al. Frequency of sodium channel genotypes and association with pyrethrum knockdown time in populations of Californian Aedes aegypti. Parasit Vectors. 2021 Mar 6;14(1):141.
17. Cornel AJ, Holeman J, Nieman CC, Lee Y, Smith C, Amorino M, et al. Surveillance , insecticide resistance and control of an invasive Aedes aegypti (Diptera: Culicidae) population in California. F1000 Research. 2016;1–16.
18. Furlong MA, Paul KC, Yan Q, Chuang Y-H, Cockburn MG, Bronstein JM, et al. An epigenome-wide association study of ambient pyrethroid pesticide exposures in California's central valley. Int J Hyg Environ Health. 2020 Aug;229:113569.
19. Gong Y, Li T, Zhang L, Gao X, Liu N. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, Culex quinquefasciatus. Int J Biol Sci. 2013 Sep 5;9(9):863–71.
20. Tao F, Si F-L, Hong R, He X, Li X-Y, Qiao L, et al. Glutathione S-transferase (GST) genes and their function associated with pyrethroid resistance in the malaria vector Anopheles sinensis. Pest Manag Sci. 2022 Oct;78(10):4127–39.
21. Wu C, Chakrabarty S, Jin M, Liu K, Xiao Y. Insect ATP-Binding Cassette (ABC) Transporters: Roles in Xenobiotic Detoxification and Bt Insecticidal Activity. Int J Mol Sci [Internet]. 2019 Jun 10;20(11). Available from: http://dx.doi.org/10.3390/ijms20112829

22. Patil NS, Lole KS, Deobagkar DN. Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes Anopheles stephensi and Aedes aegypti. Med Vet Entomol. 1996 Jul;10(3):277–82.
23. Oliver SV, Brooke BD. The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors Anopheles arabiensis and Anopheles funestus. PLoS One. 2016;11(3):1–19.
24. DeJong RJ, Miller LM, Molina-Cruz A, Gupta L, Kumar S, Barillas-Mury C. Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito Anopheles gambiae. Proc Natl Acad Sci U S A. 2007;104(7):2121–6.
25. Vijverberg HPM, vanden Bercken J. Neurotoxicological effects and the mode of action of pyrethroid insecticides. Crit Rev Toxicol. 1990;21(2):105–26.
26. Garber G, Smith LA, Reenan RA, Rogina B. Effect of sodium channel abundance on Drosophila development, reproductive capacity and aging. Fly . 2012 Jan 1;6(1):57–67.
27. Ozadam H, Tonn T, Han CM, Segura A, Hoskins I, Rao S, et al. Single-cell quantification of ribosome occupancy in early mouse development. Nature [Internet]. 2023 Jun 21; Available from: http://dx.doi.org/10.1038/s41586-023-06228-9
28. Crofton AE, Cartwright EL, Feitzinger AA, Lott SE. Effect of larval nutrition on maternal mRNA contribution to the Drosophila egg. G3 . 2018 Jun 1;8(6):1933–41.
29. Wang JL, Saha TT, Zhang Y, Zhang C, Raikhel AS. Juvenile hormone and its receptor methoprene-tolerant promote ribosomal biogenesis and vitellogenesis in the Aedes aegypti mosquito. J Biol Chem. 2017;292(24):10306–15.

30. Clifton ME, Noriega FG. The fate of follicles after a blood meal is dependent on previtellogenic nutrition and juvenile hormone in Aedes aegypti. J Insect Physiol. 2012 Jul;58(7):1007–19.
31. Bedford L, Paine S, Sheppard PW, Mayer RJ, Roelofs J. Assembly, structure, and function of the 26S proteasome. Trends Cell Biol. 2010 Jul;20(7):391–401.
32. Wang W, Yang R-R, Peng L-Y, Zhang L, Yao Y-L, Bao Y-Y. Proteolytic activity of the proteasome is required for female insect reproduction. Open Biol. 2021 Feb;11(2):200251.
33. Sun L, Ye Y, Sun H, Yu J, Zhang L, Sun Y, et al. Identification of proteasome subunit beta type 6 (PSMB6) associated with deltamethrin resistance in mosquitoes by proteomic and bioassay analyses. PLoS One. 2013 Jun 10;8(6):e65859.
34. Durcan TM, Fon EA. The three 'P's of mitophagy: PARKIN, PINK1, and post-translational modifications. Genes Dev. 2015 May 15;29(10):989–99.
35. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P, et al. Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. Proc Natl Acad Sci U S A. 2016 Apr 12;113(15):4039–44.
36. Ingham VA, Brown F, Ranson H. Transcriptomic analysis reveals pronounced changes in gene expression due to sub-lethal pyrethroid exposure and ageing in insecticide resistance Anopheles coluzzii. BMC Genomics. 2021 May 10;22(1):337.
37. Gumeni S, Papanagnou E-D, Manola MS, Trougakos IP. Nrf2 activation induces mitophagy and reverses Parkin/Pink1 knock down-mediated neuronal and muscle degeneration phenotypes. Cell Death Dis. 2021 Jul 3;12(7):671.
38. Misra JR, Horner MA, Lam G, Thummel CS. Transcriptional regulation of xenobiotic detoxification in Drosophila. Genes Dev. 2011 Sep 1;25(17):1796–806.

39. Laurence BR, Simpson MG. Cell replication in the follicular epithelium of the adult mosquito. J Insect Physiol. 1974 Apr;20(4):703–15.

40. Wang AD, Agrawal AF. DNA repair pathway choice is influenced by the health of Drosophila melanogaster. Genetics. 2012 Oct;192(2):361–70.

41. Preston CR, Engels W, Flores C. Efficient repair of DNA breaks in Drosophila: evidence for single-strand annealing and competition with other repair pathways. Genetics. 2002 Jun;161(2):711–20.

42. Preston CR, Flores CC, Engels WR. Differential usage of alternative pathways of double-strand break repair in Drosophila. Genetics. 2006 Feb;172(2):1055–68.

43. Spies M, Fishel R. Mismatch repair during homologous and homeologous recombination. Cold Spring Harb Perspect Biol. 2015 Mar 2;7(3):a022657.

44. Galhardo RS, Hastings PJ, Rosenberg SM. Mutation as a stress response and the regulation of evolvability. Crit Rev Biochem Mol Biol. 2007 Sep-Oct;42(5):399–435.

45. Agrawal AF, Wang AD. Increased transmission of mutations by low-condition females: evidence for condition-dependent DNA repair. PLoS Biol. 2008 Feb;6(2):e30.

46. Mohafrash SMM, Hassan EE, El-Shaer NH, Mossa A-TH. Detoxification gene expression, genotoxicity, and hepatorenal damage induced by subacute exposure to the new pyrethroid, imiprothrin, in rats. Environ Sci Pollut Res Int [Internet]. 2021 Feb 27; Available from: http://dx.doi.org/10.1007/s11356-021-13044-z

47. Marinowic DR, Mergener M, Pollo TA, Maluf SW, da Silva LB. In vivo genotoxicity of the pyrethroid pesticide beta-cyfluthrin using the comet assay in the fish Bryconamericus iheringii. Z Naturforsch C. 2012 May-Jun;67(5–6):308–11.

48. Nagy K, Rácz G, Matsumoto T, Ádány R, Ádám B. Evaluation of the genotoxicity of the pyrethroid insecticide phenothrin. Mutat Res Genet Toxicol Environ Mutagen. 2014 Aug;770:1–5.

49. Fetoui H, Feki A, Salah GB, Kamoun H, Fakhfakh F, Gdoura R. Exposure to lambda-cyhalothrin, a synthetic pyrethroid, increases reactive oxygen species production and induces genotoxicity in rat peripheral blood. Toxicol Ind Health. 2015 May;31(5):433–41.

50. Su C, Gao G, Schneider S, Helt C, Weiss C, O'Reilly MA, et al. DNA damage induces downregulation of histone gene expression through the G1 checkpoint pathway. EMBO J. 2004 Mar 10;23(5):1133–43.

51. Burkhart KB, Sando SR, Corrionero A, Horvitz HR. H3.3 Nucleosome Assembly Mutants Display a Late-Onset Maternal Effect. Curr Biol. 2020 Jun 22;30(12):2343-2352.e3.

52. Krauss V, Fassl A, Fiebig P, Patties I, Sass H. The evolution of the histone methyltransferase gene Su(var)3-9 in metazoans includes a fusion with and a re-fission from a functionally unrelated gene. BMC Evol Biol. 2006 Mar 2;6(1):18.

53. Schotta G, Ebert A, Krauss V, Fischer A, Hoffmann J, Rea S, et al. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. EMBO J. 2002 Mar 1;21(5):1121–31.

54. Krauss V, Reuter G. Two genes become one: the genes encoding heterochromatin protein Su(var)3-9 and translation initiation factor subunit eIF-2gamma are joined to a dicistronic unit in holometabolic insects. Genetics. 2000 Nov;156(3):1157–67.

55. Clemons A, Mori A, Haugen M, Severson DW, Duman-Scheel M. Culturing and egg collection of Aedes aegypti. Cold Spring Harb Protoc. 2010;5(10):1–6.

56. fastcat: Simple utility to concatenate .fastq(.gz) files whilst creating a summary of the sequences [Internet]. Github; [cited 2023 Apr 12]. Available from: https://github.com/epi2me-labs/fastcat
57. pychopper: cDNA read preprocessing [Internet]. Github; [cited 2023 Apr 12]. Available from: https://github.com/epi2me-labs/pychopper
58. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 2018 Sep 15;34(18):3094–100.
59. Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, et al. Software for computing and annotating genomic ranges. PLoS Comput Biol. 2013 Aug 8;9(8):e1003118.
60. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
61. Nowicka M, Robinson MD. DRIMSeq: a Dirichlet-multinomial framework for multivariate count outcomes in genomics. F1000Res. 2016 Jun 13;5:1356.
62. Van den Berge K, Soneson C, Robinson MD, Clement L. stageR: a general stage-wise method for controlling the gene-level false discovery rate in differential expression and differential transcript usage. Genome Biol. 2017 Aug 7;18(1):151.
63. Alexa A, Rahnenführer J. Gene set enrichment analysis with topGO. Bioconductor Improv. 2009;27:1–26.
64. Tenenbaum D MB. KEGGREST: Client-side REST access to the Kyoto encyclopedia of genes and genomes (KEGG) R package version 1.40.0. 2023.

Conclusion

Pyrethroid insecticides are broadly ineffective against *Ae. aegypti* (1,2). We know that resistance to these chemicals is developed through target site resistance and metabolic resistance, though many questions remain about the relative impact of specific components of resistance on the overall phenotype. Understanding these specifics is necessary for the development of control strategies that avoid the development of resistance.

Resistance testing in mosquitoes is standardized and is performed through phenotypic and genotypic measures. In chapter 1, we investigated how well these two forms of resistance testing align in their results. We found that genotype predicted phenotype generally, though some individuals with susceptible alleles still survived longer than individuals with fully resistant genotypes. This indicated that other resistance mechanisms aside from target site resistance were present in the strains used for this experiment.

In chapter 2, these other mechanisms were investigated across the first 24 hours of recovery from a pyrethroid exposure. We know that many genes involved in the xenobiotic response are associated with pyrethroid response in *Ae. aegypti* (3). Here, we discovered that many of these detoxifying genes are involved in the insecticide response, along with heat shock proteins and other redox metabolism genes as well. Additionally, genes involved in lipid and carbohydrate metabolism are upregulated after exposure as well. Lipid metabolism in particular is important for oogenesis in mosquitoes, so further investigation into the effects of pyrethroid exposure on oogenesis was warranted.

Chapter 3 explored transcriptomic differences between the developed ovaries of pyrethroid treated and control mosquitoes. We know that insects may deposit stress response related genes in their developing oocytes after exposure to a stress, which we saw evidence of here (4). Additionally,

we saw changes in gene expression that suggest a reduction in development rate. A reduction in development rate could be advantageous to the insects, allowing them to survive past the cessation of the stressor. Further experiments are necessary to fully explore the implications of reduced development rate.

Overall, the studies here improve understanding of resistance and pyrethroid response mechanisms. We are also presented with many questions for further exploration, particularly in regards to maternal contributions to the resistance phenotype. My personal conclusions from these studies is that regulatory agencies and companies should be fast-tracking the development of alternative pest control solutions to improve the vector control toolkit. In a changing environment, and with the range expansion of such a deadly pest, it is imperative that the issue of insecticide resistance is explored thoroughly and matched with the monetary and time investment necessary to combat such a wide spread issue.

References

1. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans. 2017;1–20.

2. VectorSurv Development Team. VectorSurv - Vectorborne Disease Surveillance System. 2023;

3. Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H. Gene amplification, abc transporters and cytochrome p450s: Unraveling the molecular basis of pyrethroid resistance in the dengue vector, aedes aegypti. PLoS Negl Trop Dis [Internet]. 2012;6(6). Available from: http://dx.doi.org/10.1371/journal.pntd.0001692

4. Castano-Sanz V, Gomez-Mestre I, Garcia-Gonzalez F. Evolutionary consequences of pesticide exposure include transgenerational plasticity and potential terminal investment transgenerational effects. Evolution . 2022 Nov 1;76(11):2649–68.