Investigating *Aedes aegypti* in California: Genetic Diversity, Insecticide Resistance, and Metabolic Adaptations

By

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DISSERTATION

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DEDICATION

I dedicate my dissertation to my parents, Donald and Sharon Kelly, my husband, Lucas Hill, and my first entomological mentor, Dr. Janice Edgerly-Rooks. Thank you.

ABSTRACT

The world's foremost arboviral vector, *Aedes aegypti*, has recently been established in California, with detections in 28 of 58 counties as of 2023(*Aedes Aegypti* and *Aedes Albopictus* Mosquitoes, n.d.). This dissertation explores population dynamics within the state, and the biology of insecticide resistance mechanisms using populations derived from Greater Los Angeles, Fresno, and Tulare counties.

In Chapter 1, we utilize genomic data to investigate the population dynamics of *Ae. aegypti* in California. We report evidence of multiple introductions into the state, with distinct genetic clusters identified. We investigate a specific hypothesis: that a population of *Ae. aegypti* detected in Exeter, CA, in 2014 was successfully eradicated, and the region was then reinvaded in 2017. We find evidence to support this hypothesis and posit that the region was reinvaded by a population from Southern California. This chapter also explores varying levels of resistance to pyrethroid insecticides, facilitated by mutations in the Voltage-Gated Sodium Channel (VGSC), a primary mechanism of resistance.

Chapter 2 delves into a phenotypic comparison between the Rockefeller laboratory strain and wild California *Ae. aegypti* populations. By integrating lifespan, transcriptomic, and metabolomic data, we uncovered significant differences in metabolic pathways, particularly those related to oxidoreductase activity. Notably, we observed baseline differences in oxidative stress response, energy metabolism, and lipid profiles between the populations. Our findings suggest that larval nutrition and metabolic resistance to pyrethroids significantly impact mosquito physiology and longevity.

Finally, Chapter 3 investigates metabolic shifts in response to pyrethroid (Deltamethrin) exposure in two *Ae. aegypti* populations with distinct genetic backgrounds collected from Los Angeles and the Central Valley. By examining metabolic changes in two near-wild populations, we identified significant alterations in amino acid, lipid, and nucleotide metabolism following

exposure. This study highlights the rapidity and variability of metabolic responses to insecticides, underlining potential targets for novel synergists in mosquito control.

Overall, this dissertation uses a diversity of tools to investigate the spread, genetic diversity, and insecticide resistance mechanisms in *Ae. aegypti*, with a focus on California's evolving situation. The findings advance our understanding of *Ae. aegypti* resistance biology, vector management, and suggest avenues for more targeted and effective mosquito control measures

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INTRODUCTION

The mosquito *Aedes aegypti* is the major vector of chikungunya, Zika, yellow fever, and dengue, viral illnesses of which nearly half of the world's population is at risk (Soni et al., 2023). The past decade has seen the rapid expansion in the range of *Ae. aegypti* and more cases of the viruses they carry (Kraemer et al., 2019). Despite earlier documented introductions, *Ae. aegypti* had failed to establish in California until its detection in the Central Valley region in 2013 (Gloria-Soria et al., 2014; Jewell & Grodhaus, 1984). As of 2023, it has been detected in twenty-eight of California's fifty-eight counties, as far north as Shasta County (*Aedes Aegypti* and *Aedes* Albopictus Mosquitoes, n.d.).

Aedes aegypti's recent successful establishment in California, and globally, has been facilitated by the expansion of suitable urban and suburban microclimates, climate change, and widespread resistance to pyrethroid insecticides used for adult control (Catherine L. Moyes et al., 2017; Kasai et al., 2014; Kraemer et al., 2019; Smith et al., 2016; William C. Black et al., 2021). Pyrethroid insecticides are organic compounds that share structural similarity to pyrethrins naturally produced by Chrysanthemums (Soderlund, 2010). These compounds act as neurotoxins by prolonging the opening of insects' Voltage Gated Sodium Channel (VGSC) and have been favored for indoor and outdoor spraying because of their effectiveness and safety (Vais et al., 2001). In California, vector control for public health purposes has relied heavily on pyrethrins and pyrethroids, though usage of organophosphate insecticides (acetylcholinesterase inhibitors) is on the rise due to widespread pyrethroid resistance in vectors (Liebman et al., 2019; Matthews, 2011; Overview of Mosquito Control Practices in California, 2008; Yang et al., 2020; Yoshimizu et al., 2020).

The primary mechanism by which insects have evolved to resist pyrethroids involves mutations in the insects' VGSC, which prevents pyrethroids from effectively binding their target site. The specific sites of mutations can vary regionally, and many populations have multiple complementary VGSC mutations (Catherine L. Moyes et al., 2017; Chen et al., 2020). Multiple

target side modifications have been reported in California populations of *Aedes aegypti* (Liebman et al., 2019; Mack et al., 2021). They include V410L, which was first detected in Brazil (Haddi et al., 2017) and is present in the transmembrane segment 6 of domain I (IS6). This mutation has been demonstrated to confer resistance to a broad swath of pyrethroids, and frequently co-occurs with the mutation F1534C, which is present in the transmembrane segment 6 of domain III (IIIS6) and has be found to have evolved independently in *Ae. aegypti* multiple times (Cosme et al., 2020). Additionally V1016I, present in IIS6, is present and further protects against pyrethroids (Linss et al., 2014). Additional mutations have been identified in the state, and are described in Chapter 1 and a companion paper, though their impact on pyrethroid resistance has not been thoroughly investigated (Lee et al., 2019; Mack et al., 2021).

In addition to target site mutations, a variety of other mechanisms of resistance have been reported in mosquitoes. These include behavioral resistance, including avoidance (Amelia-Yap et al., 2018), penetration resistance mediated by modifications of the insect cuticle to decrease insecticide uptake (Balabanidou et al., 2018; Jacobs et al., 2023), and a striking diversity of metabolic mechanisms of detoxification (William C. Black et al., 2021). In *Aedes aegypti*, Cytochrome P450 monooxygenases play important roles in pyrethroid resistance (John Vontas et al., 2020; Nauen et al., 2022; Smith et al., 2016). Cytochrome p450s are a heme-containing enzyme superfamily found in all kingdoms of life (Nelson, 2018) and act on both endogenous and exogenous substrates with notable roles in xenobiotic detoxification, lipid metabolism, and hormone metabolism, among other roles (Balabanidou et al., 2016; Domanitskaya et al., 2014; Gong et al., 2017; Sieglaff et al., 2005).

Cytochrome p450 mediated pyrethroid resistance has been identified in California Central Valley mosquito populations using bottle bioassays with piperonyl butoxide (PBO), a mixed function oxidase inhibiting insecticide synergist (Cornel et al., 2016). The exact cytochrome P450 genes associated with pyrethroid resistance vary by population and can be difficult to assay. Cytochrome p450s can be controlled at the level of transcription with groups

reporting modifications in regulatory regions, coding regions, and gene duplication events in mosquitoes (Cattel et al., 2020; Itokawa et al., 2013; Liu et al., 2011; Smith et al., 2018). While biochemical methods of assaying monooxygenase activity exist and are utilized, they assay levels of all monooxygenases without targeting specific dynamics with resistance conferring P450s (McAllister et al., 2012; Yang et al., 2020). Bioassays with synergists are valuable in this regard, however they are relatively time intensive and require large numbers of insects, which makes them impractical for routine surveillance.

Given the regional variability of pyrethroid resistance mechanisms (Chen et al., 2020; John Vontas et al., 2020), understanding invasive population origin can provide important insights into resistance status. Following the detection of *Ae. aegypti* in the Central Valley, microsatellite data was utilized to investigate population origin (Gloria-Soria et al., 2014), which suggested relationships to populations in the southeastern USA and Mexico. This study was followed by additional projects that identified multiple distinct populations in CA, with a southern Mexico, as well as multiple distinct Central Valley clusters related to southeastern US populations (Lee et al., 2019; Pless et al., 2017). The clear finding that the spread of *Ae. aegypti* in California was attributable to multiple distinct introductions rather than simple in-state expansion provided the opportunity to further investigate population dynamics and variability in resistance status and mechanisms.

In chapter one, we utilize genomic data to create a 37 SNP multiplex assay to study population dynamics of invasive CA *Ae. aegypti*. We investigate a specific hypothesis; that *Ae. aegypti* was introduced, eradicated, then reintroduced to the Central Valley city of Exeter, CA (Kelly et al., 2021). We report the population genomic analysis of 243 *Ae. aegypti* from California, Arizona, Florida, and Mexico, and evaluate the ability of our multiplex assay to discern population clustering compared to whole-genome sequencing and report frequencies of pyrethroid resistance associated voltage-gated sodium channel mutations. We find evidence that the city of Exeter, California, had *Ae. aegypti* introduced in 2014 from Florida. After

aggressive eradication efforts, the city then had no detection events until 2017. Samples collected following that period show no relationship to the 2014 samples, but do cluster alongside samples from southern California, suggesting a separate introduction event. We report increases in the allele frequencies of two well-known VGSC mutations (V410L and V1016I) and two novel mutations (S723T and I915K) reported in a companion paper (Mack et al., 2021), and the detection (but not increase) of a novel mutation, Q1853R (Kelly et al., 2021).

In chapter two, we perform a thorough comparison of an F2 Central Valley derived pyrethroid-resistant population of *Ae. aegypti* against our insecticide susceptible lab reference strain, Rockefeller. We integrate transcriptomic and metabolomic data along with lifespan data to investigate whether metabolites could be used as markers of pyrethroid resistance status. We also investigate hypotheses about how metabolic resistance to pyrethroids may impact various aspects of mosquito physiology and metabolism. We find evidence of constitutive upregulation of oxidoreductase activity and glutathione metabolism in Clovis, our Central Valley population. We find significant differences in lipid profiles, and evidence of increased flux through the pentose phosphate pathway in Central Valley mosquitoes, which is likely required to support oxidoreductase activity. We also describe signs of metabolic inefficiency in our laboratory reference strain.

In chapter three, we analyze metabolic shifts in two near-wild populations of *Ae. aegypti* with different genetic backgrounds in response to pyrethroid (Deltamethrin) exposure. We collect samples as they show symptoms of pyrethroid intoxication, and at the seventy-minute survival mark, after which mortality becomes less likely, and survivors are designated as resistant. We find for resistant insects that lysolipids, fatty acids, and carnitines are elevated following sublethal deltamethrin exposure, and variable flux through ammonia metabolic pathways between the two populations. Lastly, we demonstrate the essential role B-vitamins play in recovery from pyrethroid exposure and discuss potential targets for pyrethroid synergism.

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CHAPTER 1. Evidence of Local Extinction and Reintroduction of *Aedes aegypti* in Exeter, California

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AUTHOR CONTRIBUTIONS

YL, GA, GL, and AC conceived experimental design. EK, LM, and GA conducted SNP genotyping analysis. AR-W, T-YC, and KK conducted genomic DNA library preparations for whole genome sequencing. YL, MC, and TC conducted genome data analysis. EK, LM, KB, CG, RR-C, KS, LC, and EB conducted sample collection or arranged sample acquisition and provided the sample metadata. All authors contributed to the article and approved the submitted version.

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Evidence of Local Extinction and Reintroduction of *Aedes aegypti* in Exeter, California

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Established populations of *Aedes aegypti*, a mosquito vector of multiple major arthropod-borne viruses, were first found in three California (CA) cities in 2013. From 2013 to April 2021, *Ae. aegypti* thwarted almost all control efforts to stop its spread and expanded its range to 308 cities, including Exeter, in 22 counties in CA. Population genomic analyses have suggested that multiple genetically distinct *Ae. aegypti* populations were introduced into CA. However *Ae. aegypti* collected for the first time in 2014 in Exeter, appeared to be different from three major genetic clusters found elsewhere in CA. Due to intense control efforts by the Delta Vector Control District (DVCD), *Ae. aegypti* was thought to have been eliminated from Exeter in 2015. Unfortunately, it was recollected in 2018. It was not clear if the reemergence of *Ae. aegypti* in Exeter was derived from the bottlenecked remnants of the original 2014 Exeter population or from an independent invasion from a different population derived from surrounding areas. The goal of this work was to determine which of these scenarios occurred (recovery after bottleneck or reintroduction after elimination) and if elimination and reintroduction occurred to identify the origin of the invading population using a population genomic approach. Our results support the reintroduction after elimination hypothesis. The source of reintroduction, however, was unexpectedly from the southern CA cluster rather than from other two geographically closer central CA genetic clusters. We also conducted a knockdown resistance mutation profile, which showed Exeter 2014 had the lowest level of resistant alleles compared to the other populations, could have contributed towards DVCD's ability to locally eliminate *Ae. aegypti* in 2014.

Keywords: mosquito, *Aedes aegypti*, California, population genomics, mosquito control, reintroduction assessment

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INTRODUCTION

Aedes aegypti serves as a major vector of four human disease-causing viruses, including yellow fever, dengue, chikungunya, and Zika viruses, posing a major threat to public health. Records indicate this species became established in the southeastern United States of America between the 15-18th centuries (1) and then spread throughout the east coast and southern states (2). California (CA) had remained free from *Ae. aegypti* until the summer of 2013 (3, 4) when the species were first detected in Menlo Park, Clovis, and Madera (4). Since then, this species has expanded its range to 308 cities in 22 counties, as of April, 2021 (5).

Attempts to locally eliminate and even control this highly invasive species has proven to be extremely challenging. For example, the Consolidated Mosquito Abatement District (CMAD) implemented labor intensive integrated vector control management in the city of Clovis where *Ae. aegypti* were first detected in 2013. Their efforts involved extensive public education, thorough property inspections, sanitation, insecticide treatment at larval sources, and residual barrier spraying with pyrethroids. Despite these efforts, *Ae. aegypti* successfully overwintered and continued to persist in Clovis (6).

Delta Vector Control District (DVCD), which covers northern Tulare County, just south of the area covered by CMAD, first detected *Ae. aegypti* in Exeter in August of 2014. Following detection, the DVCD initiated an intensive control campaign involving thorough, routine property inspections and barrier applications, public education, breeding site treatment or removal, as well as hand and truck-mounted adulticide fogging through October. DVCD encountered significant pushback from the public due to the regular adulticide applications, mandatory property inspections, and confiscation of container habitats. However, they reported that residents followed instructions and were able to control mosquito breeding. Exeter remained free of *Ae. aegypti* from the beginning of 2015 through the summer of 2017. DVCD detected *Ae. aegypti* again in 2017 in the neighboring cities of Visalia and Farmersville and, in 2018, at multiple sites in Exeter. In response to this detection, the district attempted to mount a response similar to 2014. Despite frequent, mandatory property inspections, breeding site elimination and sanitation, and barrier spraying, the infestation persisted.

Population genetic/genomic analyses suggest that multiple genetically distinct *Ae. aegypti* populations were introduced in CA (7, 8). The *Ae. aegypti* populations in California could be largely grouped into three major genetic clusters (8). One cluster includes samples from Fresno, Madera, and Menlo Park in Central CA (8). Another cluster includes samples from Clovis in central CA adjacent to Fresno. The third cluster includes all southern CA samples, as well as 2014 Exeter samples (8). Further clustering revealed that 2014 Exeter samples are similar to an *Ae. aegypti* population from Florida rather than the populations from southern CA. However, it is important to note that the data from Lee et al. (8) does not provide definitive evidence that Exeter *Ae. aegypti* were introduced from Florida because the cluster analysis included only Florida samples for comparison. *Aedes aegypti* from southeastern states such as Louisiana and Texas are genetically similar to Florida populations based on microsatellite analysis (3).

The genetic dissimilarity of 2014 Exeter to other California *Ae. aegypti* provided an opportunity to investigate the hypothesis that (1) the Exeter mosquito population went through a severe bottleneck due to intensive insecticide spraying and control followed by a recovery after a couple of years (bottleneck and recovery) or (2) DVCD successfully eliminated its initial introduction but later new mosquitoes migrated or were reintroduced to Exeter from other cities (local extinction and reintroduction). If bottleneck and recovery occurred, then we expected 2018 Exeter samples to have similar genetic profile as 2014 Exeter samples. If localized extinction and reintroduction occurred, then we expected 2018 Exeter samples to have a similar genetic profile to any of the other three CA genetic clusters. To investigate this hypothesis, we report the population genomic analysis of 243 *Ae. aegypti* from California, Arizona, Florida, and Mexico in this paper.

MATERIALS AND METHODS

Sample Collection

Genome data was obtained from deposited sequences available for specimens originating from earlier collections in Clovis, Fresno, Madera, Menlo Park, 2014 Exeter, East Los Angeles, San Diego (CA), Vero Beach, and Key West, Florida (FL) - NCBI BioProject PRJNA385349 (8, 9). Further samples from St. Augustine (FL), Naples (FL), St. Lucie (FL), Arizona, and Mexico were collected as adults using BG Sentinel traps and the remaining samples as eggs in ovicups, which were then reared to the adult stage in the laboratory and stored in >70% alcohol prior to DNA extraction. The global positioning system (GPS) coordinates from where each site specimens originated from as well as the number of samples sequenced and genotyped are provided in **Table 1**. The sample locations map is provided in **Figure 1**.

Genome Sequencing

Protocols for sequenced specimens available in the NCBI Bioproject database are described in Lee et al. (8) and Schmidt et al. (9). For the other specimens, DNA was extracted from individual mosquitoes using a magnetic-bead based DNA extraction protocol described in Chen et al. (10). DNA concentrations for each sample were measured using the Qubit dsDNA HS Assay Kit (Life Technologies) on a Qubit instrument (Life Technologies). A genomic DNA library was constructed for each individual mosquito with the QIAseq FX DNA Library UDI kit (Qiagen, Valencia, CA) using 20 ng DNA. Enzymatic fragmentation was conducted at 32°C for 11 minutes followed by 65°C for 30 minutes. Adapter ligation was conducted at 20°C for 2 hours. PCR amplification of the constructed library was carried out for 8 cycles [(98°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 second) x 8]s. Library cleanup was done using PCRClean DX (Aline Biosciences, Woburn, MA). Library concentrations were measured using Qubit (Life Technologies).

TABLE 1 | Sample collection data. Number of *Ae. aegypti* sequenced are provided in N_{WGS} column. N_{KDR} denotes the number of samples genotyped for kdr and population specific SNPs using iPLEX assay.

Location	State	District	latitude	longitude	N_{WGS}	N_{KDR}
Clovis	California	Consolidated	36.81342	-119.66665	10	60
Fresno	California	Fresno	36.83998	-119.90485	3	
Madera	California	Madera	36.92671	-120.05016	3	
Menlo Park	California	San Mateo	37.43305	-122.19881	3	
Exeter (2014)	California	Delta	36.30385	-119.15797	3	24
Exeter (2018)	California	Delta	36.30385	-119.15797	4	24
East Los Angeles	California	Greater Los Angeles	34.03515	-118.15410	3	67
San Diego	California	San Diego	32.55557	-117.05128	4	
Phoenix	Arizona	Maricopa	33.51389	-112.47583	4	
St. Augustine	Florida	Anastasia	29.90119	-81.31262	1	
Miramar	Florida	Broward	25.98629	-80.24622	2	
Naples	Florida	Collier	26.15504	-81.75737	1	
Tampa	Florida	Hillsborough	27.96606	-82.49508	2	
Fort Myers	Florida	Lee	26.65284	-81.81183	1	
Miami	Florida	Miami-Dade	25.75458	-80.22354	2	
Key Largo	Florida	Monroe	25.08723	-80.44773	2	
Key West	Florida	Monroe	24.55684	-81.78290	1	
Haverhill	Florida	Palm Beach	26.68861	-80.11346	2	
Holiday	Florida	Pasco	28.18634	-82.74527	2	
Auburndale	Florida	Polk	28.04973	-81.77675	2	
Sarasota	Florida	Sarasota	27.31105	-82.46285	3	
Sanford	Florida	Seminole	28.82482	-81.33626	2	
St. Lucie	Florida	St. Lucie	27.52948	-80.31699	1	
Vero Beach	Florida	Indian River	27.58721	-80.37340	3	
Cuernavaca	Mexico	Morelos	17.59358	-100.84823	3	
				TOTAL	68	175

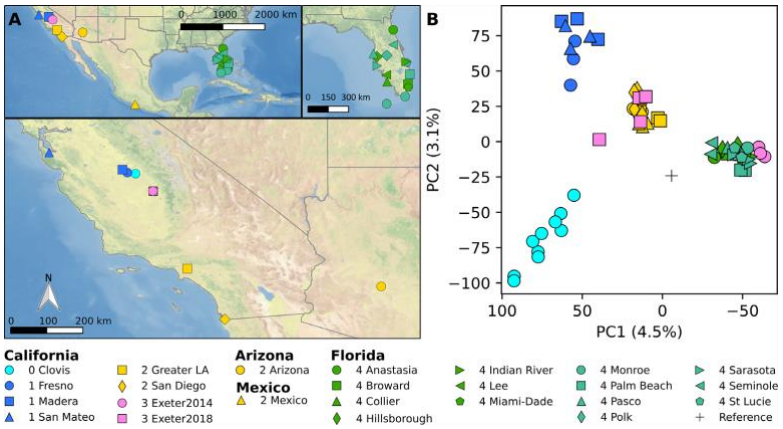

FIGURE 1 | (A) Map of sample location. ClearnTOPO2, a public domain dataset, was used as a basemap. **(B)** PCA analysis based on genome-wide biallelic SNPs.

Libraries were sequenced as 150 bp paired-end reads using a NovaSeq instrument (Illumina) at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) Nextgen DNA Sequencing Core.

SNP Genotyping

Eggs were collected from the field in Clovis and Greater LA and reared in the lab under existing protocols (11). Adult collections from Exeter were obtained from Delta Vector Control District. Sixty individuals from Clovis, 67 individuals from Greater LA, 24 individuals from Exeter 2014 and 24 individuals from Exeter 2018 were collected (**Table 1**, N_{KDR}). DNA was extracted using the Zymo Quick-DNA/RNA Mini Prep Kit (#D7001) using the protocol for Solid Tissue. DNA quality and quantity were determined using a Qubit instrument (Life Technologies) and approximately 4 ng/μL was extracted from each individual.

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DNA was submitted to the UC Davis Veterinary Genetics Laboratory for SNP genotyping using the iPLEX MassARRAY analysis following the protocol described in Lee et al. (12). Thirty-seven SNPs were selected from the published whole genome sequences (8), 29 of which were chosen due to their association with specific genetic clusters and eight within the Voltage Gated Sodium Channel gene. Data for 5 knockdown resistance (*kdr*) SNPs (F1534C, V410L, S723T, I915K, and V1016I) were included from (13). A full list of SNPs and primers can be found in **Table S1**. Populations were clustered using a Principal Component Analysis (PCA) performed in R v4.0.5, removing individuals with no calls for any SNPs.

Genome Sequence Data Analysis

Raw reads were trimmed using fastp (14) version 0.20.1. Trimmed reads were mapped to the Ae13CLOV028MT (Genbank ID: MH348176) first using BWA-MEM (15) version 0.7.15 following recommendation from Schmidt et al. (16) to minimize the impact of mitochondrial reads mapping to the nuclear genome due to presence of pseudogenes (17). Unmapped and mate-is-unmapped reads from mitogenome mapping were filtered using sambamba, converted to fastq files using samtools version 1.12, and mapped to AaegL5 reference genome (18) using BWA-MEM (15) version 0.7.15. Mapping statistics were calculated using Qualimap version 2.2 (19) (**Table S2**). Joint variant calling using all samples was done using Freebayes (20) version 1.0.1 with standard filters and population priors disabled.

The repeat regions were soft-masked in the AaegL5 reference genome and SNPs in these regions were excluded from analysis. Only biallelic SNPs with a minimum of 6X coverager were used for further analysis. A missing data threshold of 10% was used to filter SNPs. Hudson FST (21) and Principal Component Analysis (PCA) analyses was done in Python version 3.6.6 using the scikit-allel module version 1.2.0 (22). A phylogenetic tree based on the SNPs was constructed using the neighbor-joining algorithm as implemented in PHYLIP (23) version 3.696. Bayesian clustering method implemented in ADMIXTURE v1.3.0 (24) was used to estimate ancestry components for each individual. For this analysis, a total of 10 iterations were performed for values of *K* clusters from 1 to 10 with no prior population assignment. The results for each *K* were compiled using the online version of CLUMPAK and plotted in R. Windowed population genetic statistics such as F_{ST} and nucleotide diversity (π) were calculated using scikit-allel library with a 1Mbp window size and half-step overlapping windows.

RESULTS

Thirty-eight *Ae. aegypti* genomes were sequenced, originating from Florida (N=24), Arizona (N=4), Exeter 2018 (N=4), and Clovis (N=6). These genomes were analyzed together with 30 sequenced genomes used in other publications (N=68) (**Table 1**, N_{WGS}) (8, 9). Each sample was sequenced with a mean nuclear genome coverage of 11.9X per sample (**Table S2**; range = 8.3-37.9X; std = 5.0).

Principal component analysis (PCA) of genome-wide biallelic SNP genotypes reveal four major genetic clusters: (1) Clovis 2013 (cyan in **Figure 1**), (2) Fresno, Madera and Menlo Park (blue in **Figure 1**), (3) southern CA, Arizona, Mexico (yellow in **Figure 1**), and (4) Florida (green and sage in **Figure 1**). The *Ae. aegypti* population from Exeter 2014 cluster with Florida populations while 2018 Exeter samples mostly cluster together with southern CA samples. One sample from the 2018 Exeter group occupying the intermediate genetic space between southern CA and two other central populations appears to have genotypes intermediate between southern CA and Clovis populations (**Figure 2**).

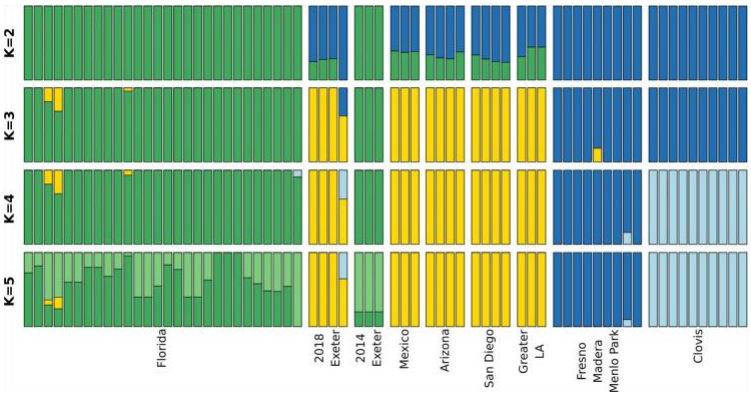

FIGURE 2 | Admixture plot of individual *Ae. aegypti* mosquitoes. Each column represents an individual with probability of each cluster represented by different colors. The total probabilities add up to 1. Individuals with high likelihood of belonging to one cluster would appear as a single color column while individuals with mixed ancestry would have multiple colors in each column.

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Consistent with PCA results, Admixture analysis of genome-wide SNPs clusters 2014 Exeter with Florida mosquitoes while 2018 Exeter clusters together with southern CA, Mexico and Arizona mosquitoes (**Figure 2**). One Exeter 2018 mosquito appears to have a signature of an admixture with the Clovis cluster. This particular sample corresponds to the outlier individual in the PCA that occupied a space between the southern CA and central CA clusters (**Figure 1**). The likelihood values increase continually with higher K (**Figure S1**) indicating that further substructure may be present beyond the K=5 clusters presented in **Figure 2**.

Exeter 2014 and 2018 were the most distant population pairs (F_{ST} = 0.152; **Figure 3** and **Table S3**). Consistent with Admixture results, East Los Angeles, Arizona, Mexico, and Florida form a closely related group with F_{ST} < 0.05 (**Table S3**). Fresno, Madera, and Menlo Park also showed no differentiation with F_{ST} = 0 (**Figures 2**, **3** and **Table S3**). The closest population pair with Exeter 2014 was Florida (F_{ST} = 0.055) while the closest population pair with Exeter 2018 was East Los Angeles (F_{ST} = 0.038).

Based on published genome sequences (8), we selected a set of biallelic SNPs most informative for separating individuals into each of the major three genetic clusters found in CA. We designed a multiplex SNP genotyping assay using the iPLEX MassARRAY system to screen 37 SNPs simultaneously for their genetic background (**Table S1**). Thirty-one of these SNPs differentiate *Ae. aegypti* into each of the four genetic clusters present in California and 6 of these SNPs are associated with permethrin resistance. We genotyped 2018 Clovis, Greater Los Angeles, Exeter 2014, and Exeter 2018 samples using this new multiplex SNP assay. Our PCA analysis of SNP genotypes (**Figure 4**) shows Clovis and Greater Los Angeles forming separate genetic clusters, although the boundary is not as clear as genome-wide SNP data shown in **Figure 1**. Consistent with genome-wide SNP data, Exeter 2018 has large overlap with southern CA samples with two samples potentially having mixed ancestry from the southern CA and Clovis clusters. Exeter 2014 *Ae. aegypti* clearly clustered separately from the Clovis 2018, Exeter 2018, and Greater Los Angeles *Ae. aegypti* mosquitoes.

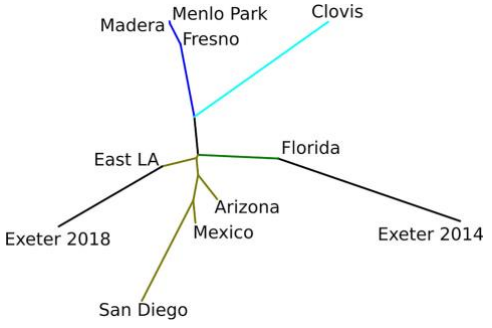

FIGURE 3 | Neighbor-joining tree based on pairwise F_{ST} distance.

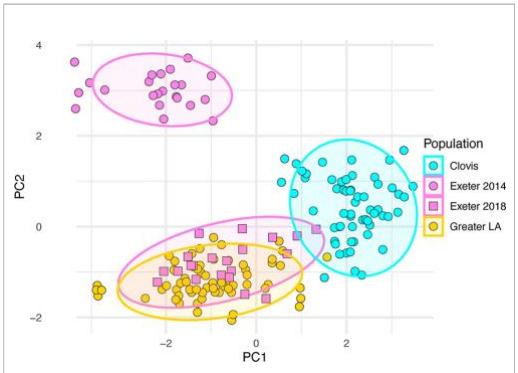

FIGURE 4 | Principal component analysis based on 37 SNPs in *Ae. aegypti* from Central and Southern California using the iPLEX MASSarray genotyping.

We calculated the windowed F_{ST}, nucleotide diversity (π), and change in π between 2018 and 2014 Exeter samples (**Figure 5**) to identify any hotspots of divergence. Any F_{ST} greater than 0.1 (red line in **Figure 5A**) indicates high genetic distance in the corresponding genomic region between two years. The genomic divergence between the two years appears to be genome-wide except in limited locations such as in the immediate vicinity of the centromeres. Overall elevated π was observed near the centromeres of Chromosome 1 and 3 (**Figure 5B**). While 2018 Exeter samples had higher π in Chromosome 1, 2014 Exeter samples had higher π in Chromosome 3 (**Figure 5C**).

We also analyzed 6 SNPs in the voltage-gated sodium channel gene, typically known as knockdown resistance gene (*kdr*) in mosquito literature (AAEL023266) located on Chromosome 3 using the method described in Mack et al. (13). Five of these 6 SNPs were previously used in Mack et al. (13). The SNP Q1853R (3:315931672) is a new addition to this study as it was only detected in the mosquitoes derived from the Exeter 2014 population. None of the other Californian populations possess this SNP and it appears to have disappeared with the eradication of that population. More than 60% of Exeter 2014 and 2018 *Ae. aegypti* had a V1016 resistant allele, while >90% of Clovis mosquitoes had this resistant allele present. Other nonsynonymous mutations in the *kdr* gene also occurred at the highest frequencies in Clovis. Except for allele F1534C, which was almost fixed in Clovis, alleles V410L, S723T and I915K were at much lower frequencies in Exeter 2014, 2018 and Greater LA *Ae. aegypti* (**Table 2**).

DISCUSSION

Our results strongly suggest that control actions taken by Delta Vector Control District personnel eliminated the local *Ae. aegypti* population in Exeter in 2014-2015. *Aedes aegypti* reinvaded Exeter in 2018 originating from different location/s than what originally invaded in 2014 and became established and are still present. We were able to confirm elimination and reinvasion in Exeter by comparative genetic analyses on 2014 and 2018 Exeter individuals.

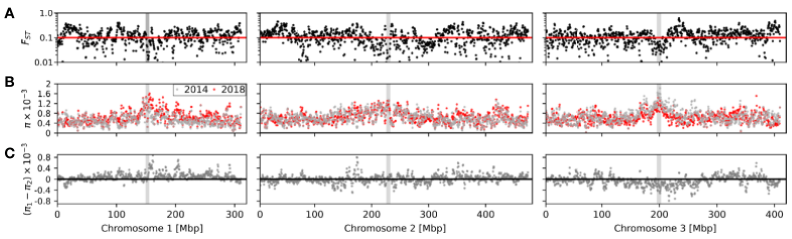

FIGURE 5 | **(A)** Windowed F_{ST}, **(B)** nucleotide diversity (π), and **(C)** change in nucleotide diversity (π_{2018} - π_{2014}) between 2014 and 2018 Exeter samples. The gray bar in the middle of each chromosome indicates location of the centromere.

TABLE 2 | Knockdown resistance (kdr) mutation genotypes per population.

Coord.	V410L 3:316080722				S723T 3:316014588				I915K 3:315999297				V1016I 3:315983763				F1534C 3:315939224				Q1853R 3:315931672			
	SS	SR	RR	%R	SS	SR	RR	%R	SS	SR	RR	%R	SS	SR	RR	%R	SS	SR	RR	%R	SS	SR	RR	%R
Clovis 2013	0	2	8	90%	0	2	8	90%	0	2	8	90%	0	2	8	90%	0	0	19	100%	10	0	0	0%
Clovis 2018	0	9	51	92.5%	0	9	51	92.5%	0	0	60	100%	0	1	59	99%	0	1	60	99%	60	0	0	0%
Exeter 2014	6	14	4	46%	6	14	4	46%	3	12	9	63%	3	12	9	63%	0	0	24	100%	17	7	0	15%
Exeter 2018	3	7	12	70%	3	7	12	70%	0	0	22	98%	3	7	12	70%	0	0	22	100%	22	0	0	0%
Greater LA 2018	11	37	19	59%	11	37	19	59%	0	15	52	88.8%	11	37	19	59%	0	1	66	99%	67	0	0	0%

Clovis 2013 data is genome sequencing data. Clovis 2018 data and Greater Los Angeles (LA) 2018 data are from Mack et al. (13). S stands for susceptible (reference) allele and R for resistant (alternate) allele. Genomic coordinates of kdr SNPs are provided on the 2nd row of this table.

Had the Exeter population experienced a severe reduction due to DVCD control efforts, followed by 3-4 years of recovery before detection again, then the 2018 Exeter individuals would be expected to be genetically similar to those from Exeter 2014. However, the 2018 Exeter samples form a genetic cluster separate from that of the 2014 Exeter cluster. Genetic differentiation between 2014 and 2018 Exeter samples appears genome-wide with the majority of genomic regions exceeding $F_{ST} > 0.1$ (**Figure 5**). This pattern is in contrast to the level of genetic differentiation we observed between Clovis 2013 and 2016 (8). Such a drastic change in genome structure would be extremely unlikely to develop within a few years simply by genetic drift or natural selection, adding additional evidence toward the local elimination and reintroduction hypothesis for *Ae. aegypti* population in Exeter, CA.

The Exeter 2018 cluster more closely aligns with the samples from the Southwestern USA and Mexico indicating that 2018 samples likely resulted from a reintroduction to Exeter. The most likely source of reinvading individuals would intuitively come from neighboring cities like Clovis and Fresno, which experience high abundances of *Ae. aegypti* in the middle to late summer. However, the genetic similarity of the Exeter 2018 to southern CA *Ae. aegypti* suggests that the founding Exeter 2018 mosquito/es were likely escapees traveling in a vehicle traveling from southern CA, demonstrating the propensity of *Ae. aegypti* for human-mediated dispersal across long distances. Our study is the first report of *Ae. aegypti* from a Central CA location sharing the majority of its genetic profile with *Ae. aegypti* from southern CA. Importantly, when *Ae. aegypti* were detected in 2018 in Exeter, they were also detected in several Visalia suburbs (unpublished data), and it remains to be learned whether the *Ae. aegypti* that are now present in Visalia and other towns neighboring Exeter are genetically similar or different to those from Exeter and whether they are migrants from the second Exeter invasion or from other neighboring central California locations. The success in eliminating the population detected in 2014 is likely due to the limited geographical scope of the population and the intensive control efforts that were implemented. However, the possibility of eliminating the new populations of *Ae. aegypti* in Exeter and suburbs of Visalia is unlikely as resources are stretched too thin to mount such an intense control effort across such an extensive area.

Southern CA *Ae. aegypti* had different frequencies of non-synonymous SNPs or mutations in the VGSC gene than in Central CA populations, including the ubiquitous V410L mutation that is known to confer resistance to pyrethroids (**Table 2**). If all six of the mutations in the VGSC gene included in this study confer some levels of resistance to pyrethroids (*kdr* resistant alleles) then Southern CA populations have a higher proportion of susceptible alleles relative to central CA populations which is consistent with a previous report (13). Although Exeter 2014 and 2018 samples had different overall genetic profiles, they both contained a greater number of pyrethroid susceptible *kdr* alleles than the Clovis population. Presence of higher frequencies of *kdr* susceptible alleles may have been a factor leading to elimination of *Ae. aegypti* in Exeter in 2014, while other neighboring central CA districts struggled to manage dispersal of their *Ae. aegypti* populations.

These findings confirm that implementation of intensive control efforts can eliminate *Ae. aegypti* locally. However, the

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replacement of the Exeter 2014 population with individuals related to the Southern CA population that had a higher proportion of pyrethroid resistance alleles adds to the difficulty of adulticide control that could be further exacerbated when they hybridize to CA Central Valley populations that have an even greater number of *kdr* alleles. Elimination strategies require intensive efforts and resources, including public consent and establishment of a strong public relations program. Anticipating challenges resulting from the dispersal of resistant populations throughout California will be critical to comprehensive and sustainable control strategies.

This is the first record of southern CA-like *Ae. aegypti* genetic cluster found north of Kern county [latitude 35.21N; (25)]. The results from our study suggest that the population of southern CA *Ae. aegypti* continues to expand its range northwards and will hybridize with the existing Clovis population, corroborating previous indications of this possibility presented in Lee et al. (25). Additional statewide surveillance on the geographic distribution of *Ae. aegypti* genetic clusters combined with socio-environmental parameters may inform the nature and potential mechanisms of *Ae. aegypti* dispersal pathways within CA. Our relatively low-cost SNP genotyping assay or a similar approach could be a cost-effective way to screen a larger number of samples than current whole genome sequencing approaches and would be a useful tool to elucidate genetic mixing, origin of introductions, and pyrethroid resistance status of local populations.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI BioProject repository with accession number PRJNA725510.

AUTHOR CONTRIBUTIONS

YL, GA, GL, and AC conceived experimental design. EK, LM, and GA conducted SNP genotyping analysis. AR-W, T-YC, and KK conducted genomic DNA library preparations for whole genome sequencing. YL, MC, and TC conducted genome data analysis. EK, LM, KB, CG, RR-C, KS, LC, and EB conducted sample collection or arranged sample acquisition and provided the sample metadata. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2021.703873/full#supplementary-material

Supplementary Table 1 | iPLEX SNP primers and assay information

Supplementary Table 2 | Whole genome sequence metadata.

Supplementary Table 3 | Pairwise F_{ST} values.

Supplementary Figure 1 | Log likelihood values of Admixture runs.

Supplementary Figure 2 | Admixture plots for higher K values.

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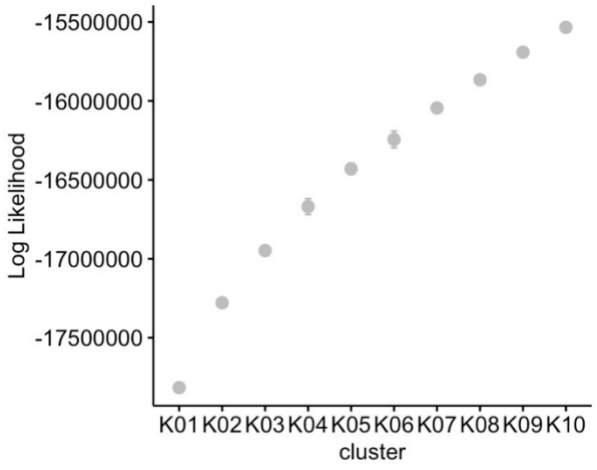
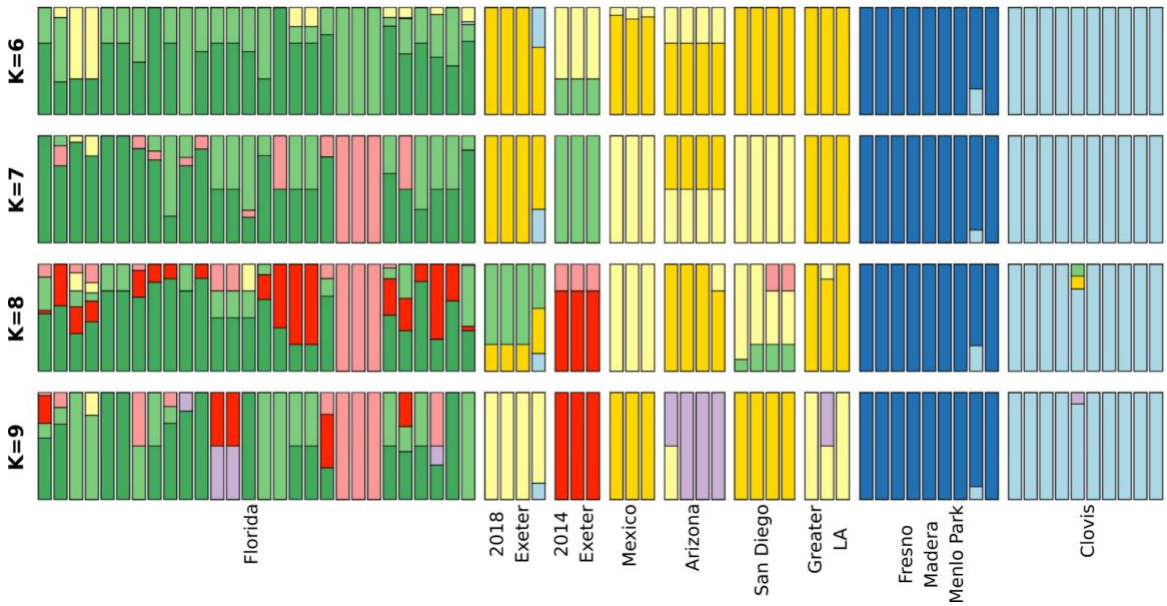
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Figure 1. Log likelihood values of Admixture runs



Supplementary Figure 2 | Admixture plots for higher K values.



CHAPTER 2. Exploring the Wilderness Within: An Integrative Metabolomics and Transcriptomics Study on Near-Wild and Colonized *Aedes aegypti*

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ABSTRACT

Comparisons between field derived insect colonies and insecticide susceptible laboratory strains can provide valuable insight in insect research, tempered by the awareness that colonization and other life-history differences can make phenotypic differences difficult to attribute. Laboratory strains are essential benchmarks in monitoring insecticide resistance status, and can play important roles in attempts to understand aspects of field population physiology. This is especially true when researching pyrethroid resistance in the yellow fever mosquito *Ae.aegypti*, as wild meaningfully pyrethroid susceptible populations are exceedingly rare. As such, we endeavor to conduct a thorough phenotypic comparison of a wild derived F2 Central Valley mosquitoes compared to the susceptible lab reference colony, Rockefeller (Rock), using metabolomic data, gene expression data and lifespan data in order to better understand resistance physiology in this invasive mosquito population and investigate whether metabolites could be developed as diagnostic biomarkers of metabolic resistance status. We compare Rockefeller and Central Valley mosquitoes using gene expression and metabolomic data. We additionally compare the impacts of larval diet on lifespan for multiple Central Valley mosquito populations with variable levels of metabolic resistance to pyrethroids to Rockefeller mosquitoes. From lifespan analysis we find more metabolically resistant wild derived mosquitoes lifespan is sensitive to restricted larval nutrition. From metabolomic and gene expression analysis we find Central Valley mosquitoes have constitutive upregulation of oxidoreductase activity, glutathione metabolism and increased flux through the pentose phosphate pathway, likely to support these processes. We find Rock, on the other hand,

demonstrates evidence of metabolic inefficiency and mitochondrial dysregulation, which may be tolerated in a laboratory environment. We review how Central Valley *Ae. aegypti* P450 and GSTE profiles compared to other insecticide resistant groups, and conclude that while metabolomic data can classify our study groups, few detected markers meet high fold change thresholds that would make them good candidates for biomarker development.

INTRODUCTION

Insect reference strains play essential roles in insect research. Reference strains enable reproducible experimentation, and can serve as important baselines for comparative analyses. These reference strains differ from colonies in that strains are bred continuously in the lab for many generations without “replenishment” with field collected mosquitoes (Kuno 2010). These strains become genetically homogenous and may change significantly as they proliferate without the selective pressures of the field. The Rockefeller (Rock) strain of *Aedes aegypti (Ae. aegypti)* has a history nearly 140 years long, and is frequently used as a reference strain in insecticide resistance evaluations of *Ae. aegypti* due to its susceptibility to the insecticides typically applied for adult mosquito control, mainly pyrethroids and organophosphates.

Rock is frequently utilized in insect physiology and resistance studies, where comprehensive physiological research comparing Rock to wild *Ae. aegypti* populations provides important context. Baseline differences in stress response physiology, energy metabolism, and chemoreception have important implications for mosquito research in areas like viral competence, metabolism, and insecticide resistance. In this study we provide a comprehensive phenotypic comparison of Rock and a near wild colony derived from the Central Valley of California by integrating metabolomic and transcriptomic analyses with phenotypic assays.

Several studies have used transcriptomic data in attempts to identify shared pyrethroid detoxification pathways that could be candidates for surveillance of metabolic resistance (Jean-Philippe David et al. 2014; Frédéric Faucon et al. 2017; Muhammad

Riaz et al. 2013; Strode et al. 2008; Poupardin et al. 2012; Saavedra-Rodriguez et al. 2019). However, no previous research has integrated metabolomic data.

California was free of *Ae. aegypti* until 2013, when the mosquito was detected in the city of Clovis within Fresno County in the heart of the San Joaquin Valley (Metzger et al. 2017). Its initial persistence through the Valley's winter months was a surprise, and it has since been detected throughout the state. There are multiple population groups of *Ae. aegypti* in California. The *Ae. aegypti* in the southern part of the state appear to resemble surrounding populations in the southwestern US, while the origins of *Ae. aegypti* in the San Joaquin Valley are less clear and appear derived from multiple introductions, though one group bears genetic similarities to those found in the Southeastern US (Pless et al. 2017). Deployment of pesticides for control of these populations in Clovis, CA and surrounding cities revealed that they demonstrate a strong resistance to pyrethroids (Cornel et al. 2016; Mack et al. 2021). Early eradication efforts failed, and these mosquitoes have remained a persistent problem. This area was even selected as a candidate for the evaluation of Wolbachia-infected mosquito release program(Crawford et al. 2020).

This study endeavors to investigate how wild, insecticide resistant populations of *Aedes aegypti* in California compare physiologically to a susceptible lab reference strain (Rockefeller) by integrating transcriptomic and metabolomics analyses. In addition, we use near wild (F2) populations with similar background genetics and variable resistance profiles to explore hypotheses related to the trade offs between resistance and fitness parameters such as lifespan and fecundity. These studies elucidate the importance of the pentose phosphate pathway in metabolic resistance and highlight important

alterations in cellular metabolism between a wild and colonized mosquito line. We also explore the potential for use of metabolites as markers of the insecticide resistance phenotype.

METHODS

Insect Colonies

Lifespan, fecundity, metabolomic and transcriptomic studies were conducted using near wild (F2) colonies of *Aedes aegypti* collected from cities in Fresno and Tulare county and maintained in our insectary. The Rockefeller (Rock) mosquitoes are an inbred laboratory strain (Kuno 2010). The wild derived colonies are F2 colonies generated from field collections conducted by the Cornel lab at the Kearney Research and Extension center in three cities in the San Joaquin valley of California; Clovis, Dinuba, and Sanger. This region has a high prevalence of *Aedes aegypti*, and was the site of first detection in 2013 when *Aedes aegypti* were introduced into the state (Gloria-Soria et al. 2014).

Mosquito Rearing

Metabolomic and Transcriptomic Analyses: Samples were reared on a standard diet composed of Tetramin fish food. Samples were age-matched by pupation date and collected by aspiration 5 days post eclosion. The 10% sucrose solution used to feed adult mosquitoes was withdrawn 36 hours prior to sample collection, and replaced with water. Samples were flash frozen on liquid nitrogen, and then stored at -80 until they were submitted to the West Coast Metabolomics core for analysis. The collection period for all samples was restricted to a 1.5 hour window from 1 to 2:30pm on a single day.

Lifespan and Fecundity Assays: Study mosquitoes were reared at a density of 200 larvae per tray with 1000mls of tap water. Larvae were fed two diet treatments consisting of FLUVAL Cichlid pellets: our standard *Ae. aegypti* culture diet (full diet) and a restricted diet (half) (Table 2.1). Pupation was tracked daily from 5-9 days post eclosion. All treatments were blood-fed at 25 days post-eclosion. Adults were placed into cages by tray and dead individuals were counted and removed daily.

Table 2.1. Larval Diet Treatments for Lifespan and Fecundity Assays. A pinch is a measurement equal to 1/16 teaspoons, while a drop is equivalent to 1/64th teaspoons.

Day	Full Diet (ground fish flakes)	Half Diet
0 (Hatch Day)	2 pinches	1 pinch
1	1 drops	No food
2	2 drops	1 drops
3	4 drops	2 drops

4	4 drops	2 drops
5	8 drops	4 drops
6	6 drops	3 drops
7	4 drops	2 drops

Metabolomic Profiling

Frozen samples were submitted to the University of California, Davis West Coast Metabolomics Center for analysis using a set of 3 complementary metabolomic mass spectrometry (MS) based assays, designed to measure primary metabolites, lipids, and biogenic amines. Primary metabolites, including carbohydrates, amino acids, fatty acids, nucleotides and aromatics, were detected using a gas chromatography-time-of-flight (GC-TOF) mass spectrometer. Lipids were analyzed on a Quadrupole Time of Flight Mass Spectrometer (QTOF-MS) and with MS-Dial 3.98, after filtering for a minimum peak intensity of 1000. Biogenic amines, including acylcarnitines, TMAO, nucleotides and nucleosides, methylated and acetylated amines, di- and oligopeptides, were measured using a Hydrophilic Interaction Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry with Tandem Mass Spectrometry(HILIC QTOF MS/MS). Data was analyzed using Metaboanalyst 5.0 and ChemRich (Pang et al. 2021; Barupal and Fiehn 2017). Samples were normalized by the sum of internal standards, log transformed and mean centered prior to performing principal component analysis. For each assay panel t-tests were performed followed by false-discovery rate. Throughout the chapter p values refer to FDR adjusted p values. Accurate peak annotation is a

significant hurdle to interpretation of untargeted metabolomics data, so we used the Mummichog algorithm within Metaboanalyst to investigate pathway activity, and generate insight from both annotated and unannotated peaks in our dataset (Pang et al. 2021). Mummichog maps peaks to predefined metabolic networks or pathways using retention time and mass to charge ratio.

Library Prep and Transcriptome Sequencing

RNA was extracted using a Zymo RNA Cell and Tissue Kit, and submitted to the UC Davis Genome Center for library prep and 3' Taq-seq analysis. 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 (LifeTechnologies, 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.Reads were checked for quality using FastQC v0.11.9, then trimmed using bbduk, a function within bbmap (v37-50). Resulting reads were aligned to the *Aedes aegypti* LVP_AGWG-50 genome, indexed with an –sjdbOverhang 99 [76] using STAR v2.7.2a. Read files were then indexed using samtools v1.3.1.

Differential Gene Expression and Enrichment Analyses

Differential gene expression analysis was performed using edgeR(Robinson, McCarthy, and Smyth 2010). Additionally iDEP (integrated differential expression and pathway analysis) was used for exploratory data analysis(Ge, Son, and Yao 2018). Samples were filtered to only include genes with a minimum of 2 Counts per million (CPM) in 12 of 19 libraries. Of the 19804 genes in 19 samples, 9195 genes passed filtering. Principal Component analysis was employed to evaluate sample clustering. The differential gene expression threshold was set at 1.5 minimum fold change, with a false-discovery rate cutoff of 0.05. The differentially expressed genes (DEG) were used to perform gene set enrichment analysis (Table 2). PGSEA (parametric gene set enrichment analysis) was performed using the PGSEA package with all samples (Figure 2.5)(Furge and Dykema, n.d.). Gene annotations were downloaded from Vectorbase 65. For genes with unspecified products Computed GO Functions and Components were used to infer function, alongside cross referencing of mosquito and drosophilid orthologs.

RESULTS

More resistant field lines live longest, except for females under nutritional stress.

Median time-to-knockdown and voltage gated sodium channel mutation frequency for all populations are reported in Mack et al (Mack et al. 2021). The V410L, F1534C and V1016I mutations are nearly fixed in these populations (Liebman et al. 2019; Mack et al. 2021). The median knock-down time in response to pyrethrum for Clovis mosquitoes was 82 minutes, 11 times greater than that of the susceptible

reference colony, Rockefeller (5 minutes). The median knock-down time of Dinuba mosquitoes was 53 minutes, just 1.35x greater than that of Sanger at 39 minutes, and 7.5x greater than that of Rockefeller. The median knock-down time of Sanger was 5.5x greater than Rockefeller. The diagnostic time for pyrethrum is 15 minutes, the time by which 100% mortality would be observed for a susceptible population. On a standard larval diet female body size did not differ significantly between groups and survivorship was only significantly different by survival analysis for Sanger vs. Rockefeller mosquitoes, with Sanger mosquitoes exhibiting a shorter median survival time but a larger portion of long lived mosquitoes (>60 days) (Figure 2.2). Females of each population outlived their male counterparts in both treatments. When mosquitoes were subjected to a restricted larval diet, both Sanger and Rockefeller females outlived their standard diet counterparts. This was not the case for the more metabolically resistant Dinuba, which experienced a decrease in life expectancy. For males, the restricted larval diet was only life-extending for Sanger males, and non-significant for Rockefeller and Dinuba. On the full larval diet we measured first clutch size and observed a slight general trend of decreased fecundity with increasing resistance to pyrethrum, but sample sizes were small and the results were not statistically significant (Supplementary Figure 2.1).

Metabolomics Panels and Transcriptome Profiles Classify Populations in Principal Component Analysis

Of the 133 annotated primary metabolite features just 29 were differentially enriched with a fold change over 1.5 and $p<0.05$ (9 up, 20 down in Clovis relative to Rock). Of the annotated metabolites only sucrose levels differed by greater than 10 fold

change (up 16x Rock, p =0.001) From the biogenic amine panel 15 compounds were up, 29 down out of 161 annotated features. The dipeptide Gly-Pro was up 10x in Clovis ($p < 0.005$). From the lipid panel of 590 annotated features 77 were up, 32 were down in Clovis relative to Rock with a 1.5 fold change cutoff and FDR adjusted $p<0.05$, and no annotated compounds met our 10x, sub 0.005 FDR adjusted p threshold. Principal component analysis was performed to investigate sample clustering for both transcriptome data and the three metabolomics panels (lipids, biogenic amines and primary metabolites). All four datasets separated samples by population, with the greatest overlap in the biogenic amine panel (Figure 2.3). Gene expression data resulted in the clearest separation by population, but PC1 and PC2 explained just 35% of the variance in the data. Lipid metabolite data, on the other hand, still grouped samples well by population, and PC1 and PC2 explained 62% of the variance in the dataset. Notably Rock samples appeared to cluster more tightly, likely reflecting the lower diversity in this laboratory strain. We utilized Random Forest analysis to select features that differentiate between our population groups, the ten top features arranged by feature importance are included in Figure 2.3. From the gene expression data CYP9J26, a cytochrome P450 repeatedly associated with insecticide resistant groups was a top distinguishing feature(Frédéric Faucon et al. 2017; Strode et al. 2008). Notably across two assays (primary metabolites and biogenic amines) Guanosine and Threonine were top features, elevated in Clovis. Phosphatidyl-inositols were distinguishing features abundant in Clovis, while ceramides were enriched in Rockefeller.

Metabolomic Profiles Reveal Enrichment in Pentose Phosphate Pathway Metabolites, Glutathione Metabolism and Lysolipids in Wild *Ae. aegypti* relative to Rockefeller

In order to gain insight from unannotated metabolites LC-MS peaks were analyzed using the Metaboanalyst functional analysis module within Metaboanalyst. From the biogenic amine data 2694 features were analyzed, and 31% were significant with a p value threshold of 0.005. The lipid dataset included 16,841 peaks, 10556 peaks were detected in positive ESI mode, and 6285 were detected in negative ESI mode of which 18% and 45% of peaks were significant respectively. Pathway enrichments are represented in Figure 2.5. Metabolic networks are relatively less-well annotated with regards to metabolites, and pooling metabolomics panels resulted in fewer significant pathway hits compared to gene set enrichment analysis.

We had predicted maintenance of enzymes conferring pyrethroid resistance like CYPs and GSTs may result in decreased energy stores for Clovis mosquitoes (Hardstone et al. 2010), yet we instead observed that Clovis mosquitoes had relative enrichment of saturated and unsaturated triacylglycerols. We observed Clovis mosquitoes had enrichment of unsaturated fatty acids (arachidonic acid being the key compound, Supplemental Table 2.1), but lower amounts of ceramides and phosphatidylethanolamines, which play essential roles in the modulation of membrane fluidity in insect cells (Dawaliby et al. 2016). Ceramides, enriched in Rock, also play important roles in mediating fecundity in insects, and are associated with mitochondrial dysfunction in mammals (Shi et al. 2021; Roszczyc-Owsiejczuk and Zabielski 2021). Fatty acids and lysolipids, common stress biomarkers, were enriched in Clovis, which aligned with our hypothesis that Clovis may have elevated markers of oxidative stress

(Tan et al. 2020; Stanley and Kim 2020), as were levels of oxidized Glutathione (Figure 2.6). Histidine was enriched in Clovis, and plays an important role in normal mosquito egg development (Hansen et al. 2011). We observe significant under-enrichment of amino acids in Clovis, with Threonine, Histidine, Proline and Lysine as exceptions. Differential enrichment of certain b vitamins and their derivatives was also observed, with biotin and folinic acid enriched in Rock, while nicotinamide and 4-pyridoxic acid were enriched in Clovis. We observed subtle alterations in sugar profiles; sucrose and ribose were elevated in Rock (1.6 FC and 1.4 FC), while glucose was very slightly elevated in Clovis (1.3 FC) and glucose-6-phsophate, fructose-6-phosphate, ribose-5-phosphate, phosphogluconic acid and fructose-1-phoshphate, metabolites in glycolysis and the PPP, were all elevated in Clovis(Figure 2.6).

We observed differential enrichment of several neuro-active metabolites and urea cycle metabolites(Figure 2.6). Histamine levels were moderately elevated in Clovis (1.6FC, $p < 0.0005$), and histamine acts as a neurotransmitter in insects, with histamine receptors active in mosquito brains and peripheral tissues(Matthews et al. 2016). We found 3-Hydroxykynurenine was elevated in Rock (2.5 FC, $p<0.005$), as was Kynurenic acid (1.6FC, $p<0.005$), both important metabolites of tryptophan metabolism to xanthurenic acid(1.2FC up in Rock, $p = 0.006$) in mosquitoes, a processes essential for normal eye development and mediating oxidative stress from blood-feeding in mosquitoes(Han, Beerntsen, and Li 2007; Lima et al. 2012). Gamma-Aminobutyric acid(GABA) plays an important role in mediating immunity to dengue infection, and was enriched in Rock (GABA, 1.6 FC, $p<0.005$)(Zhu et al. 2017). Components of the urea

cycle including ornithine(1.5 FC, p<0.005) and urea (2.2 FC, p<0.005) were moderately elevated in Rock.

Genes Associated with Detoxification are Overexpressed in Clovis, while Immune and Catabolic Processes are up In Rock

Sequencing generated 3' Taq-Seq single end reads, with an average library size of 1498609(min:826843, max:1928446) across samples an average of 87% of reads mapped to the reference genome. Gene annotations were derived from Vectorbase (Release 65). Over 900 genes (493 up in Clovis, 419 down) were differentially expressed between the two groups with a FDR cutoff of 0.05, and a minimum fold change of 1.5, and 383 (204up, 179 down) were differentially expressed with a 2x fold change threshold. Detoxification genes, particularly Cytochrome P450s were, unsurprisingly, among the most differentially expressed genes (DEGs). The most overexpressed in Clovis was CYP6AG4 (p <0.005, 29 FC), which was associated with a pyrethroid susceptible strain in (Strode et al. 2008), while CYP9J26(p <0.005, 17 FC) was second. Others included CYP6AG7(p <0.005, 7 FC) associated with Deltamethrin resistance (Saavedra-Rodriguez et al. 2019), CYP6BB2 (p <0.005, 5 FC) overexpressed in permethrin, imidacloprid and propoxur selected resistant larvae (Jean-Philippe David et al. 2014) and insecticide resistant mosquitoes in Puerto Rico relative to Rock (Derilus et al. 2023). Additionally CYP6Z8 (5 FC, p <0.005) and GSTE6,4,3 (p <0.005) were overexpressed in Clovis and trend towards enrichment in resistant groups in (Frédéric Faucon et al. 2017). GSTE6 was also enriched in Puerto Rican mosquitoes that survived lambda-cyhalothrin exposure(Derilus et al. 2023). Enrichment analyses (Table 2.2, Figure 2.5) reveal that genes related to monooxygenase activity, antioxidant

activity and response to oxidative stress are upregulated in Clovis. Additionally, enrichment analyses, complimented by metabolomic data, support enrichment of the pentose phosphate pathway and NADP metabolic processes. While Central Valley *Ae. aegypti* were the subject of a thorough pyrethroid exposure response study, we find little overlap in the detoxifying genes identified in the study, with the exception of AAEL006829, a microsomal glutathione-s-transferase, and significant overlap in pathway enrichment (Figure 2 within, (Mack and Attardo 2023). This may reflect that the detoxifying genes upregulated in our study are involved in more immediate insecticide response (<6 hours post exposure), or play other roles such as mediating cytotoxic stress induced by xenobiotic challenge and maintenance of the metabolic resistance phenotype.

When broadly comparing disparate mosquito populations the reasons for observed differences are often impossible to know conclusively. That said, we hypothesized that some of the differences we may observe in Clovis and Rock may be related to environmental adaptation. Rockefeller, as a reference strain, is typically maintained in high-temp, high-humidity insectaries. Our Clovis mosquitoes, on the other hand, are near-wild mosquitoes collected from the Central Valley, USDA zone 9b and parent generations experienced hot, dry summers and winter lows reaching 20-25 degrees celsius. Recent work investigating genomic signatures of local adaptation in CA *Ae. aegypti* resulted in a list of 112 candidate genes as putative candidates of local adaptation (Soudi et al. 2023). Of these, 18 were differentially expressed as transcripts in our study, 11 up in Clovis ($p < 0.05$) and 7 were up in Rock. Up in Clovis were Synotropin-like 1, involved in adapting cellular homeostasis (Carney et al. 2023)

(AAEL019820, 2.2 FC), and fringe, which is involved in modulating Notch signaling (Grammont and Irvine 2001)(AAEL002253), lipophorin receptor 2 (AAEL019755), unpaired 3, involved in tissue repair and development (Wang et al. 2014) (AAEL024562), SoxNeuro, a transcription factor involved in central nervous system development, (AAEL000584) were all 1.6 fold up. Bloated tubules (AAEL010883) was up just 1.2 fold, but notably encodes a member of the sodium and chloride dependent neurotransmitter family. In Rock javen-like(AAEL004209), Rab23 (AAEL001532) and Tenascin major (AAEL000405) were up (FC of 1.4, 1.3 and 1.3) and notably all involved in embryonic development. We identify additional genes involved in ion balance not identified in the local adaptation study, including differential expression of inward-rectifying potassium channel genes, with Kir2B up in Clovis (1.8 FC, p= 0.001) and Kir2A up in Rock(1.4 FC, 0.01), and AAEL005575, a putative transient receptor potential channel 4, (3.1 FC, p<0.005).

In Rock, peptidases, cholesterol transport and genes involved in nucleotide and lipid catabolic processes were up-regulated. Many of the top up-regulated genes in Rockefeller were unspecified products, with computed GO functions as structural components of the cuticle (AAEL020471, 11FC p= 0.005), chitin binding (AAEL023490, 3.7 FC, p= 0.007) and multiple predicted serine endopeptidases and protein kinases. Antimicrobial genes cecropin (AAEL029047, 3.7 FC, p= 0.02) and defensin antimicrobial peptide (AAEL003832, 3.7 FC, p= 0.007) were also up-regulated, along with the leucine-rich immune proteins (LRIM) 8, 10A, 10B, 13, 17 and 24, though LRIM18 was up in Clovis. Mitochondrial genes were highly differentially expressed(ND6 11 FC, mRpL37 7 FC). While differentially expressed genes were generally dispersed

throughout the genome, using iDEPs Genome tool we find a significant cluster of DEGs on the mitochondria genome. We also observed mild upregulation of genes associated with differentially enriched metabolites such as AAEL012955, a phosphatidylethanolamine binding protein (2 FC, $p < 0.005$), and a sucrose transport protein (AAEL011519, 2 FC, $p < 0.005$). Additionally we find a protein phosphatase-2a (AAEL004288, 1.5 FC, $p < 0.005$) perhaps related to the elevated ceramide levels observed in Rock.

Table 2.2. Pathway Enrichment Results.

GO Molecular Function			
Direction	**adj.Pval**	**Genes (n)**	**Pathways**
Up in Rock	3.8E-04	21	Peptidase activity
	2.6E-03	25	Transition metal ion binding
	6.6E-03	34	Hydrolase activity
Up in Clovis	6.5E-05	13	Tetrapyrrole binding/Iron Binding/Monooxygenase activity
	2.4E-03	2	Farnesol dehydrogenase activity
	3.1E-03	28	Transition metal ion binding/Oxidoreductase activity
GO Biological Processes			
Direction	**adj.Pval**	**Genes (n)**	**Pathways**
Up in Rock	2.3E-03	21	Proteolysis
	2.3E-03	3	Sterol transport, Intracellular lipid transport
	2.6E-03	3	Defense response to bacterium

Metabolites Clarify Pathway Level Gene Expression Differences in Essential Metabolic Processes and Nervous System Organization

Pathway analysis and metabolite enrichment overlap in hits on the pentose phosphate pathway, with transaldolase and transketolase up in Clovis, and enrichment of metabolites throughout the pathway(Figure 2.6). Clovis mosquitoes may be using the non-oxidative branch to increase flux through glycolysis to the TCA cycle, though these pathways are not as ubiquitously altered as the PPP. Within glycolysis the phosphopyruvate hydratase complex (AAEL024228, 3.5 FC, $p<0.005$) and an NAD+ dependant aldehyde dehydrogenase (AAEL01480, 2.8 FC, $p <0.005$) are up in Clovis, while in the TCA cycle we only see mild alteration of malate dehydrogenase which catalyzes the malate to oxaloacetate step (AAEL008166, 1.4 FC, $p= 0.04$), and the isocitrate to oxalosuccinate conversion which precedes amino acid metabolic pathways (AAEL000746, 1.4 FC, $p=0.002$) though this enzyme also acts in glutathione metabolism.

In Clovis we observe hormone changes relative to Rock, particularly Farnesol dehydrogenase activity, potentially indicating relatively higher levels of JH synthesis(Mayoral et al. 2009), while in Rock we see evidence of elevated levels of 20E based on elevated expression of AAEL027264 (2.4 FC, $p<0.005$), a putative Phantom (CYP306a1) ortholog. The balance of these hormones can mediate fecundity and metabolic flux (Ekoka et al. 2021; Gruntenko and Rauschenbach 2008). In Rock translation initiation complexes (eIF3h, 2 FC, $p < 0.005$), are active along with lipid transport and localization processes (Figure 2.5A). AAEL007899, found up in non-blood fed ovaries, is up slightly in Rock(1.4 FC, $p = 0.04$) (Matthews et al. 2016). Lysosomal

activity, mannosidase activity and mitochondrial activity are all enriched in Rock relative to Clovis, potentially representing the breakdown of materials to liberate cellular resources, potentially for reproduction(Figure 2.5A).

DISCUSSION

Reference mosquito strains play essential roles in insect research, serving as essential benchmarks in bioassays. Here, we combine lifespan data, transcriptomic, and metabolomic assays to provide a thorough phenotypic comparison of Rockefeller and California populations of wild *Aedes aegypti.* We observe differences in levels of metabolic enzymes associated with pyrethroid resistance, and fundamental alterations in metabolic pathways mediating lifespan and response to oxidative stress (Figure 2.5,2.6). In lifespan assays, when comparing our wild populations with conserved V410L, 1016, and 1534 genotypes, we observe wild pyrethroid tolerant groups to have modestly longer lifespans, and for females lifespan is extended by larval diet restriction, with the exception of our more metabolically resistant group. These results shed light on how nutrition may modulate the impact of pyrethroid resistance on longevity, as previous reviews have reported variable relationships between pyrethroid resistance and adult longevity (Freeman et al. 2021). Notably previous work that isolated the Val1016Ile and Phe1534Cys KDR mutations found little impact on adult longevity (Brito et al. 2013), while studies incorporating comparisons of KDR mutations and CYP mediated resistance phenotypes found significant impacts on longevity (Freeman et al. 2021; Smith et al. 2021). We speculate that the pathways that mediate the oxidative

effects of constitutive maintenance of CYPs and GSTs involved in metabolic pyrethroid resistance can be life-extending when nutritional conditions are favorable.

While restricted diets have life extending impacts for a wide variety of organisms(Partridge, Gems, and Withers 2005), in drosophila amino acid balance is found to modulate this dietary effect, with methionine supplementation alone supporting prolonged lifespan and undiminished fecundity(Grandison, Piper, and Partridge 2009). We find amino acids generally enriched in Rockefeller mosquitoes, particularly Methionine, and speculate the balance of these amino acids may be under unique selective pressure in lab environments, and groups naturally select for high fecundity in lab mosquito strains.

We hypothesized that we would observe baseline differences in expression of transcripts of enzymes associated with pyrethroid resistance, such as cytochrome P450s, GSTs and esterases based on substantial prior research associating these with insecticide resistance(Derilus et al. 2023; Frédéric Faucon et al. 2017). Additionally, we predicted these enzymes may raise the oxidative state of the insect, which may be compensated with alteration in antioxidant pathways to combat oxidative stress. We found evidence for these hypotheses at the metabolite, transcript and phenotype level. We see elevated glutathione metabolism and antioxidant activity (Figure 2.5, Supplemental Table 2.2 and 2.3) as well as greater activity in the pentose phosphate pathways, an essential source of NADPH required to “recharge” oxidized CYPs and glutathione.

In Rockefeller mosquitoes, pathways involved in protein turnover and cellular transport and communication are significantly upregulated. Colonization in laboratories removes pressure from adult mosquitoes to be resilient to significant alterations in environmental conditions such as temperature and humidity. Laboratory colonization may remove pressure to maintain efficient cellular processes, as calorically rich diets are continuously available, and mates and laying substrates located conveniently nearby. Possible evidence of metabolic dysregulation in our study include high rates of catabolism and mitochondrial activity(Figure 2.5A). In humans ceramides play diverse regulatory roles, stimulating uptake of free fatty acids, triggering autophagy, and can trigger mitochondrial fragmentation and reduced efficiency (Hammerschmidt and Brüning 2022; Roszczyc-Owsiejczuk and Zabielski 2021) and may also have impacts on our observed differential mitochondrial gene expression.

Relative to Clovis, Rock appears to have lower levels of JH synthesis at the point of collection. In adult insects JH supports energy storage, perhaps contributing to the TAG enrichment observed in Clovis (Baumann et al. 2013). Our detection of differential lipid profiles and flux through JH and 20E synthetic pathways may reflect modest alterations in early adulthood, pre-blood meal development. It is interesting to note that both tryptophan metabolism and phosphatidylethanolamine homeostasis play essential roles in insect eye health and development, and metabolites within these pathways are differentially regulated between our two populations, perhaps suggesting differences in eye health (Zhao and Wang 2020; Han, Beerntsen, and Li 2007).

We report novel differences in transcripts related to synapse organization and ion balance, which may be compensatory mechanisms of resistance to pyrethroid and other

nerve-targeted xenobiotics. We also observe elevated Histamine levels in Clovis, and histamine receptors have been found to operate in mosquito brains and peripheral tissues, though histamine receptors were not differentially expressed in our study(Matthews et al. 2016), nor was the voltage gated sodium channel transcript in our populations.

Taken together, we see that Rockefeller and our wild Clovis mosquitos demonstrate robust alterations in fundamental metabolic pathways. While our study cannot conclusively attribute differences to specific aspects of life history, it does represents the first inclusion of metabolomic data in a baseline comparison of mosquito populations, and we sought to pilot an exploration of whether metabolites may present viable biomarkers of phenotypes like metabolic pyrethroid resistance, by identifying features that may be altered broadly across a phenotype despite unique gene set alterations (such as unique resistance conferring cytochrome 450 profiles). We find few markers with the high (>10fold) changes that would best support this aim, but describe interesting metabolic signatures of each population and demonstrate clearly that metabolomic information can powerfully clarify the downstream impacts of differential gene expression data.

CONCLUSION

In this work we found Central Valley mosquitoes relative to the lab reference strain Rock have elevated expression of enzymes associated with pyrethroid resistance including CYPs, GSTs ETC and enrichment of triacylglycerides, fatty acids, lysolipids and nucleotides. In Central valley mosquitoes antioxidant pathways appear to be

constitutively upregulated, which may play important roles in mediating context dependent pyrethroid related fitness costs. Rock shows evidence of increases in proteolytic pathways and significant alterations in mitochondrial metabolism relative to our wild population, which may support fertility and/or reflect inefficiencies in cellular metabolism that may have arisen from laboratory colonization. Rockefeller and Clovis populations have significant differences in nervous system gene expression and metabolites.

FIGURES

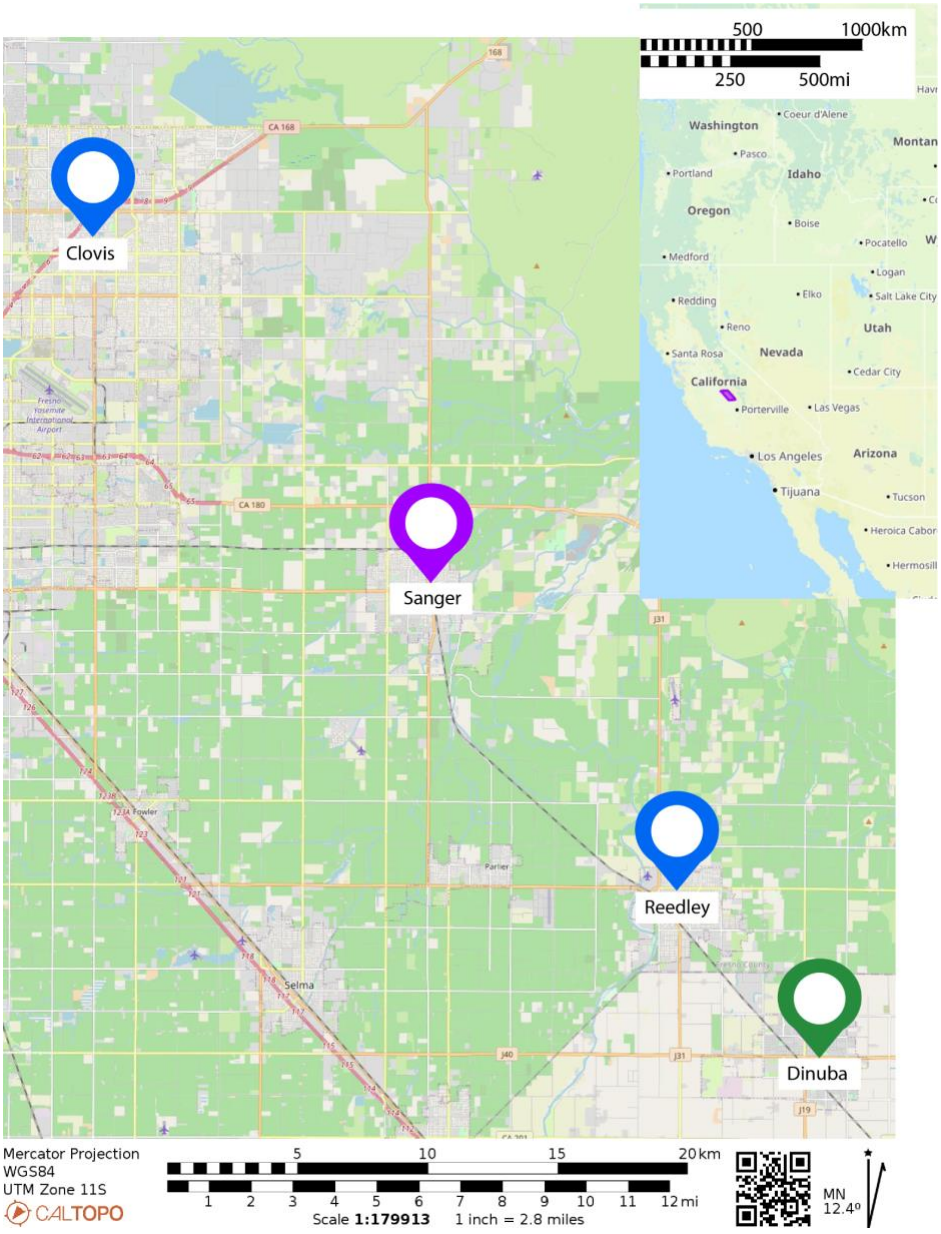


Figure 2.1. Mosquito Regional Collection Map*. Mosquitoes were collected at sites throughout the annotated cities in the summer (July-September) of 2018.*

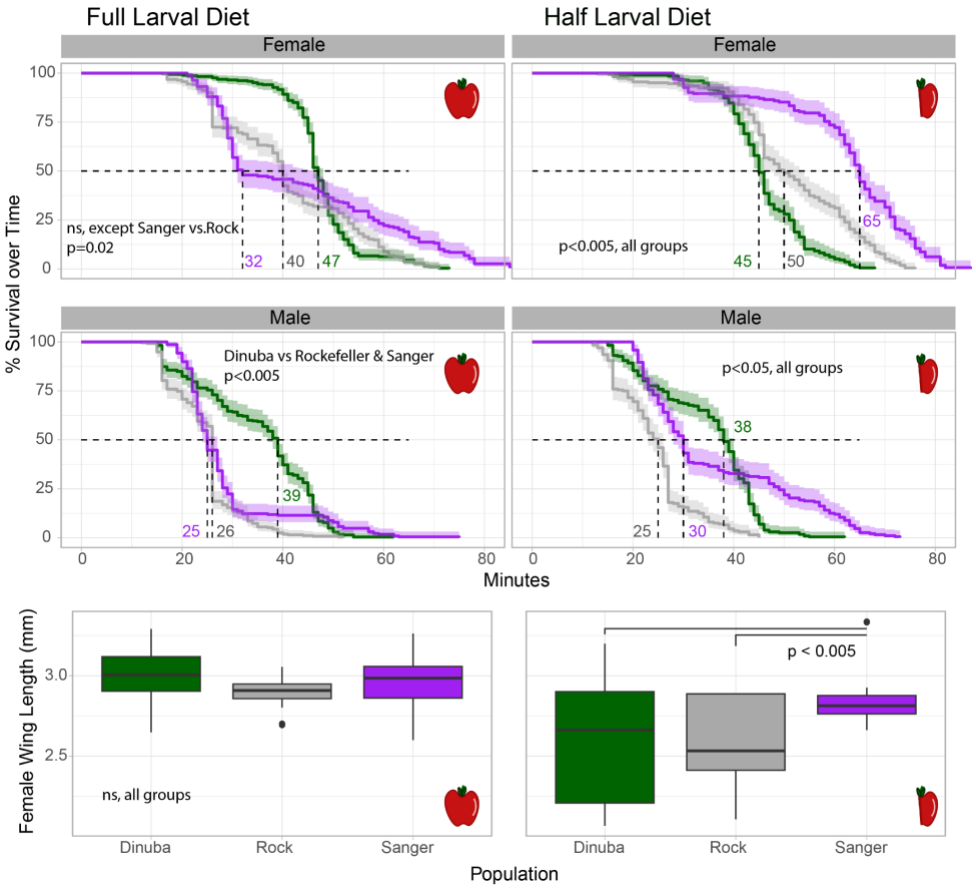


Figure 2.2. Dietary Impacts on Lifespan and Body Size For Near Wild Aedes aegypti with variable resistance phenotypes. Statistical analysis for life span was done using log-rank survival analysis, with Hochberg correction for multiple tests. Wing lengths were tested using a two-way anova followed by Tukey HSD. P values on graphs represent differences within graph quadrants only. Males lived significantly shorter than their female counterparts for all populations, and restricted diet reduced female body size significantly for all groups ($p<0.0005$). Further results are described in the text.

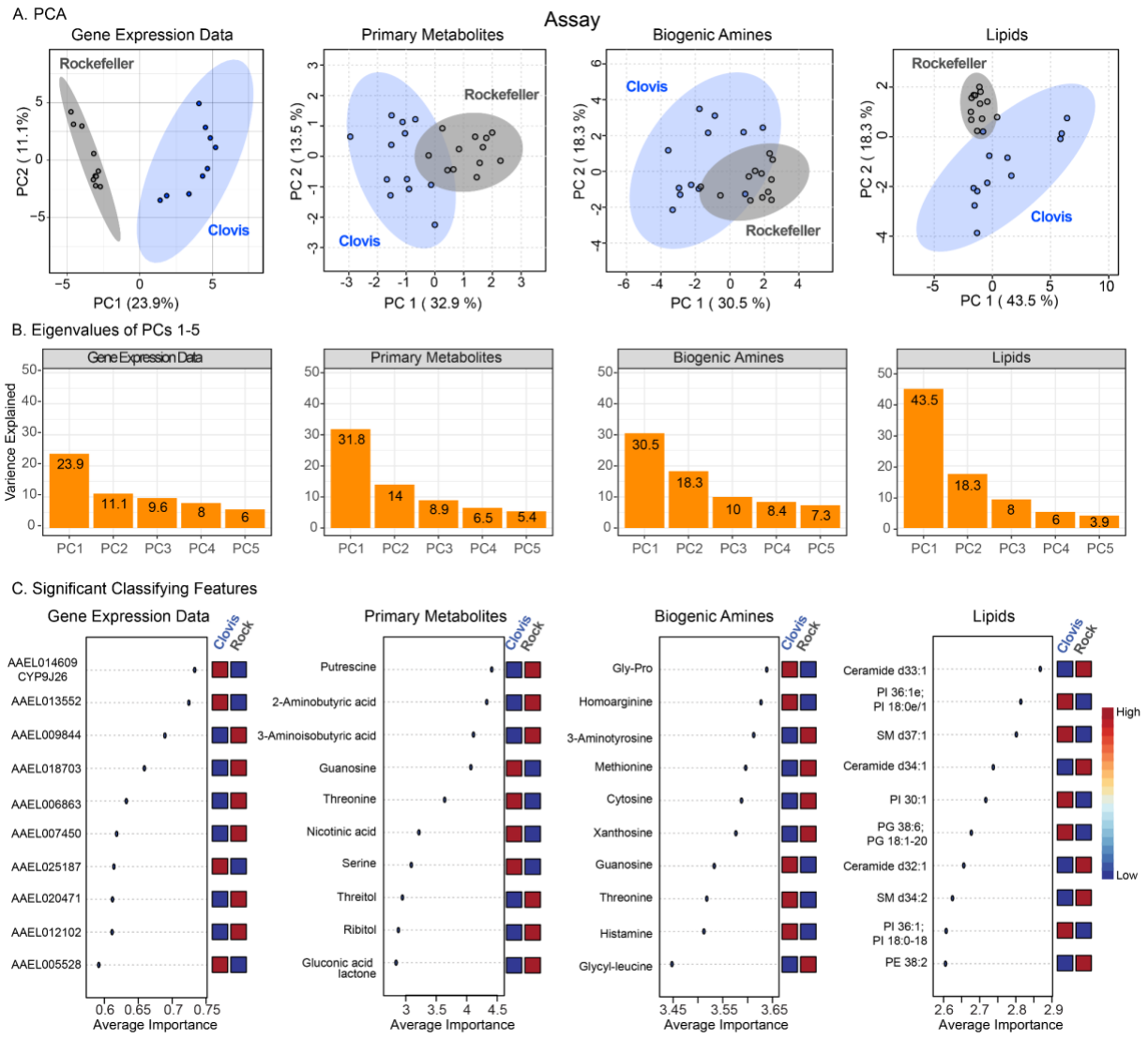


Figure 2.3. A. Principal component analysis of metabolomic assays and transcriptome data B. Scree plots and C. Top 10 classifying features by random forest analysis

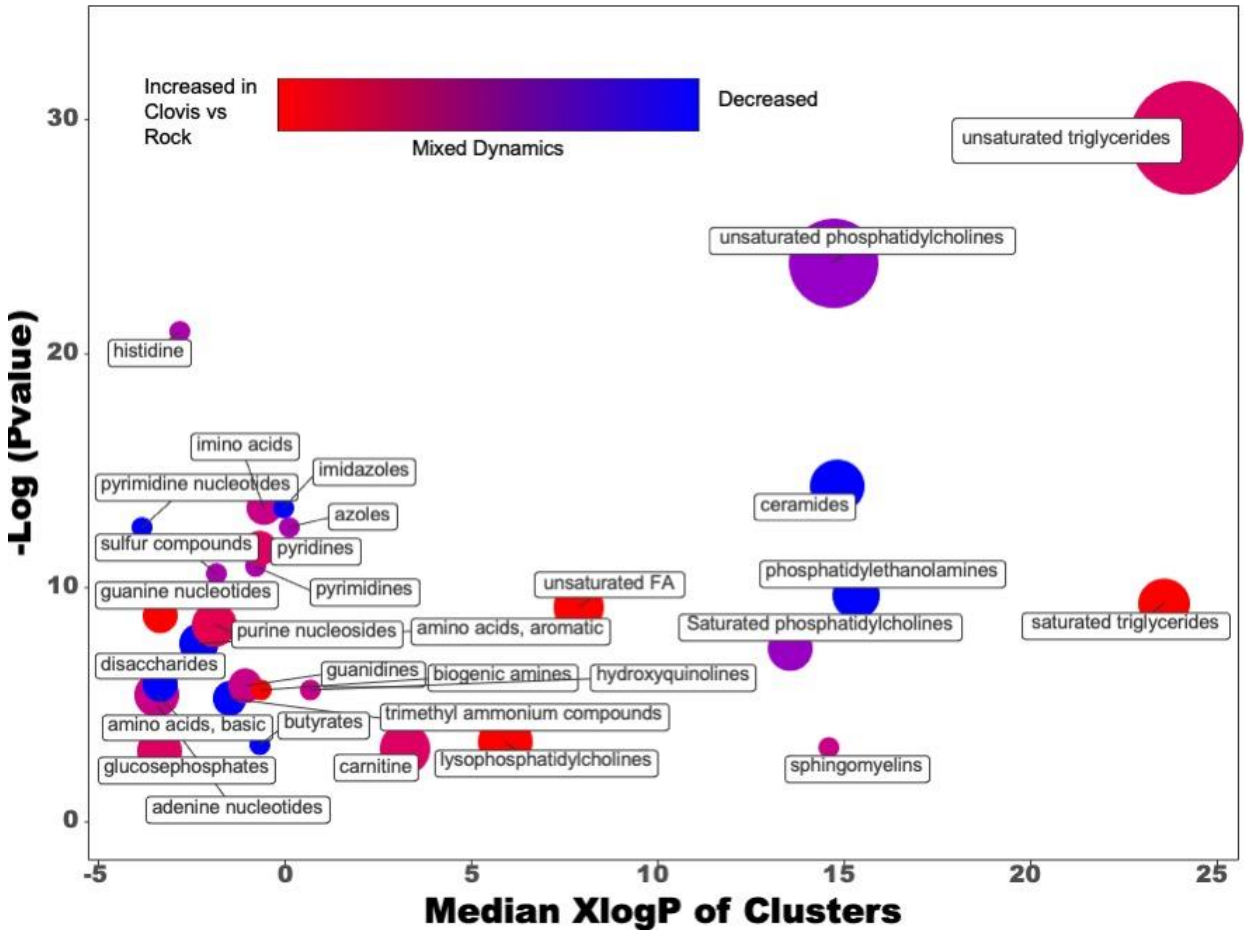


Figure 2.4. Chemrich Chemical Set Enrichment Analysis Plot

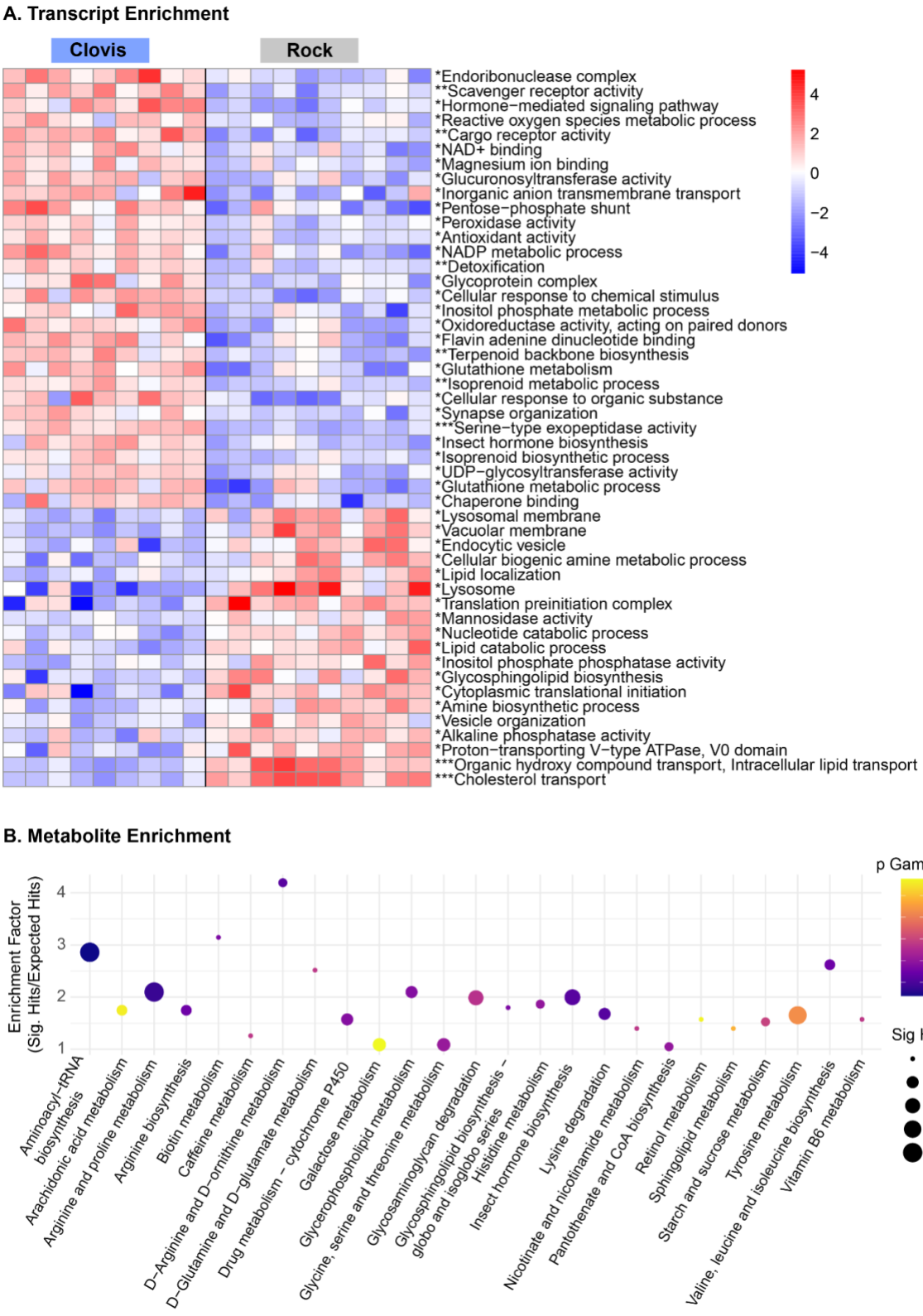


Figure 2.5. Gene and Metabolite Enrichment Plot. A. represents enrichment from PGSEA with a FDR cutoff of 0.05. B. represents metabolite enrichment with a FDR adjusted cutoff of 0.05.

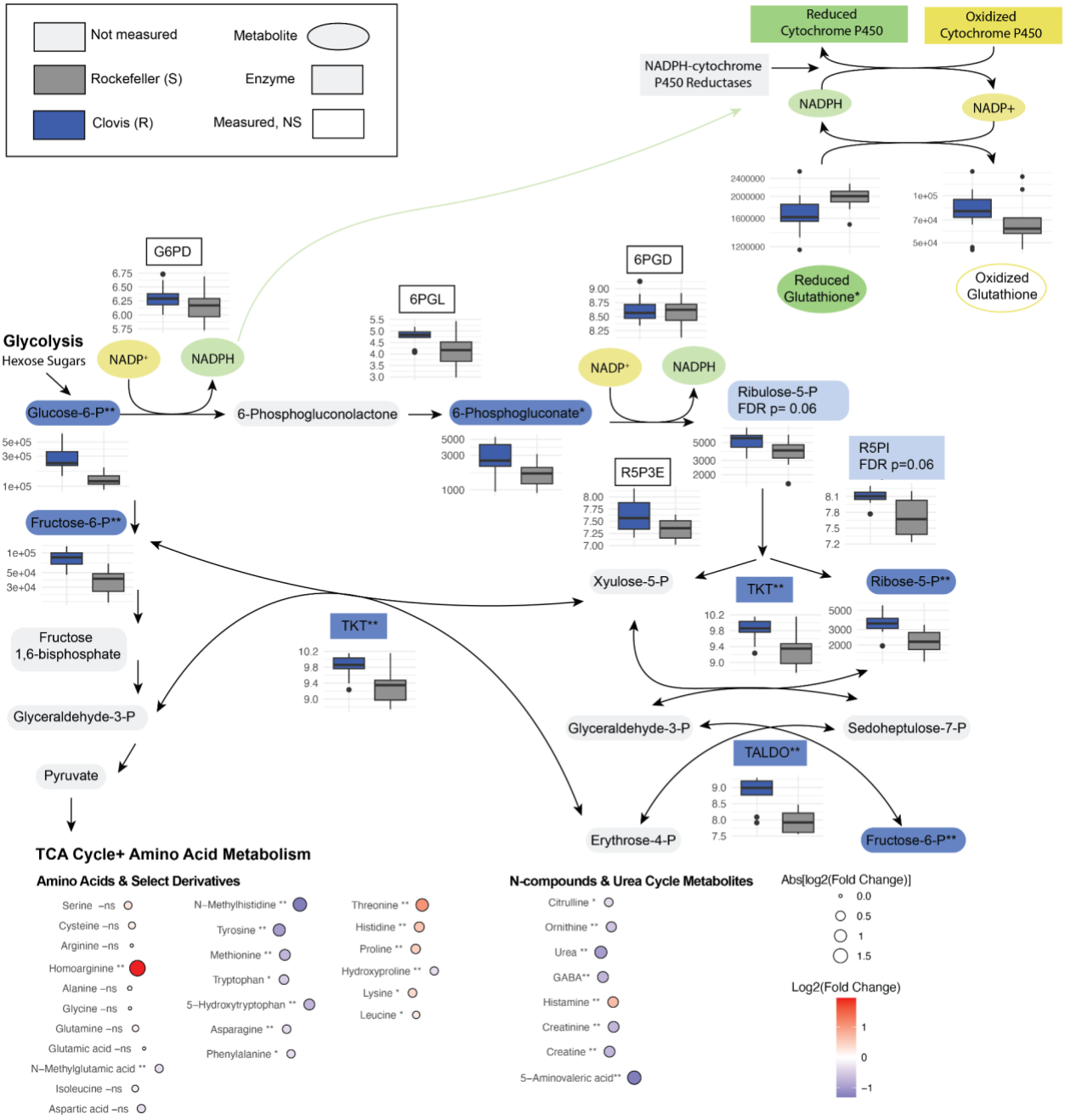
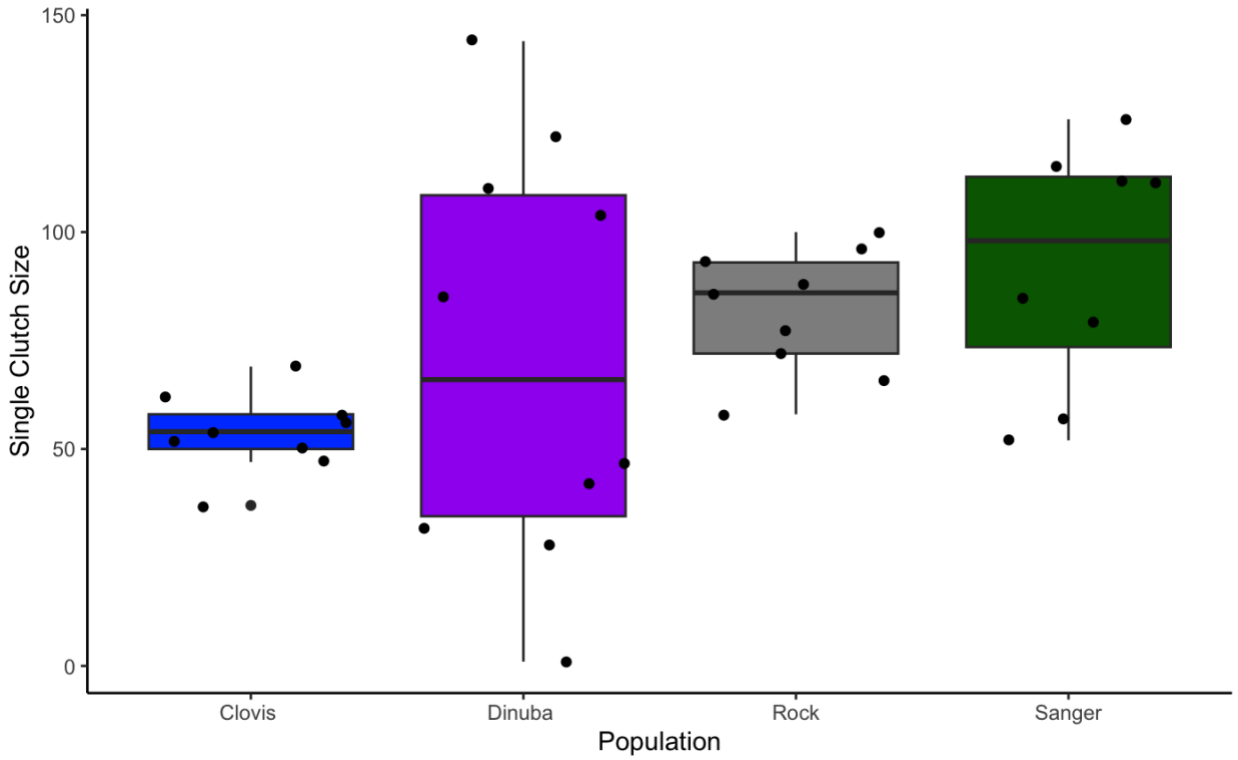

Not measured
Metabolite
Rockefeller (S)
Enzyme
Clovis (R)
Measured, NS
Reduced Cytochrome P450
Oxidized Cytochrome P450
NADPH-cytochrome P450 Reductases
NADPH
NADP+
Reduced Glutathione*
Oxidized Glutathione
G6PD
6PGL
6PGD
Glycolysis
Hexose Sugars
NADP+
NADPH
Glucose-6-P**
6-Phosphogluconolactone
6-Phosphogluconate*
Ribulose-5-P FDR p= 0.06
R5P3E
R5PI FDR p=0.06
Fructose-6-P**
Xylulose-5-P
TKT**
Ribose-5-P**
Fructose 1,6-bisphosphate
Glyceraldehyde-3-P
Sedoheptulose-7-P
TALDO**
Pyruvate
Erythrose-4-P
TCA Cycle+ Amino Acid Metabolism
Amino Acids & Select Derivatives
Serine –ns
Cysteine –ns
Arginine –ns
Homoarginine **
Alanine –ns
Glycine –ns
Glutamine –ns
Glutamic acid –ns
N–Methylglutamic acid **
Isoleucine –ns
Aspartic acid –ns
N–Methylhistidine **
Tyrosine **
Methionine **
Tryptophan *
5–Hydroxytryptophan **
Asparagine **
Phenylalanine *
Threonine **
Histidine **
Proline **
Hydroxyproline **
Lysine *
Leucine *
N-compounds & Urea Cycle Metabolites
Citrulline *
Ornithine **
Urea **
GABA**
Histamine **
Creatinine **
Creatine **
5–Aminovaleric acid**
Abs[log2(Fold Change)]
0.0
0.5
1
1.5
Log2(Fold Change)
1
0
–1

Figure 2.6. Differential expression of pentose-phosphate pathway genes and metabolites, and downstream metabolite features. * Indicated a FDR adjusted p between 0.05 and 0.005, ** indicates FDR adjusted p below 0.005.

SUPPLEMENT

A.



B.

P Value Table	Clovis	Dinuba	Rockefeller

Dinuba	0.57		
Rockefeller	0.21	0.88	
Sanger	0.057	0.47	0.89

Supplemental Figure 2.1. A. Box plot of egg clutch size by population. B. Table of p-values for pairwise comparisons for one-way ANOVA followed by Tukey's post-hoc test.

Supplemental File 2.1. Measured Metabolite Data

Supplemental File 2.2. ChemRich Results

Supplemental File 2.3. Annotated Gene Expression Data

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CHAPTER 3. Comparative metabolomic profiling of two populations of California *Aedes aegypti* following deltamethrin exposure

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Declaration of interests: The authors declare no competing interests.

ABSTRACT

Resistance to pyrethroid insecticides has facilitated the rapid spread of *Aedes aegypti* mosquitoes throughout California. *Aedes aegypti* demonstrate both metabolic and target site resistance mechanisms to insecticides, but precise mechanisms of pyrethroid detoxification are not yet well described and appear to vary between and within populations. For our study, we produced F2 generation colonies representing two genetically distinct populations of *Aedes aegypti* from the San Joaquin Valley and from Southern California. These two populations were then subjected to a modified CDC bottle-assay with Deltamethrin, a pyrethroid insecticide. Following Deltamethrin exposure, we observed knock-down times and collected samples from the upper and lower knock-down quartiles, representing susceptible and resistant insects. We apportioned 10 pools of 5 adult female *Aedes aegypti* organized by population and resistance status, and submitted them to the West Coast Metabolomics center for high-throughput metabolomic analysis. This technique offers a snapshot of the insects' metabolomes, revealing different levels of activity and demands on metabolic pathways. We analyzed the data in Metaboanalyst to observe how the mosquitoes metabolomes changed following insecticide exposure. We looked for differences between the susceptible and resistant insects and shared features between the two study populations. These analyses revealed that remodeling of the mosquito metabolome following insecticide challenge is rapid, significant increases in lyso-lipids, free fatty acid, and carnitines are observed just 90 minutes after exposure. The metabolomes of unexposed mosquitoes did not vary significantly by population, but the populations did have different metabolomic profiles following insecticide challenge. This analysis highlights the essential role B-vitamins play in stress response, physiology, and reveals potential targets for insecticide synergists.

INTRODUCTION

Aedes aegypti, the primary vector of dengue, Zika, yellow fever and chikungunya viruses, successfully established in California in 2013 in Fresno and Madera county in the San Joaquin Valley (Gloria-Soria et al., 2014). It has since been detected in 25 of 58 counties throughout the State. Genomic analyses by multiple research groups have revealed evidence of multiple distinct introductions of the vector into the state(Kelly et al., 2021; Pless et al., 2017, 2020). At present two population groups seem to predominate. One genetic group is dominant in the southern part of the state, while the other dominates in the Central part of the state around the San Joaquin Valley(Kelly et al., 2021; Lee et al., 2019; Pless et al., 2017). The successful establishment and spread of this vector is due, in part, to widespread resistance to pyrethroids, the primary insecticide class used for adult control (Liebman et al., 2019; Mack et al., 2021; J. Singh & Yadav, 2020; F. Yang et al., 2020).

Resistance to pyrethroids in *Aedes aegypti* involves multiple mutations in the voltage gated sodium channel, the pyrethroid target site, and metabolic mechanisms of resistance(Chen et al., 2020; Smith et al., 2016; Strode et al., 2008; William C. Black et al., 2021). Resistance in California populations is relatively well characterized, due to the combination of monitoring programs run by local vector control districts, state level initiatives conducted by the California Department of Public Health (CDPH) and partnerships with research institutions.Target site mutations are widespread in California *Ae. aegypti(Liebman et al., 2019),* but previous research has demonstrated significant variability in resistance phenotype among individuals with shared target site phenotypes (Mack et al., 2021).

Metabolic mechanisms of insecticide resistance, including detoxification, inactivation, and sequestration, also play an important role in resistance, reviewed in (William C. Black et al., 2021). California *Aedes aegypti* metabolic mechanisms have been assessed for the Central

Valley using synergists such as PBO (Cornel et al., 2016) and across the state by CDPH using metabolic assays(F. Yang et al., 2020), which found CA populations to have elevated esterase, mixed function oxidase and acetylcholinesterase relative to susceptible Rockefeller mosquitoes. Additional physical and behavioral resistance mechanisms such as cuticular thickening(David G. Lilly et al., 2016; O. R. Wood et al., 2010; Yahouédo et al., 2017) and pyrethroid avoidance (Meyers et al., 2016; Reddy et al., 2011; Russell et al., 2011) have also been reported in insects, but are not well characterized in California populations. Mechanisms of metabolic detoxification appear to be incredibly diverse and difficult to monitor (Saavedra-Rodriguez et al., 2019; Smith et al., 2016). The diversity and redundancy of metabolic detoxification genes and pathways has hampered the development of molecular assays for surveillance of metabolic resistance mechanisms.

Recent advances in mass-spectroscopy instrumentation and compound annotation tools have ushered in a resurgence of biochemical research in entomology, following an era denominated by genomic and gene expression studies(Dettmer et al., 2007; Johnson et al., 2016). High throughput metabolomics has significant promise in elucidating important biological processes underlying phenotypes of interest, like pyrethroid resistance. Metabolites shift rapidly in response to physiological changes and stress challenges, and investigating these processes can assist in the discovery of response mechanisms, important biological dynamics, and novel targets for insecticides or synergists, which may have been obscured in analyses by other methods.

This study uses a complementary panel of three metabolomic assays to comprehensively compare the metabolomes of two genetically distinct, near-wild populations of *Aedes aegypti* pre and post insecticide exposure. Samples were additionally grouped by resistance status, in order to facilitate investigations into unique metabolic features associated with the resistance phenotype. These results provide a new layer of information about

resistance phenotypes, and highlight important metabolic processes initiated rapidly after insecticide exposure. To our knowledge this is the only study that looks at time points early following exposure (30 to 70 minutes), and highlights a variety of potential targets for pyrethroid synergists.

METHODS

Insect Colonies

The *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) mosquitoes used in this study were taken from wild-derived colonies from the San Joaquin Valley(referred to as Central Valley hereafter) and Greater Los Angeles County in the summer of 2020. Greater LA Vector Control District personnel collected container breeding sources in backyards. Eggs were then hatched and sorted as adults. For the San Joaquin Valley population, members of the Kearney Agricultural Research and Extension Center in Parlier, CA collected adult mosquitoes from Reedley and Clovis, CA. Larvae were raised on a diet of tetramin fish food, and adult females were bloodfed using bovine blood on hemotek feeders. Mosquitoes were maintained in our Darwin insectary on a 12:12 day/night light cycle at 28 ℃ and 80% RH. Rockefeller mosquitoes were used as a susceptible reference colony in resistance evaluation assays.

Resistance Evaluation

Mosquitoes from the F2 populations had their resistance status evaluated using the CDC Bioassay protocol with permethrin and deltamethrin(*CONUS Manual for Evaluating Insecticide Resistance in Mosquitoes Using the CDC Bottle Bioassay KitCS330338-A*, n.d.). Bottles were treated with 1 ml acetone alone (controls), 43ug/ml Permethrin, or 0.75 ug/ml deltamethrin. Additionally, a modified CDC Bioassay protocol that incorporated a 24 hour hold was used for survivorship assessment. Knockdown curves for all mosquito strains were analyzed using Kaplan-Meier survival analysis (Therneau, 2023).

Insecticide Exposure

Insecticide coated bottles were prepared according to methods described in the CDC Bioassay Manual(*CONUS Manual for Evaluating Insecticide Resistance in Mosquitoes Using the CDC Bottle Bioassay KitCS330338-A*, n.d.). Pesticide coated bottles were prepared with deltamethrin in acetone at a concentration of 0.75 ug/ml. Control bottles were prepared with acetone alone. All bottles were allowed to dry in a dark fume hood for two hours. Mosquitoes were introduced into bottles at a density of 16-20 mosquitoes per bottle, and left in the bottles for 15 minutes. Exposed insects were then transferred into holding cages and monitored every 10 minutes for signs of pyrethroid intoxication. A mosquito was determined to be intoxicated if it was unable to right itself for at least three minutes. Intoxicated mosquitoes were transferred directly into tubes on a dry ice ice-block before being transferred into the -80 freezer.

Sample classification

Samples were then sorted into tubes according to onset of intoxication symptoms. Only adult females were collected. Females were prioritized due to their longer lifespan and ability to act as vectors. Mosquitoes treated with acetone coated bottles were classified as Controls. Mosquitoes that showed symptoms of intoxication prior to 30 minutes after exposure were classified as susceptible to Deltamethrin. This threshold was chosen as 30 minutes is the designated diagnostic time for Deltamethrin susceptibility according to the CDC bioassay protocol. Samples that did not exhibit any symptoms of intoxication 70 minutes after exposure were classified as resistant. Adult females were pooled into sets of 6 individuals per tube. We generated 5 control tubes per population, and 10 treatment tubes per population and classification (susceptible or resistant).

Metabolomics Data Acquisition and Data Processing

Frozen samples were submitted to the University of California West Coast Metabolomics center for analysis with three complementary mass spectrometry based untargeted

metabolomic assay profiling 1)primary metabolites, 2)lipids, and 3)biogenic amines. Samples are extracted according to protocols published by Matyesh *et al.*, 2008 (Matyash et al., 2008).

The primary metabolite assay targets include carbohydrates, amino acids, free fatty acids, aromatics and nucleotides. Samples are analyzed by injection with an Gerstel automated linear exchange cold-injection system on a gas-chromatography time-of-flight mass spectrometer (GCTOF MS). Data acquisition parameters are described in detail in Fiehn et al., 2008 (Fiehn et al., 2008). Data are processed with ChromaTOF vs. 2.32 and further processed by a filtering algorithm implemented in BinBase. Samples are then normalized by the average sum of peak heights for identified metabolites.

The lipid panel detects ceramides, sphingomyelins, cholesteryl esters, lyso- and phospholipids, mono-, di- and triacylglycerols, galactosyl- and glucuronyllipids. Lipids are analyzed with Liquid-Chromatography Electrospray Ionization Quadrupole Time-of-Flight Charged Surface Hybrid Mass Spectrometry (CSH-ESI QTOF MS/MS). Data are processed using MS-DIAL using default parameters with adjustments for peak height and width(Tsugawa et al., 2015). Blank subtraction is then performed based on maximum peak height relative to blank average height, the average of all non-zero peaks and whether or not the feature is found in multiple samples. Potential duplicates and isotopes are checked and deleted with MS-FLO(DeFelice et al., 2017). Next, MS/MS spectra are checked before combining adducts. Peaks are annotated manually and compared with the Fiehn laboratories LipidBlast library(Kind et al., 2013). MassHunter Quant software is then used to verify peaks. Samples are then normalized by the average sum of peak heights for internal standards.

The biogenic amine panel targets cylcarnitines, TMAO, cholines, betaines, SAM, SAH, nucleotides and nucleosides, methylated and acetylated amines, di- and oligopeptides. Biogenic amines are analyzed by hydrophilic interaction chromatography on a electrospray quadrupole

time-of-flight mass spectrometer tandem mass spectrometer (HILIC-ESI QTOF MS/MS). Samples are then analyzed by the metabolomics core in a 4 step process. Raw data are initially processed by mzMine 4.0. Selected peaks can be identified with Agilent's MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the NIST14 / Metlin / MassBank libraries to identify metabolites with manual confirmation of adduct ions and spectral scoring accuracy. MassHunter then allows the quantifications for peaks that were missed in the primary peak finding process. Samples are then normalized by the average sum of peak heights for internal standards.

Statistical analysis

Statistical analyses were performed using a combination of the statistical programming language R (version 4.2.2), Metaboanalyst version 5.0 (Pang et al., 2021),and Chemical Similarity Enrichment Analysis (ChemRich) from the Fiehn Lab(Barupal & Fiehn, 2017). The metabolomic data sets were each normalized and statistically analyzed independently in Metaboanalyst. For primary metabolites and biogenic amines samples were median normalized, while lipids were normalized by sum. Features were log transformed and median normalized prior to statistical analysis. ANOVA results for each population by status (Control, Susceptible, Resistant) are reported in Supplemental File 3.1, results from linear modeling are summarized in Supplemental File 3.2. Each dataset was analyzed primarily using Metaboanalyst's metadata capabilities, so that metabolites could be analyzed by both population and status. Principal component analysis and random forest classification was performed following normalization.

Chemical Enrichment Analysis for Metabolomics

Biological interpretations of metabolomics data face significant challenges due to constraints with regard to pathway mapping and statistical analysis of pathway impact. Insect metabolic pathways are poorly annotated relative to mammalian metabolic pathways,

metabolomic assays may fail to detect important intermediates, many metabolites function in multiple pathways, and many metabolites play unknown biological roles. Chemical similarity enrichment analysis allows for statistical analysis of non-overlapping chemical sets. Chemical Enrichment analysis was performed both using ChemRich and the Metaboanalyst Enrichment module(Barupal & Fiehn, 2017; Pang et al., 2021). For chemrich the Susceptible and Resistant mosquitoes from both populations were pooled and compared to calculate fold change and use t-tests and fdr adjustments to obtain p values.

The Metaboanalyst functional meta-analysis module uses Mummichog and accepts unannotated metabolomics data generated from high-resolution mass spectrometry methods such as those used to acquire the biogenic amine and lipid data(Lu et al., 2023). Numeric mass (m/z) and retention time (rt) were supplied for all peaks in the dataset, including unknowns. Data was analyzed against the *Drosophila melanogaster* KEGG library, as well as chemical class sets. The meta-analysis pooled peaks functionality allows for the simultaneous analysis of datasets generated from multiple instruments using the same samples.

RESULTS

Central Valley and Greater LA Mosquitoes Demonstrate Strong Resistance To Permethrin

The two populations differ significantly in their response to both permethrin and deltamethrin, type I and type II pyrethroids (Table 3.1). The Central Valley population demonstrates particularly strong resistance to type I pyrethroids, though both populations knock-down fully within one hour of continuous exposure to 0.75ug deltamethrin. Both populations however had similar responses to a 15 minute duration exposure.

Table 3.1. Resistance Phenotyping with CDC bottle bioassay exposures. Diagnostic time represents the time at which 100% knock-down is expected.

Insecticide	Population	LT50 (minutes)	LT90 (minutes)
Permethrin (Diagnostic Time: 10 minutes)	Central Valley	110	NA
	Greater LA	30	60
	Rock	5	5
Deltamethrin (Diagnostic Time: 30 minutes)	Central Valley	20	30
	Greater LA	15	25
	Rock	5	5

Pyrethroid Exposure Induces Rapid Metabolome Remodeling

A total of 618 metabolite features were scored from the primary metabolite assay, of which 175 were annotated. Following normalization and linear modeling with Unexposed as the reference group with a p value cutoff of 0.005, 100 of the 175 annotated features were found to differ significantly by status, while just 15 differed by population when adjusting for status (Table 3.2). ANOVA results are summarized in Supplemental File 3.1, covariate analysis results are reported in Supplemental File 3.2.

From the primary metabolite assay amino acids were elevated, along with organic acids including cysteinylglycine, pyruvic acid, phosphoenolpyruvic acid, urea, malonic acid and azelaic acid. Several organic acids were decreased in resistant samples, including cis-aconitic acid, citric acid, glutamic acid, oxoglutaric acid, phosphoserine, ureidosuccinic acid and allantoic acid. Elevated carbohydrates included glycerol, ribose and gluconic acid, but carbohydrates

were generally depleted such as xylose, xyulose, glyceric acid, threonic acid, trehalose-6-phosphate, pectin, Erythritol, and fructose-1-phosphate.

Elevated nucleic acids included xanthine, uridine, pseudouridine and uracil, while adenine, adenosine monophosphate, and adenosine were reduced in resistant samples post exposure. Several benzenoids were elevated including myo-Inositol; ribitol; terephthalic acid; and epsilon-Caprolactam.

Pyrethroid Exposure Increases Levels of Deoxynucleic Acids and Organic Acids

A total of 4303 peaks were scored from the biogenic amine panel, of which 421 were annotated. Following linear analysis with Unexposed as the reference group and a p value cutoff of 0.005, 186 of the annotated features were significantly different by status. Of the 421 annotated features 21 varied by population when adjusting for status. There is redundancy in metabolome coverage between the biogenic amine assay and the primary metabolite assay, but similar compound class level dynamics were observed. Elevated in the biogenic amine assay were additional nucleic acids including deoxyuridine, deoxycytidine, deoxyinosine, FAD, purine, citicoline, and 1-methyladenosine. Decreased nucleic acids included cyclic AMP, deoxyguanosine, cytidine monophosphate, deoxyadenosine, orotidylic acid, orotic acid, uridine 5'-monophosphate, uridine diphosphategalactose, pyrophosphate, guanosine monophosphate, N6-Methyladenosine, 1,7-dimethyluric acid and NADH.

Elevated organic acids included betaine, taurine, urea, N8-acetylspermidine, ergothioneine and 4-acetamidobutanoic acid. Of organoheterocyclic compounds 4-Pyridoxic acid; Riboflavin; Indoxyl sulfate; Nicotinic acid; Folinic acid and the organic oxygen compound and niacin synthesis metabolite kynurenine were elevated in resistant samples. Decreased levels of organoheterocyclic compounds included biotin, pyroglutamic acid, isoxanthopterin, kynurenic acid, pyridoxamine and folinic acid. Over 30 organic acids were elevated including

most amino acids, and choline, histamine and ethanolamine were also elevated. About 20 organic acid compounds were decreased including a diversity of di-peptides and carnosine, cis-aconitic acid, methylmalonic acid and succinic acid.

Lysolipids Can Classify Insecticide Exposure Status

From the lipid panel 2763 peaks were scored, of which 358 were annotated. Following linear analysis with unexposed as the reference group and a p value cutoff of 0.005, 168 metabolites varied significantly by status, and 43 varied significantly by population when adjusting for status. When comparing resistant samples to unexposed samples, resistant samples had elevated levels of fatty acids and lysolipids (Figure 3.5, Table 3.2, Supplemental File 3.1,3.2). The lipids that most effectively classify status are fatty acids and lysophosphatidylcholine 18:0 and lysophosphatidylglycerol 16:1(Figure 3.3.)

For Chemical Set Enrichment Analysis (ChemRich) The San Joaquin Valley and Greater LA populations were grouped together into susceptible and resistant groups in order to calculate fold-change between susceptible and resistant status for ChemRich analysis. The most consistent dynamics are observed in lipid groups (Figure 3.3, Supplemental File 3.2). Resistant insects have consistently elevated levels of saturated and unsaturated lysolipids as well as unsaturated and saturated free fatty acids. The “key” compounds of those classes were LPE (18:0), LPC (20:5), eicosapentaenoic acid, and myristic acid respectively. Sugar acids, sugar alcohols and carnitines were also enriched, and dehydroascorbic acid, glycerol-3-galactoside were the key sugars.

Table 3.2. Lipid Class Enrichment between Resistant and Unexposed Samples. Results are those with a FDR adjusted p < 0.05.

Lipid Group	Increased in Resistant	Decreased in Resistant
Fatty Acids and Conjugates	15	0
Fatty esters	2	1
Glycerophosphoethanolamines	9	35
Glycerophosphocholines	7	35
Glycerophosphoinositols	4	2
Glycerophosphoglycerols	4	4
Glycerophospholipids	0	4
Glycerophosphoserines	1	3
Triradylglycerols	0	26
Ceramides	0	5
Eicosanoids	1	0

Principal Component Analysis Classifies Resistant, but Not Early-knockdown Samples, From Controls

General sample clustering patterns were consistent across the three assays (Figure 3.2, Figure 3.1). Samples classified as “susceptible” are generally clustered with control samples. Despite the susceptible mosquitoes displaying signs of pyrethroid intoxication, their metabolome was insufficiently altered to cluster separately from samples that were not exposed to insecticide. Samples classified as resistant clustered separately, and there was noticeable separation between the two populations, 7 samples from the San Joaquin Valley reliably clustered as a subgroup of resistant samples. Half (5/10) susceptible samples from the San Joaquin Valley clustered with the resistant samples. This observation indicates that the biochemical changes observed in the resistant group can begin before the resistant collection time point (70 minutes). This observation also indicates that these changes alone do not represent effectively protective metabolic processes. If susceptible samples are also undergoing these processes, yet demonstrate strong symptoms of intoxication, relatively few metabolites may reflect likelihood of mortality at early such collection points (<40 minutes).

Pathway Enrichment Reveals Shared and Unique Impacts on Metabolic Pathways

The LC-MS derived data from the biogenic amine and lipid panels were analyzed using the Metaboanalyst functional meta analysis function. Data was analyzed separately for each population comparing resistant samples to unexposed samples. The central valley data had an overall 3544 features from the biogenic amine dataset, 2058 from ESI positive mode and 1856 from ESI negative mode. With a p value cutoff of 0.005 21% of features were considered significant. From the lipid dataset 2733 features were submitted, 1633 from ESI positive mode and 1100 from ESI negative mode. Of these, 7% and 38% of the features were considered significant with a p cutoff of 0.005. Susceptible samples were also compared to unexposed controls, but no features met the 0.005 p value cutoff.

For the Los Angeles population overall 4303 features were submitted from the biogenic amine dataset, 2591 from ESI positive mode and 1712 from ESI negative mode. Of these features, 49% and 60% were significant with a p value cutoff of 0.005. From the lipid dataset 1656 features were detected in ESI positive mode, of which 11% were significant and 1104 were detected in ESI negative mode, of which 40% were significantly altered. Pathway enrichment results are reported in figure 6. When comparisons were made of the susceptible and unexposed group, 0% of the biogenic amine features met the 0.005 p value cutoff, and just 3% of lipid features were significant. Of the enriched pathways (Figure 3.6) 12 were shared across populations.

DISCUSSION

In this study adult female *Aedes aegypti* representing two near-wild populations were collected following deltamethrin exposure either as they exhibited a knock-down phenotype or after showing no symptoms of intoxication for over one hour. Pooled samples were characterized using mass spectrometry. The combination of three complimentary untargeted metabolomics assays (primary metabolites, lipids, and biogenic amines) reveals the short timescale in which insects undergo metabolic changes following topical insecticide exposure, and demonstrates variability in insecticide metabolism dynamics between and within different populations. Nearly 400 metabolites differed significantly by status, 79 differed by population when adjusting for status, and pathways impacted by deltamethrin exposure play crucial roles in various cellular processes such as energy production, amino acid metabolism, lipid metabolism, and nucleotide metabolism, and nitrogenous waste excretion.

This study highlights dynamics that have also been reported in other species, pointing to shared metabolic detoxification mechanisms in response to deltamethrin exposure, and pyrethroid exposure more broadly. Research in Deltamethrin susceptible and resistant strains *Anopheles sinensis* identified differences in carboxylic acids and glycerophospholipids metabolism as dominant differences 24 hours post exposure, while a study investigating the impact of temperature and one-hour insecticide exposure in *Aedes aegypti* found impacts on energy metabolism dominate early insecticide response (Li et al., 2022; P. Singh et al., 2022). Taken along with the results described in our study, suggests that timing is likely an important feature of resistance. The ability to respond more rapidly to pyrethroid damage and oxidative stress, to mobilize energy stores and modify damaged lipids, may increase survival. For feasibility reasons many studies compare multiple populations at set time-points, like 24 hours, and may then miss interesting detoxification ramp-up dynamics. In general, altered metabolites followed the patterns that early-knock down (susceptible) samples were trending in the same direction as in resistant mosquitoes relative to unexposed controls. Exceptions were particularly interesting, cases where a metabolite was up in the susceptible group relative to resistant and control mosquitoes were often markers of significant cellular distress, such as 8-Oxo-2'-deoxyguanosine, 2'-Deoxyadenosine, 2'-Deoxyguanosine, important markers of DNA oxidation (Figure 3.3, Supplemental File 3.1)(Cadet et al., 1999). Inversely, metabolites up in resistant samples and down in susceptible samples relative to controls were involved in excretion, lipid modification and metabolism such as urea, pantothenic acid, LPG16:1. Others are known to have cytoprotective properties such as taurine and histidine (Figure 3.3, Supplemental File 3.1)(Abdel-Rahman Mohamed et al., 2021; Bai et al., 2018; Surai et al., 2021).

One unique dynamic revealed in this study is the impact of pyrethroid exposure on nitrogenous waste metabolism, which appears to be differentially impacted between the two populations included in this study (Figure 3.6,3.9.). Previous research has demonstrated that

Aedes aegypti utilizes an amphibian-like uricolytic pathway to excrete urea and can excrete waste both as uric acid and urea throughout development, as an adaptation to blood-feeding (Isoe & Scaraffia, 2013). Functional analysis with mummichog uses the *Drosophila melanogaster* metabolome for reference, so enrichment of this specific pathway is not noted, though alanine and arginine metabolism are top pathway hits (Figure 3.6). This detoxification pathway could be a particularly useful target for synergism, due to it being a unique adaptation of female mosquitoes to their nitrogen rich diet from blood feeding. Metabolites in this pathway have other important roles in stress response physiology, such as glutamine, glutamic acid and alanine. Research in exercise physiology has identified alanine and glutamine as useful supplements following the oxidative damage generated by intense exercise, as alanine can help improve glutamine pools, and glutamine can then participate in the glutathione homeostasis(Petry et al., 2014; Rogero et al., 2006). Elevated levels of alanine in resistant insects following deltamethrin may be facilitating the maintenance of redox homeostasis.

We find that B vitamin levels are significantly impacted by deltamethrin exposure (Figure 8). B vitamins are essential cofactors in many of the metabolic pathways that facilitate recovery from pyrethroid exposure, and reliably classify status in our study (Figure 3.3,3.8.). Pyridoxic acid, the end product of vitamin B-6 metabolism, is frequently reported as an important exposome metabolite and biomarker for stress, and was elevated in resistant insects (Cao et al., 2016; Zeng et al., 2021). Pantothenic acid (B5) levels are elevated following insecticide exposure, and B5 levels can effectively classify status in our study (Figure 3.3, 3.8.) Pantothenic acid is an essential cofactor in lipid metabolism and acetylation reactions, and increases in levels of B5 may result from increased demands for coenzyme A following pyrethroid exposure, as is observed following exposure to various stressors in other animal systems (Miller & Rucker, 2020) and supplementation with B5 can increase stress tolerance(Hu et al., 2022). Pantothenic acid is likely protective against the damaging effects of pyrethroids. Resistant insects, by

upregulating coenzyme A synthesis, could more rapidly and effectively respond to lipid oxidation and disruptions in membrane fluidity. Similarly increases in Niacin (B3) may be compensatory following deltamethrin exposure as B3 is an essential precursor to NAD+, which participates in oxidation reduction reactions. Depletion of NADH in resistant insects is likely the result of consumption of NADH in reducing reactions following the state of oxidative stress induced by pyrethroid exposure. Biotin levels decrease following exposure in our study. These dynamics are particularly interesting in the context of previous literature, which found that a biotin deficient diet increased resilience against the DNA damaging agent hydroxyurea, and increased resistance to heat stress in insects fed a biotin deficient diet. Biotin is synthesized using alanine, which is elevated in resistant samples following deltamethrin exposure, and alanine metabolism is enriched in both populations following deltamethrin exposure (Figure 3.6,3.9). It may be that prioritizing alanine liberation and delivery to alternate pathways is important to stress resistance, which may be important to consider as biotin binding proteins are deployed for insect control, as interesting cross resistance dynamics may emerge (Christeller et al., 2010). Biotin restriction has repeatedly been shown to negatively impact fecundity in insects, a phenotype repeatedly associated with pyrethroid resistance phenotypes(Freeman et al., 2021), so tradeoffs around this vitamin may be interesting to explore in the context of insecticide resistant lines.

Changes in lipid composition were particularly pronounced in our study. Pyrethroid exposure is well known to induce lipid oxidation in many species(Brinzer et al., 2015; Kale, 1999; Sreejai & Jaya, 2010; C. Yang et al., 2020). In resistant insects collected 70 minutes post exposure, we see significant increases in fatty acids, lysolipid classes and decreases in corresponding phospholipid groups (Figure 3.5.) Interestingly, we also see shifts in lipid metabolites in susceptible samples from the central valley. These mosquitoes are collected at less than 30 minutes post exposure, indicating lipid composition shifts can be rapid, and that Central Valley mosquitoes may have unique physiology that facilitates these shifts. Pyrethroid

exposure produces lipid peroxides(Terhzaz et al., 2015), which can trigger apoptotic cascades (Su et al., 2019). Degradation of these damaged lipids may support survival after insecticide challenge, though fatty acids and lysolipids can have membrane destabilizing properties when incorporated into membranes, reviewed in Arouri & Mouritsen (Arouri & Mouritsen, 2013). Lysolipids play important signaling roles which may mediate responses to stress challenges and calcium signaling, though their roles are best characterized in mammals(Frasch & Bratton, 2012; Mehta, 2005; Wang et al., 2015).

Overall, our study finds that the major metabolic changes following deltamethrin exposure align with changes observed in other populations and other insect species exposed to pyrethroids, though there are differences in timing dynamics (Brinzer et al., 2015; Li et al., 2022; P. Singh et al., 2022). In our analysis of impacted pathways we uncover interesting dynamics with regards to b-vitamins, amino acid metabolism, nitric oxide signaling and oxidative stress metabolism. We suggest metabolic pathways that may be interesting targets for further investigating with regards to insecticide resistance, and describe impacts on nitrogenous waste production pathways, which could be targeted for synergism in mosquito control products.

CONCLUSION

In conclusion lysolipids, fatty acids and carnitines are elevated by 70 minutes following sublethal deltamethrin exposure, along with sugar acids, amino acids and sugar alcohols. Butyrates decrease, and we see mixed dynamics for dipeptides, oligopeptides and oligosaccharides. Nucleic acids appear to play important detoxification roles, and unique amino acid metabolism pathways in mosquitoes may provide effective targets for novel synergists and synergisms that could reduce impacts on non-target species.

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FIGURES

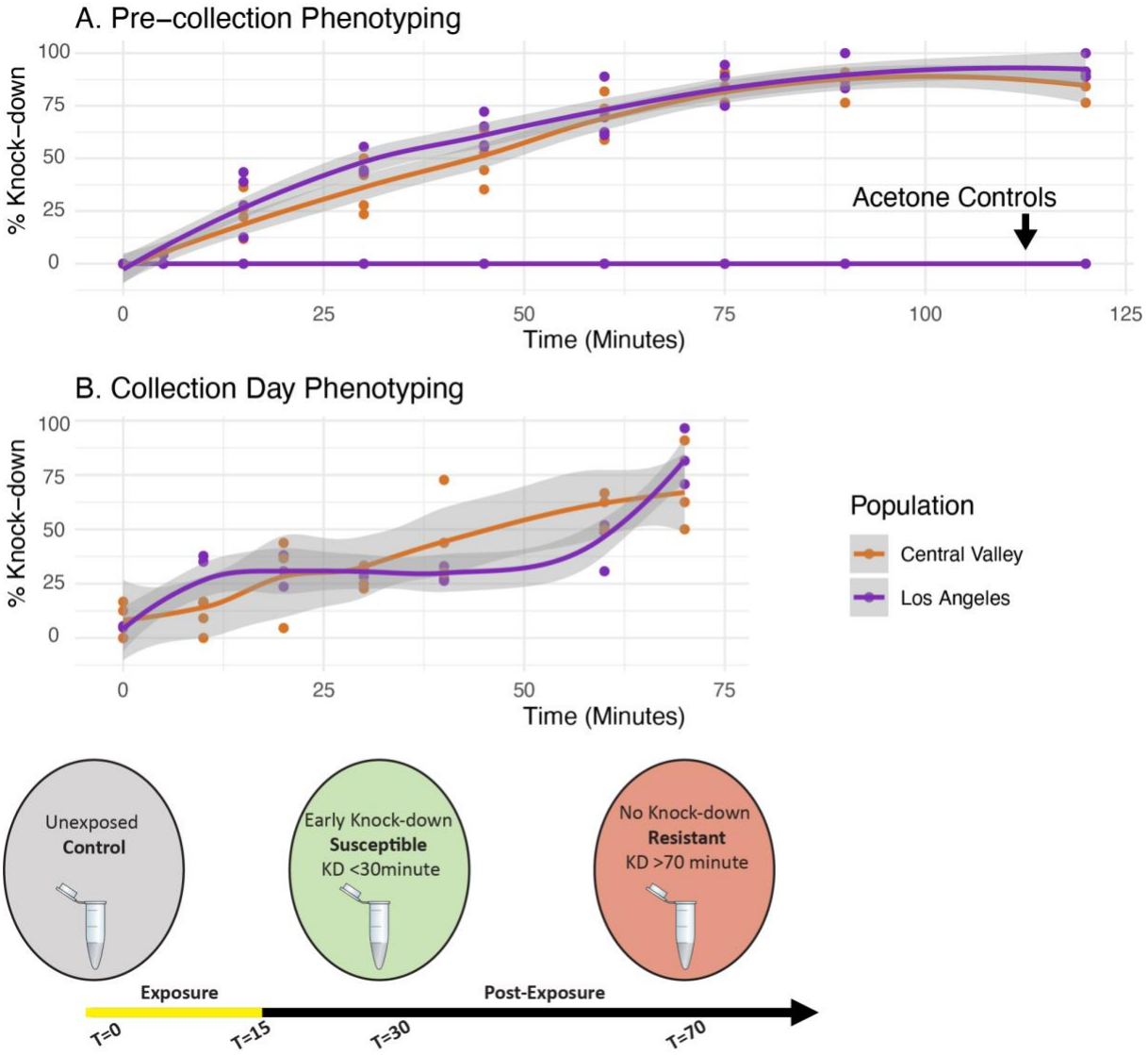


Figure 3.1. Sample Collection Parameters. 1A. Knock-down curves for both populations from pre-collection method evaluation. Mosquitoes were monitored for up to 120 minutes, then checked at 24 hours. 1B. Sample collection knock-down curves. 1C.The diagram Illustrates sample collection regime following insecticide exposure and pre-collection method evaluation data.

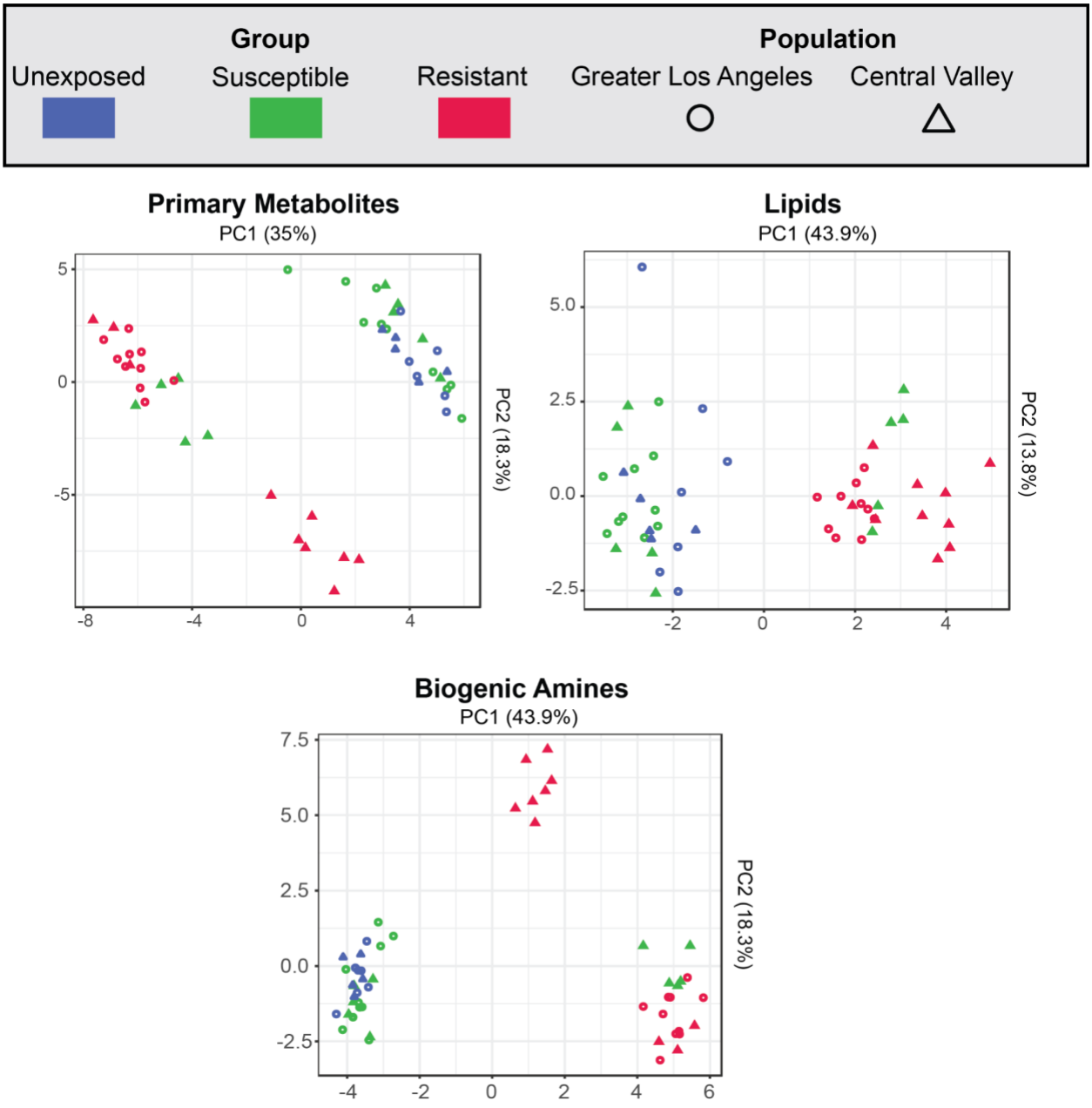


Figure 3.2. Principal Component Analysis. Principal component analysis (PC1xPC2) for the three metabolomic assays.

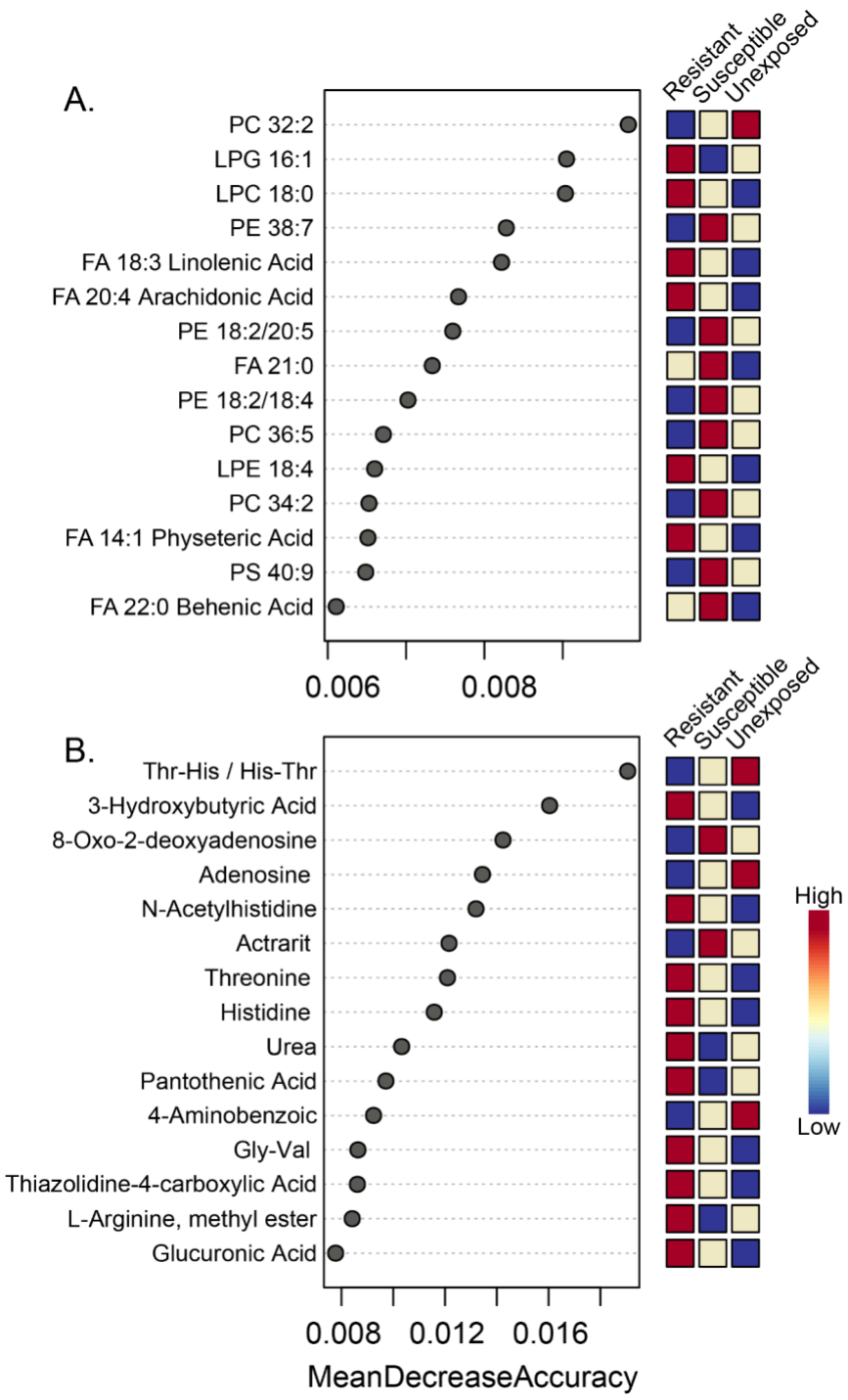


Figure 3.3. Random forest feature of importance ranking for A. Lipids and B. Biogenic Amines. Results for primary metabolites were not included due to redundancy in top hits with the Biogenic Amine panel. Feature abundance is depicted on the right.

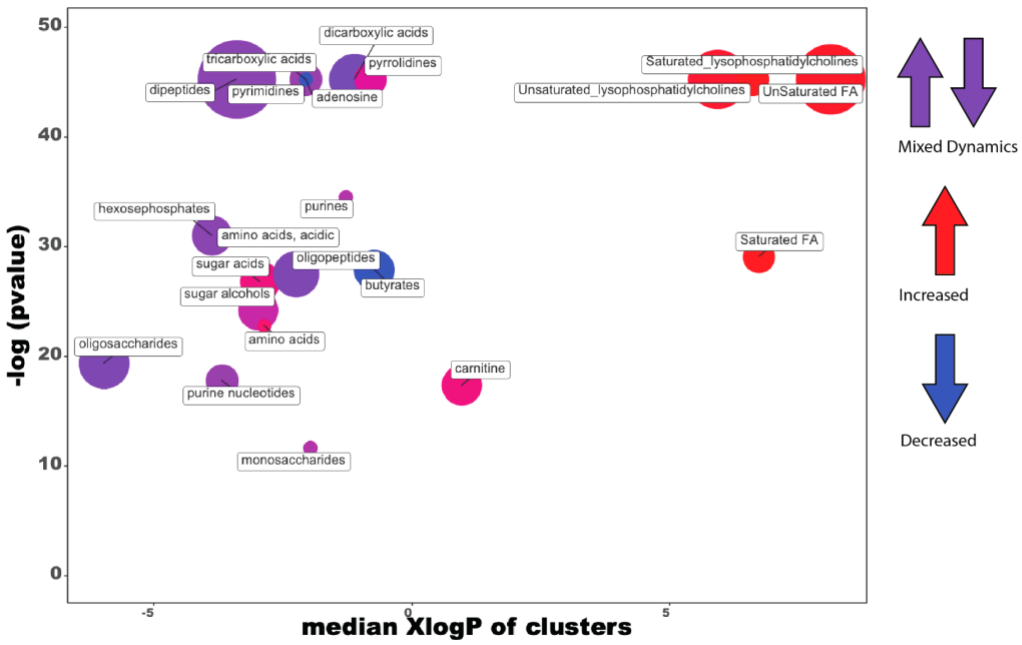


Figure 3.4. ChemRICH enrichment result. FDR adjusted t-tests and fold changes were calculated comparing the susceptible and resistant groups from both populations together to reveal broad patterns in metabolite changes.

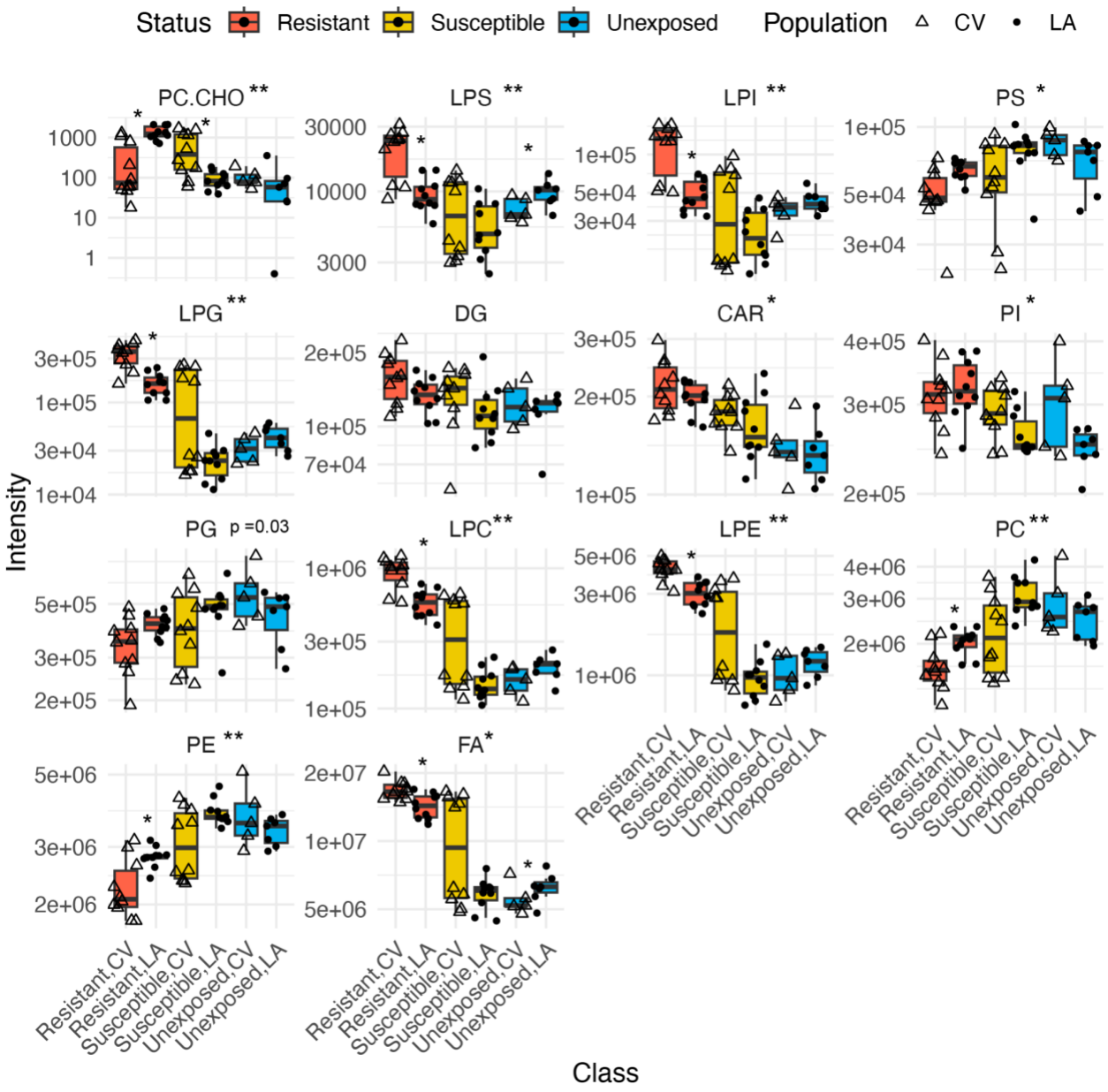


Figure 3.5. Lipid class level Intensity by status and population. Two way ANOVA significance annotation by lipid class indicates difference by status * or interaction between status and population ** with a p <0.05. Significance between populations annotated with * over boxplots.

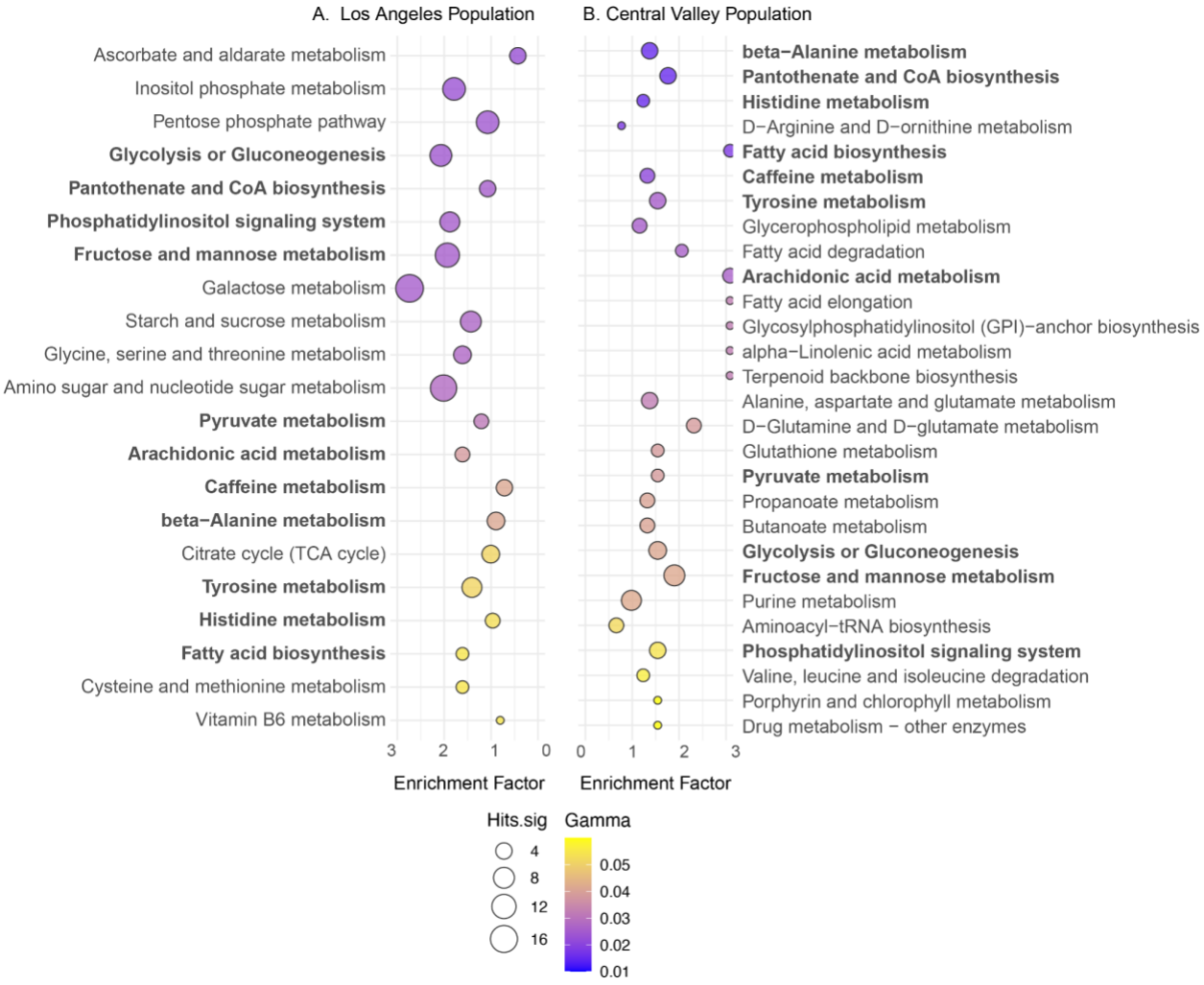


Figure 3.6. Mummichog KEGG Pathway Enrichment Plots by Population. Enrichment is the ratio of significant hits (p<0.001) over expected hits. Point color and size correspond to gamma and 1/gamma respectively. Pathways with a gamma values less than 0.06 are displayed.

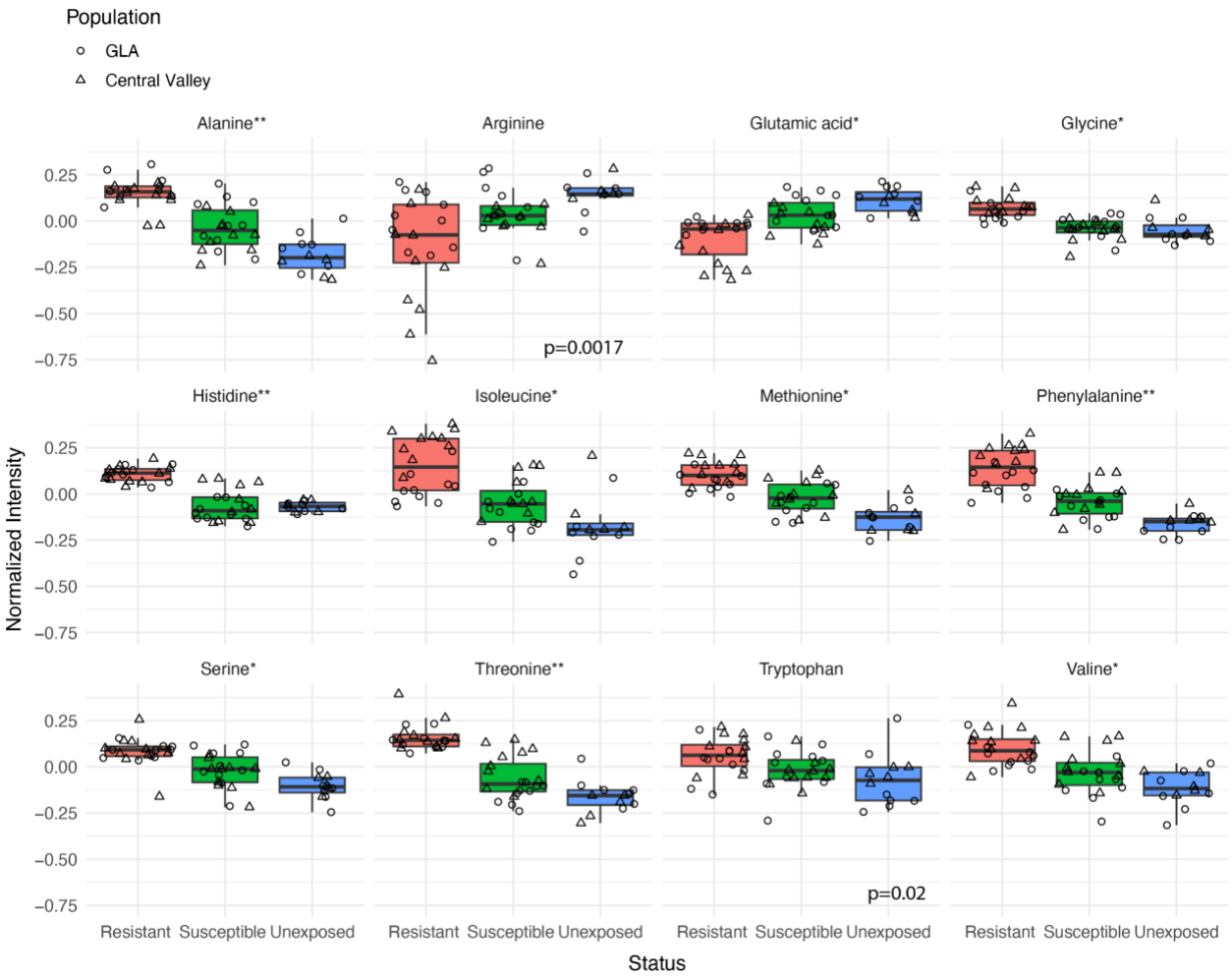


Figure 3.7. Differential Peak Intensity of Essential Amino Acids. P values derived from linear modeling with FDR adjustment. **P < 5e-10, *P<5e-5, P listed if larger than 5e-5. Amino acids that don't vary by Intensity in a significant way are omitted.

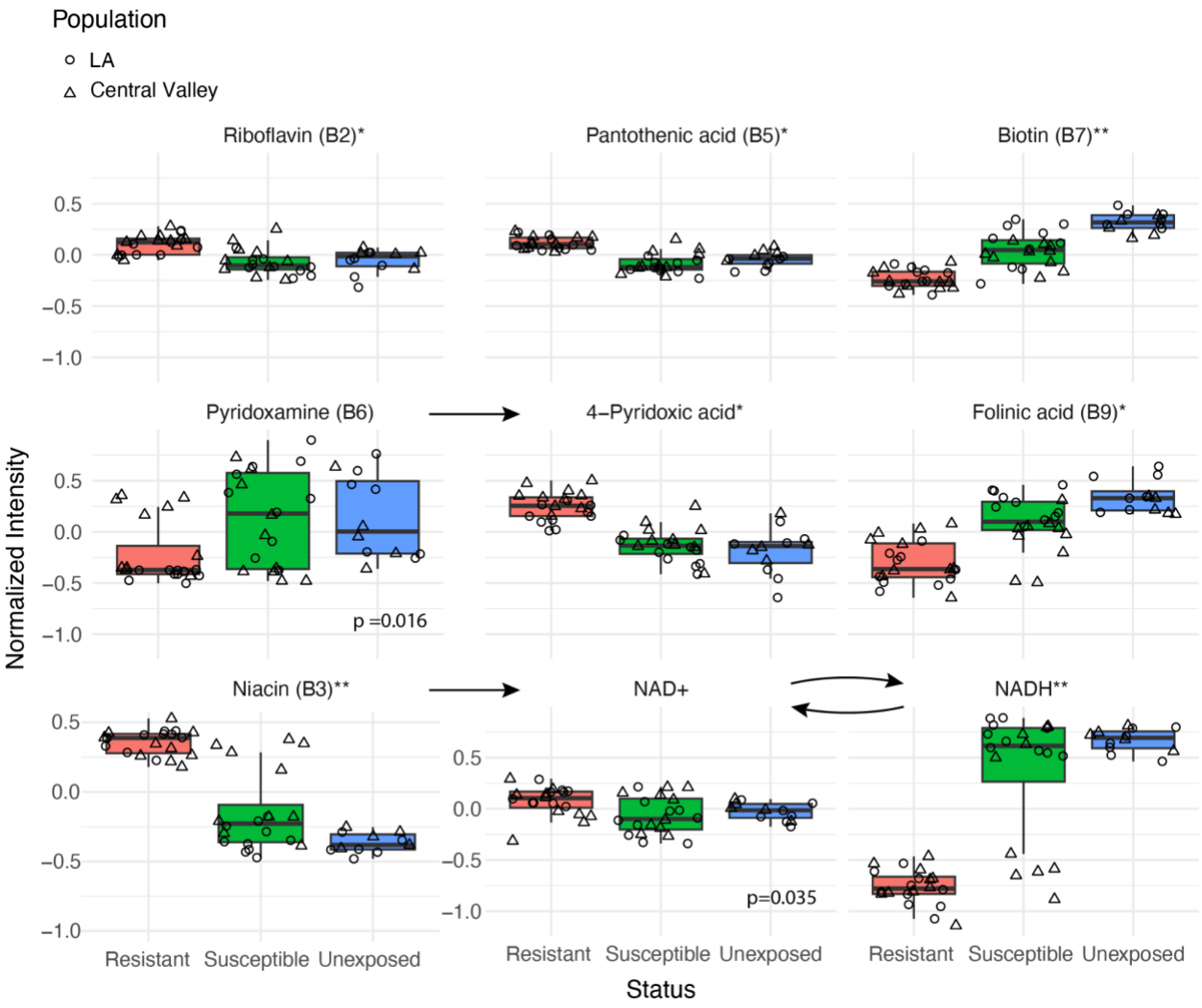


Figure 3.8. Differential Peak Intensity of B Vitamins. P values derived from linear modeling with FDR adjustment. **P < 5e-10, *P<5e-5, P listed if larger than 5e-5. 4-Pyridoxic acid is a metabolite of vitamin B6, and B3 is a precursor to the electron carrier NAD+ and its reduced form NADH.

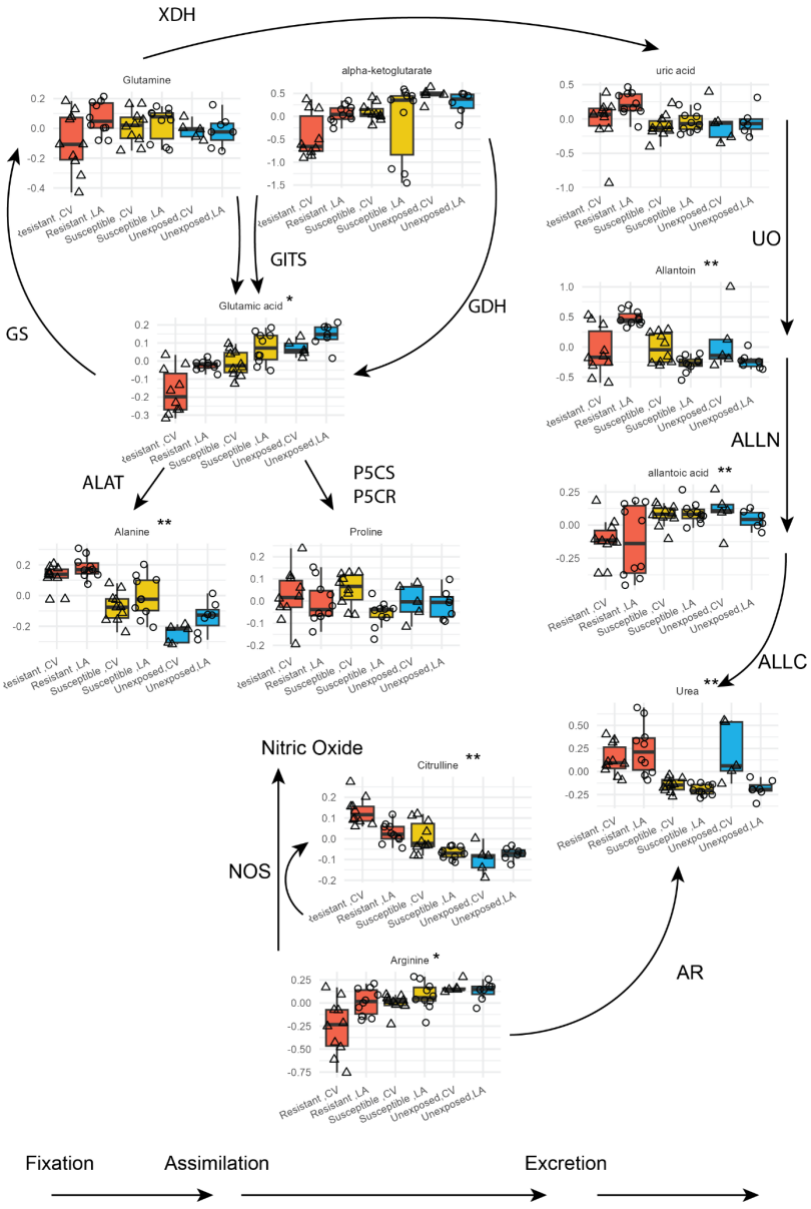
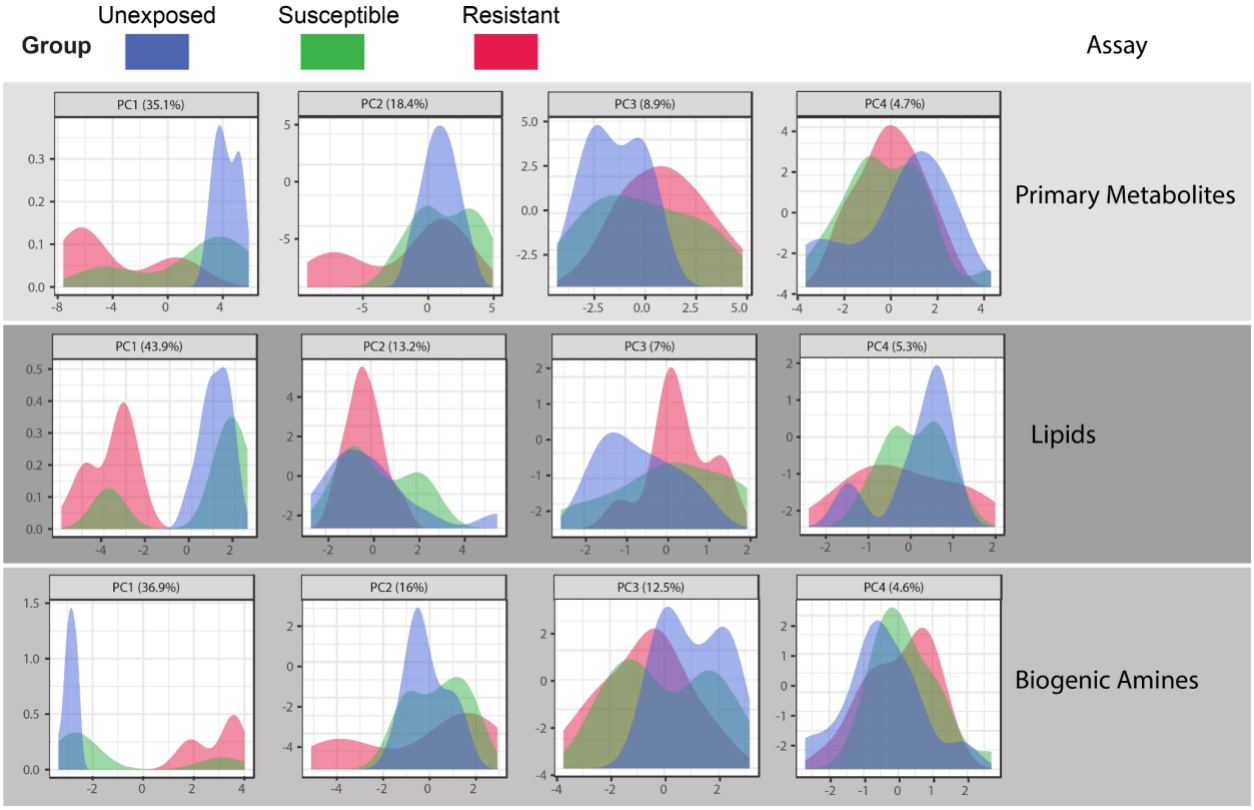


Figure 3.9. Metabolite dynamics within *Ae. aegypti* ammonia metabolism following deltamethrin exposure. Significance for each metabolite is indicated by * for significant difference by status (Resistant-Control) and ** for significant differences by status and population. Figure is modeled off of the pathway crosstalk network proposed in Isoe & Scaraffia 2013. Abbreviations: Glutamine synthetase (GS), glutamate synthase (GltS), glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT), pyrrolidine-5-carboxylate synthase (P5CS), pyrrolidine-5-carboxylate reductase (P5CR), xanthine dehydrogenase (XDH), urate

oxidase (UO), allantoinase (ALLN), allantoicase (ALLC), arginase (AR) and nitric oxide synthase (NOS).

Supplementary File S3.1. ANOVA Summary Table. This large summary table compiles statistical results and attempts to make information about metabolic features easily viewable. The table includes feature name, InchIKey, feature ID, median normalized intensity of peaks by group (control, susceptible, resistant) and includes sparklines plots to make data patterns viewable. Ratios of median Resistant/Control intensity and median Susceptible/Control Intensity are also included. Columns D:I represents the Central Valley/SanJoaquin Valley Population and Columns J:O represent the Greater LA population. F.value, P.value,negative log10P, FDR adjusted P are included, along with Tukey HSD pairwise comparison results(column T and then U:Z.). Metrics of peak abundance are included in columns AA,AC and AD. Median Pool Intensity (AA), the ratio of Pool Intensity to Blank intensity (if available)(AC), and percentage of known metabolites as a % of total intensity (AD) are included.

Supplementary File S3.2. Covariate Table. Summary of linear modeling results for all three assays.



Supplemental Figure 3.1. Sample distribution densities for principal components 1-4 for each metabolomic assay. Primary metabolites and biogenic amines were median-normalized, log transformed and features were median normalized prior to principal component analysis. Lipids were analyzed using nearly the same procedure, but were instead normalized by the sum of peak heights.

Data and code availability

The data files used for this study are available as supplemental attachments. Code available upon request to lead author.

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CONCLUSION

The global expansion of *Aedes aegypti* presents a significant threat to public health. In 2023, there were more than one hundred locally acquired dengue cases in the mainland US, including cases in Pasadena and Long Beach, California. The majority of locally-acquired dengue cases occurred in Florida (CDC 2023).

In chapter one, we investigated population dynamics within the state of California. We demonstrated, alongside others, that California has experienced multiple successful invasion events (Kelly et al. 2021; Lee et al. 2019; Pless et al. 2020, 2017). We found that the frequency of voltage-gated sodium channel mutations appears to increase over time, and that invasive populations often arrive resistant to the tools available for adult control. We demonstrated that, with favorable conditions and thorough treatments, small invasions can be cleared. However, suitable habitats are likely to see *Ae. aegypti* established eventually.

In chapters two and three, we viewed pyrethroid resistance physiology through a new lens, by capturing a snapshot of metabolite levels in mosquitoes representing different phenotypes and following pyrethroid challenge. Monitoring pyrethroid resistance in adult mosquito populations is a challenge for vector control entities. Some vector control entities may not have the facilities required to rear insects or mix pesticide solutions. For those that do, the time required to collect, sort, and rear sufficient numbers of adult mosquitoes for toxicology studies is substantial and tends to conflict with the other duties, like conducting inspections and treating breeding sources. Mutations in the voltage gated sodium channel can be assayed by a variety of sequencing methods, but we demonstrate that even among mosquitoes with a given target site genotype, insecticide tolerance can vary considerably (Mack et al. 2021). Teasing out metabolic mechanisms of resistance is complex, and we sought to identify markers of resistance status downstream of alterations in specific detoxifying enzymes.

In Chapter two, we compared a wild, pyrethroid-resistant population of *Aedes aegypti* collected from Clovis, CA to our pyrethroid susceptible lab reference strain, Rockefeller, using

gene expression data and metabolomic panels for lipids, biogenic amines and primary metabolites. We found metabolite differences were generally modest, less than 10 fold, with the exception of a dipeptide Gly-Pro. However, we found that metabolite data did help us to identify important pathways supporting the constitutive maintenance of elevated activity of cytochrome P450s and Epsilon Glutathione S-Transferases. We found evidence of elevated flux through the pentose phosphate pathway in Clovis, and increased demand on antioxidant pathways. Pathways that support maintenance of detoxifying enzymes are likely important vulnerabilities for insecticide synergism, which can be explored further.

In Chapter three, we studied metabolite alterations in response to deltamethrin exposure in two CA populations of Ae. *aegypti* with genetically distinct backgrounds, collected from the Central Valley and LA. We described the essential roles of B-vitamins in surviving insecticide challenge, and differential utilization of pathways producing nitrogenous wastes.
Through this dissertation, we document pyrethroid resistance status, describe new facets of resistance physiology, and identify targets for synergism. The discussion of pyrethroid resistance status, given the documented fitness costs (particularly of metabolic resistance mechanisms) (Freeman et al. 2021; Smith et al. 2021), begs the questions; will resistance naturally fade for these invasive populations over time? And, where is the selective pressure for resistance coming from?

In the current landscape, it appears that insecticide resistance may not naturally fade. Recent work in California has found widespread pyrethroid contamination in urban catch basins, which are important urban vector breeding grounds (Sy et al. 2022, 2024; Surendran et al. 2019). Other work has found proximity to agriculture can be selective for resistance in Anopheles mosquitoes (Hien et al. 2017), and work in Brazil reported persistence of pyrethroid resistance for ten years following the cessation of government pyrethroid applications, an outcome attributed to elevated private pesticide usage during dengue outbreaks (de Lourdes Macoris et al. 2018). California is an agricultural state, and *Aedes* populations live beside areas

that experience intense insecticide pressure, particularly in the San Joaquin Valley. Additionally, insecticide treatments are still applied for West Nile risk mitigation for Culex mosquitoes ("MVCAC IVM Whitepaper: Integrated Vector Management Is Critical for Protecting Public Health" 2020). With these dynamics in mind, it is difficult to imagine that environmental pyrethroid pressure can be meaningfully curtailed to restore efficacy of pyrethroids.

Utilizing integrated vector management strategies, partnering with research entities, and trialing innovative control strategies are all necessary as the vector control community grapples with the loss in efficacy of the main tool available for adult control and disease outbreaks. A striking variety of novel strategies are emerging, alongside vaccine candidates for major arboviruses, such as dengue and malaria (Crawford et al. 2020; Schairer et al. 2021; Utarini et al. 2021; Tully and Griffiths 2021; RTS,S Clinical Trials Partnership 2015). Following the 2016 Zika outbreaks, the CDC awarded funding to create Centers of Excellence to conduct applied research and training of professionals to respond to vector-borne disease threats across the United States ("Research and Evaluation: Prevent and Control Vector-Borne Diseases" 2023). As a trainee myself, I am both cognizant of the challenges and inspired by the exciting opportunities that lie ahead in the dynamic field of Vector Control.

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