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The adult horn fly transcriptome and its complement of transcripts encoding cytochrome P450s, glutathione S-transferases, and esterases

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ABSTRACT

The horn fly, *Haematobia irritans*, is a blood-feeding parasitic fly with a global distribution that includes Europe, Africa, Asia, and the Americas. The fly has a major detrimental economic impact upon cattle production, with losses estimated at over $800 million annually in the United States and $2.5 billion in Brazil alone. Insecticide resistance in specific horn fly populations has been a problem for many years and there are several mechanisms whereby resistance develops. Little is known about the complement of metabolic enzymes encoded by the horn fly's genome that might provide the fly with detoxification or sequestration pathways to survive insecticide treatments. The cytochrome P450, glutathione S-transferase, and esterase enzyme families contain members that are capable of sequestering and/or detoxifying xenobiotic molecules such as insecticides. We sought to develop a comprehensive dataset of metabolic enzyme-encoding transcript sequences from the adult horn fly, as this is the life stage whose actions directly impose the economic costs to cattle producers. We used an Illumina paired-end read RNA-Seq approach to determine the adult horn fly transcriptomes from laboratory and field populations of horn flies with varying levels of pesticide resistance, including untreated and pyrethroid-treated newly eclosed adult flies. We followed with bioinformatic analyses to discern sequences putatively encoding cytochrome P450, esterase, and GST enzymes. We utilized read-mapping of RNA-Seq data and quantitative real-time polymerase chain reaction (qRT-PCR) to examine gene expression levels of specific P450 transcripts in several fly populations with varying degrees of pesticide resistance.

1. Introduction

The horn fly, *Haematobia irritans*, is a blood-feeding parasitic fly with a global distribution that includes Europe, Africa, Asia, and the Americas. The horn fly has a major detrimental economic impact upon cattle production, with losses attributable to this fly estimated at over $800 million annually in the United States (Kunz et al., 1991) and $2.5 billion in Brazil (Grisi et al., 2014) alone. These losses are attributable to irritation and blood loss due to fly bites with resulting decreased milk production and weight gains in growing or weaning animals. Adult flies generally remain upon the host animal throughout both day and night, and both male and female flies feed upon bovine host blood, taking 20–30 blood meals per day (Foil and Hogsette, 1994). Horn fly control centers upon the use of insecticides, with pyrethroids and organophosphates predominating the range of commercially available products. However, insecticide resistance in specific horn fly populations has been

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a problem for many years and there are various mechanisms whereby resistance can develop. Jamroz et al. (1998) correlated pyrethroid resistance with amino acid differences at the sodium channel, the target site of pyrethroid activity. Target site resistance to cyclodiene also appears to have developed in a horn fly population in Louisiana, USA that was treated with endosulfan, a cyclodiene (Domingues et al., 2013). A multifactorial resistance mechanism seemed to be functioning in diazinon resistant horn flies studied by Li et al. (2007); metabolic esterase activity was higher in resistant flies, although a specific esterase causing resistance could not be identified. Metabolic-based pyrethroid resistance was suspected in a highly pyrethroid resistant horn fly population in Georgia, USA (Sheppard, 1995) and several populations of Brazilian horn flies that showed high pyrethroid resistance but little, if any, target site resistance-associated sodium channel mutations (Guerrero and Barros, 2006).

Little is known about the complement of metabolic enzymes encoded by the horn fly's genome that might provide the fly with detoxification or sequestration pathways to resisting insecticides. The cytochrome P450, glutathione S-transferase, and esterase enzyme families contain members that are capable of sequestering and/or detoxifying xenobiotic molecules such as insecticides (Ranson et al., 2002). A range of these metabolic enzymes has been identified from the genomes and transcriptomes of insects such as mosquito (Ranson et al., 2002), honeybee (Claudianos et al., 2006), and *Rhodnius prolixus* (Schama et al., 2016), but information from the horn fly is lacking. Torres et al. (2011) reported 3 cytochrome P450- and 1 glutathione S-transferase (GST)-encoding unigenes were found in their 992 unigene horn fly abdominal tissue transcriptome. Guerrero et al. (2008) reported 3, 9, and 7 esterases, cytochrome P450s, and GSTs, respectively, from their 2689 unigene larval transcriptome of the horn fly. They also found 1, 1, and 3 esterases, cytochrome P450s, and GSTs, respectively, in their 2668 unigene egg transcriptome of the horn fly in the same study. However, neither group's horn fly transcriptome study utilized deep sequencing protocols. Konganti et al. (2018) reported the whole genome assembly of the horn fly genome, including 165, 99, and 19 predicted transcripts putatively encoding cytochrome P450s, esterases, and GSTs, respectively. A caveat regarding these gene predictions is the DNA for the genome sequencing was sourced from eggs of a strain of fly reared in artificial laboratory conditions for over 60 years, imposing environmental and behavioral selection pressures quite different than natural populations would experience (Guerrero and Kunz, 2000).

Over the years, our team had acquired an extensive collection of horn fly samples from across the southern U. S. and Brazil. Most of these samples had phenotype information for resistance to several classes of pesticides and had been genotyped for target site-based pyrethroid resistance. Although several populations had major components of metabolic-based resistance (Sheppard, 1995; Guerrero et al., 1997; Guerrero and Barros, 2006), the gene expression of pesticide-metabolizing enzymes had not been systematically studied. Thus, we sought to develop a dataset of metabolic enzyme-encoding transcript sequences from the adult horn fly, as this is the life stage whose actions directly impose the economic costs to cattle producers. The adult stage is also the target of most current insecticidal products attempting to reduce the fly numbers below an economic threshold. The adult stage's metabolic enzyme capacity would be most directly affected by insecticide treatments and likely the most relevant to the development of resistance. We used an Illumina paired end read RNA-Seq approach to determine the adult horn fly transcriptomes from several field and laboratory populations of horn flies, varying in their phenotypic expression of pesticide resistance. We followed with bioinformatic analyses to identify transcripts putatively encoding cytochrome P450, esterase, and GST enzymes and quantitative real-time polymerase chain reaction (qRT-PCR) to examine differential gene expression.

2. Materials and methods

2.1. Fly samples

In August and September 2009, adult flies were collected with aerial sweep hand nets from pastured cattle at the St. Gabriel Research Station (St. Gabriel, LA, USA) of the Louisiana State University (LSU) Agricultural Center and held in Erlenmeyer flasks at 30 °C and total darkness to facilitate egg collection. Cattle at this Research Station had been treated with various insecticides in a mosaic treatment regime over the years and horn flies infesting this location had developed a moderate level pyrethroid resistance. Domingues et al. (2019) documented sodium channel-mediated target site resistance in St. Gabriel, also inferring the likely presence of metabolism-based resistance. Eggs were seeded onto bovine manure collected from cattle at this station and emerging adult flies collected. Unfed newly eclosed adults were segregated according to sex and a portion of untreated males and females were considered as controls and frozen at − 80 °C for RNA extractions. Groups of male and female flies were tested to quantify permethrin resistance using the impregnated filter paper method (Sheppard and Hinkle, 1987). To obtain flies that were exposed to sublethal doses of permethrin, we exposed a group of newly eclosed unfed adult flies to permethrin by placing them in the petri plates of the impregnated filter paper assay method for 2 h with a dose of 1.56 μg permethrin /cm^2. This dose was approximately one-third of the LC_{50} dose of 4.4 μg permethrin /cm^2 for this population. Dead and alive flies were sexed and separately frozen at − 80 °C for RNA extraction. We also exposed flies to permethrin + piperonyl butoxide (PBO, an organic compound known to inhibit the activity of cytochrome P450s and capable of synergizing the activity of pyrethroids) by treating flies, similarly, using a 2 h exposure to 1.56 μg/cm^2 permethrin, 1% PBO and separately freezing the dead and alive male and female flies at − 80 °C for RNA extractions.

For the differential expression and qRT-PCR experiments described below, we tested adult flies from archived samples of populations with varying levels of pyrethroid resistance (Table 1). Flies were sampled in 1997 from cattle at University of Georgia Central Branch Experimental Station in Eatonton, GA. Newly eclosed unfed and actively feeding adults from the Kerrville Susceptible reference strain were separately collected in 1997 from the *in vitro* colony maintained at the Knipling-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, TX. Newly eclosed unfed and actively feeding adult pyrethroid resistant flies were obtained in 1998 from the Resistant and Super Resistant colonies maintained in a screened pen on a bovine host at the Knipling-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, TX. We also tested flies collected from cattle at the Rosepine LSU research farm (Rosepine, LA, 2009). The resistance status and relevant references for these fly populations is described in Table 1. Collected horn flies were frozen in dry ice immediately upon collection and stored at − 80 °C until nucleic acid extraction.

2.2. RNA isolation for St. Gabriel adult fly transcriptome sequencing

Fourteen unfed, newly eclosed adult flies from the St. Gabriel population untreated Control Male, untreated Control Female, Permethrin treated alive Male, and Permethrin + PBO treated killed Male groups were used to purify total RNA in a protocol adapted for use with the FastPrep 24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA) and the FastRNA Pro Green Kit (MP Biomedicals). Frozen flies were transferred to a 1.5 ml microcentrifuge tube prechilled on dry ice and pulverized using a RNAse-free Kontes pellet pestle prechilled in liquid nitrogen. Lysing Matrix D from the FastRNA Pro Green kit and 1 ml of ice-cold RNA Pro solution was added to the fly material. The tubes were processed in the FastPrep 24 for 40 s at a setting of 6.0. The tubes were returned to ice for 3 min, then reprocessed and returned to ice for two more cycles. Following a 5 min microfuge step, the supernatant was transferred to a clean 1.5 ml tube and incubated for 5 min at room

Table 1
Resistance status of horn fly populations used in qRT-PCR gene expression analyses.

Name	Resistance Status		Known Mechanisms	Location	Reference
	Class	Phenotype			
Kerrville Susceptible	PYR:	Susceptible	–	Kerrville, TX	Guerrero et al. (1997), Jamroz et al. (1998)
	OP:	Susceptible	–		
Kerrville Resistant	PYR:	Moderate	Target site (moderate *kdr*, low *super-kdr*) + Metabolic	Kerrville, TX	Guerrero et al. (1997), Jamroz et al. (1998)
	OP:	Susceptible	–		
Kerrville Super Resistant	PYR:	Very high	Target site (very high *kdr* and *superkdr*) + Metabolic	Kerrville, TX	Guerrero et al. (1997), Jamroz et al. (1998)
	OP:	Susceptible	–		
Georgia	PYR:	High	Target site (very high *kdr* and *superkdr*) + Metabolic	Eatonton, GA	Sheppard (1995), Foil et al. (2010)
	OP:	Unknown	Unknown		
LSU St. Gabriel	PYR:	Moderate	Target site (low *kdr*, no *superkdr*) + Metabolic	St. Gabriel, LA	This study; Domingues et al. (2019)
	OP:	Unknown	Unknown		
LSU Rosepine	PYR:	Low	Target site (low *kdr*, no *superkdr*) + Metabolic unknown	Rosepine, LA	Younger (2011), Foil et al. (2010)
	OP:	Moderate	Target site + other		

temperature. One-tenth volume of 3 M sodium acetate was added and mixed, followed by addition of 300 μL of chloroform, 10 s vortexing, and 5 min incubation at room temperature. After a 5 min microfuge step, the supernatant was extracted with 1 vol of Acid Phenol-$CHCl_3$ (Thermo Fisher Scientific, Waltham, MA, USA) by 30 s vortex, 5 min incubation on ice, and a 5 min microfuge step. The aqueous phase was recovered, and 1 vol of isopropanol added to precipitate the nucleic acids. The recovered pellet was resuspended in 0.1 mM EDTA and precipitated with the lithium chloride treatment protocol of the ToTALLY RNA Isolation Kit (Thermo Fisher Scientific). Approximately 70 μg of RNA was recovered at this stage from each sample. The samples were diluted to 0.2 μg/μL and DNAse treated with the TURBO DNA-free Kit according to manufacturer's protocol (Thermo Fisher Scientific). A 2 μg sample was analyzed by 1% TBE agarose gel electrophoresis to verify the intactness of the RNA and the absence of contaminating genomic DNA.

2.3. St. Gabriel adult horn fly transcriptome sequencing and bioinformatics

Sequencing of our 4 St. Gabriel fly RNAs was performed at the National Center for Genome Resources (Santa Fe, NM, USA) using the standard Illumina RNA-Seq library preparation protocol and a single lane of the RNA-Seq 2 × 54 paired end approach. A duplex-specific nuclease (DSN) step was incorporated into the library preparation protocol for Control Male, Control Female, Permethrin treated alive Male, and Permethrin + PBO treated killed Male samples, attempting to reduce the levels of highly abundant ribosomal RNAs and enhance the detection of lower abundance transcripts to produce a more comprehensive transcriptome. We also collected sequence data from libraries of the Control Male and Control Female samples without the DSN step. Raw reads were processed and quality preassessment performed by the Illumina pipeline and the contaminant filtering pipeline developed at National Center for Genome Resources. *De novo* assembly of the transcriptome is described in Domingues et al. (2018). The collection of contigs was annotated by BlastX sequence similarity searches against the UniRef100 database using an E-value cutoff of 1×10^{-3}. Raw reads for the Control Male, Control Female, Permethrin treated Male, and Permethrin + PBO treated Male transcriptomes were submitted to NCBI's SRA (accession number SRP131897) and assembled transcriptomes are accessible under the BioProject ID PRJNA429442 (Domingues et al., 2018). To produce an adult fly transcriptome, the 4 assemblies were clustered with cd-hit-est to remove redundancy and keep the longest representative sequence of the cluster (Weizhong and Godzik, 2006; Fu et al., 2012). BUSCO version 3.1.0 was run on the pooled transcriptome transcripts using the diptera_odb9 lineage database consisting of 2799 BUSCO search groups from 25 species (Simão et al., 2015; Waterhouse et al., 2017) in transcriptome mode.

In the main Tables, we report DSN expression counts from the Control Male, Control Female, Permethrin treated Male, and Permethrin + PBO treated Male transcriptomes. These counts were normalized during the DSN protocol sequence output analysis at NCGR to account for variations in raw read outputs. These expression counts represent the total number of normalized raw reads that aligned to the assembled transcript. In the Supplementary Tables, we report expression counts from the DSN and the non-DSN RNA-Seq runs described above. These reported counts from the non-DSN RNA-Seq runs were normalized to account for the slightly higher read output from the Control Male (127,276,458 raw reads from Domingues et al. (2018)) than the Control Female (126,531,116 raw reads from Domingues et al. (2018)).

2.4. qRT-PCR

Quantitative reverse transcription PCR (qRT-PCR) experiments were designed and completed following the MIQE guidelines (Bustin et al., 2009). TaqMan probes and primers (Supplementary File 1) were designed for the CYP3073H1 (contig BPA_1033 in our pooled horn fly transcriptome, Supplementary File 2), the Progesterone receptor membrane component 1 (PGRMC1)-like (BPA_735), the CYP6EK4 ortholog (BPA_14230), the cytochrome P450 4g1-like (STEP1_C4604), cytochrome P450 6a21-like (STEP1_REP_C5674), cytochrome P450 6d1-like (BPA_16883), cytochrome P450 6a9-like (STEP1_REP_C589), glutathione S-transferase hiGSTE5 (STEP1_REP_C3807), glutathione S-transferase hiGSTD3 (STEP1_REP_C4816) contigs, and four reference genes, 18S (Accession No. EU179518), β-actin (Accession No. JN969088), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Accession No. GGLM01032021), and phosphoglycerate kinase (PGK; Accession No. GGLM01028752), using the OligoArchitect Online design tool (http://www.oligoarchitect.com/Login.jsp) and synthesized by Sigma-Aldrich Inc. (St. Louis, MO, USA). For CYP3073H1 (contig BPA_1033) and the Progesterone receptor membrane component 1 (PGRMC1)-like (BPA_735), individual flies were pulverized in microcentrifuge tubes on dry ice using liquid nitrogen-cooled pestles. Each fly was considered a biological replicate, and 5 flies from each population were used for each target's analysis. For remaining targets, 14–20 flies with an equal number of males and females were pooled to form a sample. The pooled samples were pulverized in microcentrifuge tubes on dry ice using liquid nitrogen-cooled pestles. Each pooled sample was considered a biological replicate, and four samples from each population were used for each target. Total RNA was purified using the ZR Tissue and Insect RNA Microprep Kit (Zymo, Irvine, CA, USA) or TRIzol Reagent with QIAGEN RNeasy Mini Kit (Qiagen), treated for DNA contamination with the TURBO DNA-free Kit or QIAGEN DNase I, quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and quality verified by 1% agarose gel electrophoresis. The AMV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) was used to synthesize cDNA. A control duplicate reaction without the reverse transcriptase was completed for each RNA sample to test for DNA contamination. The cDNA samples

were assessed for integrity and assayed for the presence of qPCR inhibitors with 3':5' analysis and SPUD assay, respectively (Nolan et al., 2006). Annealing temperatures and primer concentrations were optimized by temperature gradient PCRs and primer concentration matrix qPCRs, respectively. Standard curves were calculated for each target and the optimal set of reference genes identified by NormFinder version 20. The qRT-PCR reactions were completed in Low-Profile Multiplate 96-Well Unskirted PCR Plates (BioRad, Hercules, CA, USA) using 25 μL reactions, which included TaqMan Universal PCR Master Mix no AmpErase® UNG (Thermo Fisher Scientific), 900 nM each of forward and reverse primers, 250 nM probe, and 5 μL cDNA. Plates were covered with Microseal 'B' adhesive seals (BioRad). The BioRad CFX96 Real-Time System was used with cycling parameters of 95 °C for 10 min; followed by 50 cycles of 95 °C for 15 s, 60 °C for 1 min, and a plate read. All samples on the plate were run in triplicate and no template controls and inter-plate calibrators were used for each target assayed on a plate. Baseline-subtracted curve fit mode was used to analyze raw fluorescence emission data for quantification using the relative standard curve method with CFX Manager Software v3.1 (BioRad).

SYBR Green reactions were designed for cytochrome P450 reductase (STEP1_REP_C3186), CYP6GW2 (BPA_896), CYP9F30 (STEP1_REP_C2171), cytochrome P450 6a14-like (BPA_19806), and 18S transcripts using Vector NTI software (Thermo Fisher Scientific). Total RNAs were extracted from 1 g adult flies using the ToTALLY RNA Isolation Kit as described above. The reverse transcription was carried out using RETROscript® Reverse Transcription Kit (Thermo Fisher Scientific). Briefly, the 20 μL reaction mixture consisted of 1 μg Turbo DNase-treated RNAs, 2 μL random decamers, 2 μL 10X reverse transcription buffer, 4 μL dNTP mixture, 1 μL RNase inhibitor, and 1 μL MMLV reverse transcriptase, using a 60 min reaction at 44 °C followed by 10 min at 92 °C. The 10 μL qPCR reaction mixture consisted of 1 μL cDNA, 5 μL SsoAdvanced Universal SYBR® Green Supermix (BioRad), and 0.3 μM of each primer. Three technical replicates of each sample were used on each plate. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, and 58 °C for 30 s. After the reaction, a melting curve analysis from 65 to 95 °C was applied to ensure consistency and specificity of the amplified product. The expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized using the 18S reference gene. All primers and probes are listed in Supplementary File 1.

3. Results

3.1. Transcriptome characterization

The St. Gabriel horn fly population was used for this study due to the POST-2009 sample that was documented by Domingues et al. (2019) to have low target site-mediated pyrethroid resistance, with 0% and 8.8% R alleles for *super-kdr* and *kdr*, respectively. The filter paper PBO synergist bioassays were used to verify the moderate level of pyrethroid resistance (Resistance Ratio = 43) was due almost entirely to PBO-suppressible metabolic resistance (data not shown). To identify the greatest number of putative metabolic resistance-related transcripts, we pooled the transcriptomes identified in adult flies by Domingues et al. (2018) with NCBI entries for horn fly eggs (NCBI Accession Nos. FD449556-FD457982) and first instar larvae (Accession Nos. FD457983-FG466257) reported in Guerrero et al. (2008) for querying using BlastX against the UniRef database at E-value filter of 1.0E-25 to assign putative functions (Supplementary File 2). There were 42,176 unique transcript contigs and the nucleotide sequence to each can be found in Supplementary File 3. The BUSCO results were 53.7% (1504) complete BUSCO, which included 34.5% (967) single copy and 19.2% (537) duplicated, 13.9% (389) fragmented and 32.4% (906) missing.

Searching the hit descriptions for the terms "Cytochrome", "Esterase", and "Glutathione" discovered 136, 21, and 18 sequences that we considered putative cytochrome P450s, metabolic esterases, and GSTs, respectively (Supplementary File 4). Thirty-eight of the putative cytochrome P450 sequences encoded full-length or nearly full-length P450-like open reading frames and we provided those sequences to Dr. David Nelson who assigned official names using the accepted cytochrome P450 nomenclature developed out of his research program. The 18 GST-encoding sequences were assigned names by using each of the contig sequences in a BlastX search of NCBI sequences and using the resulting hit identifications to determine the proper GST family assignment, numbering each family's member consecutively. The metabolic esterases were not assigned official names, rather their BlastX hit descriptions from Supplementary File 2 were used to determine likely function of each transcript's encoded open reading frame. Our set of named adult horn fly cytochrome P450s and GSTs are listed in Table 2 and the metabolic esterases in Table 3. Of the P450 sequences in Table 2, we found 8, 17, and 2 members of the CYP 4, 6, and 9 families, respectively.

3.2. Gene expression analysis

We did a metabolic gene expression analysis comparison between our 4 St. Gabriel adult fly transcriptomes, the Control Untreated Males, Control Untreated Females, Permethrin-Treated Alive Males, and Permethrin + PBO-Treated Dead Males. Tables 2 and 3 show the normalized DSN library transcriptome expression counts associated with each P450, GST, and metabolic esterase found in our 42,176 contig pooled transcriptome (Supplementary File 2). Because we did not have appropriate replicates of our fly exposure experiments and sequenced libraries, we only used these expression counts to guide our selection of transcripts for further study and quantification by qRT-PCR (described below). Additionally, the DSN treatment may have affected transcript distribution in the 4 libraries. Indeed, one P450 6a14-like transcript (BPA_19806) and one GST transcript (hiGSTS2) that were present in our non-DSN assembled sequence output (data not shown) were not found in our DSN-treated sequenced library data (Supplementary File 4).

Selecting transcripts for closer study by qRT-PCR (Table 4), we included cytochrome P450 reductase, as Shi et al. (2015) demonstrated the important role of cytochrome P450 reductase in regulation of the activities of cytochrome P450s and resistance to the pyrethroid fenpropathrin. Our SYBR Green qRT-PCR relative gene expression profiles of selected cytochrome P450s and cytochrome P450 reductase show expression differences between horn fly populations with varying degrees of pyrethroid resistance (Figs. 1–5). For the cytochrome P450 reductase gene, upregulation of the cytochrome P450 reductase gene occurs upon exposure to a sublethal dose of permethrin in the St. Gabriel flies (Perm Male vs. Con Male, Fig. 1). Overexpression of the cytochrome P450 reductase gene was also observed in the highly pyrethroid resistant Georgia and moderately pyrethroid resistant Kerrville Resistant (Res) flies compared to the Con Male flies.

We used qRT-PCR analysis to quantify gene expression of 9 different P450s in several horn fly populations with varying pyrethroid resistance phenotypes (Figs. 2–6, Supplementary Fig. 1, and Supplementary Files 5–7). These P450s were selected based on the pattern of expression counts in the 4 St. Gabriel sequenced transcriptomes (Table 2; Supplementary File 2 and 4) and our ability to reproducibly quantify expression with either SYBR Green or TaqMan qRT-PCR. Fig. 2A shows CYP6GW2 Ortholog expression in the St. Gabriel flies was induced 7-fold by sublethal dose of permethrin (Perm Male vs. Con Male), and expression in the pyrethroid resistant Georgia and Kerrville Resistant population males and females was 4- to 5-fold elevated compared to the St. Gabriel untreated male flies (Con Male). Overexpression of the cytochrome P450 6a14-like transcript in the pyrethroid resistant Georgia and Kerrville Resistant populations was even more pronounced compared to the St. Gabriel flies (Fig. 2B). Both the Georgia and Kerrville Resistant populations expressed 40- to 100-fold higher 6a14-like transcript than the St. Gabriel Con Male, and even 4- to 10-fold higher compared to the permethrin-exposed survivors of the St. Gabriel Perm

Table 2
Expression of Cytochrome P450s and Glutathione S-transferases identified in the adult horn fly transcriptome.

Protein ID	Length[a]	BlastX Hit Information			Expression Counts[b]				Contig ID
		Description	Species	E-value	Untreated		Permethrin Males		
					Female	Male	Alive[c]	+PBO Dead[d]	
Cytochrome P450s									
CYP4D77 ortholog	**512**	probable cytochrome p450 4d14	*M. domestica*	4E-133	156	51	237[e]	242	BPA_2004
CYP4D94	**528**	cytochrome P450 4d1-like	*M. domestica*	2E-167	572	386	655	1026	STEP1_REP_C3792
CYP4G177	**555**	cytochrome p450 4g1	*M. domestica*	0	40,698	25,628	259,784[e]	300,545	STEP1_REP_C2150
CYP4GU1 ortholog	**522**	cytochrome p450 4d2-like isoform X1	*M. domestica*	1E-127	14	7	0	81	BPA_5399
CYP4P18	**520**	cytochrome p450 4p1-like	*M. domestica*	8E-127	119	33	150[e]	120	BPA_1270
CYP4P19	**528**	cytochrome p450 4p1-like	*M. domestica*	6E-146	24	17	117[e]	47	BPA_9879
CYP4S37	**490**	probable cytochrome p450 4s3 isoform X2	*M. domestica*	0	93	163	418	95	BPA_1302
CYP4S38	**490**	probable cytochrome p450 4s3 isoform X1	*M. domestica*	0	97	116	133	189	BPA_800
CYP6A83 ortholog	**503**	probable cytochrome p450 6a21	*M. domestica*	1E-165	29	11	74[e]	211	BPA_34_HORNFLY_RNASEQ_490297
CYP6A104	**506**	cytochrome p450 6a9-like	*M. domestica*	0	207	95	4	40	BPA_1000
CYP6A105	**506**	probable cytochrome p450 6a21	*M. domestica*	7E-167	289	116	221	285	BPA_1097
CYP6A106	**499**	probable cytochrome p450 6a21	*M. domestica*	3E-161	2069	513	3330[e]	4815	STEP1_REP_C2079
CYP6C4	**500**	cytochrome p450 6a18-like	*M. domestica*	0	39	60	82	75	BPA_2557
CYP6D20 ortholog	**518**	cytochrome p450 6d3-like	*M. domestica*	0	44	127	17	125	BPA_1969
CYP6D30	**504**	pyrethroid resistance cytochrome P450	*M. domestica*	5E-162	65	60	117	238	BPA_41_HORNFLY_RNASEQ_267091
CYP6D31	**518**	pyrethroid resistance cytochrome P450	*M. domestica*	0	148	111	69	244	BPA_36_HORNFLY_RNASEQ_419659
CYP6EK4 ortholog	**502**	cytochrome p450 6a18	*M. domestica*	0	12	21	320[e]	415	BPA_14230
CYP6FS4	**502**	probable cytochrome p450 6u1	*M. domestica*	1E-127	13	28	27	132	BPA_668
CYP6FT16	**513**	cytochrome p450 6g1-like	*M. domestica*	7E-125	162[f]	38	345[e]	171	STEP1_C3702
CYP6FT17	**511**	cytochrome p450 6g1-like	*M. domestica*	1E-114	20	58	517[e]	462	BPA_3176
CYP6GU1 ortholog	**501**	cytochrome p450 6a2-like	*M. domestica*	9E-144	63	22	56	131	BPA_2917
CYP6GV11	490	cytochrome p450 6a2-like	*M. domestica*	2E-117	313[f]	77	588[e]	193	BPA_1427
CYP6GV12	462	cytochrome P450 6a2-like	*M. domestica*	8E-112	206	192	240	172	BPA_4989
CYP6GW2 ortholog	**501**	cytochrome p450 6a22-like	*M. domestica*	4E-106	355[f]	44	726[e]	261	BPA_896
CYP6LH1 ortholog	**516**	cytochrome p450 6g1-like	*M. domestica*	6E-129	113	43	28	65	BPA_1750
CYP9F29	**513**	probable cytochrome p450 9f2	*M. domestica*	7E-159	2	50[g]	187	133	BPA_31_HORNFLY_RNASEQ_585313
CYP9F30	**512**	probable cytochrome p450 9f2	*M. domestica*	0	234	266	3802[e]	4078	STEP1_REP_C2171
CYP12A33	575	cytochrome p450 CYP12A2-like	*M. domestica*	0	51	47	330[e]	401	BPA_1504
CYP28J2	353	probable cytochrome p450 28d1	*M. domestica*	1E-106	37	75	2	36	BPA_8179
CYP304A1	515	probable cytochrome p450 304a1	*M. domestica*	0	120[f]	29	0	3	BPA_2887
CYP313B6	**509**	probable cytochrome p450 313b1	*M. domestica*	1E-159	343	144	973[e]	1026	BPA_9150
CYP317A5	**534**	probable cytochrome p450 317a1	*M. domestica*	1E-124	146[f]	4	101[e]	369	BPA_6990
CYP437A7	**506**	probable cytochrome p450 28d1	*M. domestica*	1E-70	99	30	145[e]	181	STEP1_C4097
CYP3073E2	**501**	probable cytochrome p450 313a4	*M. domestica*	4E-73	632	204	116	97	BPA_845
CYP3073G1	**505**	probable cytochrome p450 313a2 isoform X1	*M. domestica*	7E-67	230	184	0	23	BPA_36_HORNFLY_RNASEQ_758995
CYP3073G1	512	probable cytochrome p450 313a2 isoform X1	*M. domestica*	4E-67	215	159	0	25	BPA_5616

(*continued on next page*)

Table 2 (*continued*)

Protein ID	Length[a]	BlastX Hit Information			Expression Counts[b]				Contig ID
		Description	Species	E-value	Untreated		Permethrin Males		
					Female	Male	Alive[c]	+PBO Dead[d]	
CYP3073H1	**508**	probable cytochrome p450 313a4	*M. domestica*	6E-74	260	89	3305[e]	2447	BPA_1033
Glutathione S-transferases									
hiGSTE1	223	PREDICTED: glutathione S-transferase 1-like	*M. domestica*	2E-38	18	28	67	15	BPA_6355
hiGSTE2	**223**	PREDICTED: glutathione S-transferase 1-like	*M. domestica*	5E-38	113	166	608[e]	400	STEP1_C3974
hiGSTE3	**227**	PREDICTED: glutathione S-transferase 1-like	*M. domestica*	6E-98	28	23	93[e]	51	GI=164672134|GB=FD455114.1
hiGSTE4	**223**	PREDICTED: glutathione S-transferase 1	*M. domestica*	1E-105	114	56	100	273	STEP1_C3776
hiGSTE5	**222**	PREDICTED: glutathione S-transferase 1-like	*M. domestica*	4E-97	493	467	2647[e]	2718	STEP1_REP_C3807
hiGSTD1	**208**	PREDICTED: glutathione S-transferase 1	*M. domestica*	4E-97	820	649	1348	1951	STEP1_REP_C419
hiGSTD2	**214**	PREDICTED: glutathione S-transferase 1-1	*M. domestica*	6E-74	16[f]	0	1	0	STEP1_REP_C4544
hiGSTD3	172	PREDICTED: glutathione S-transferase 1-1	*M. domestica*	3E-74	2	16[g]	10	95	STEP1_REP_C4816
hiGSTD4	**215**	glutathione S-transferase 1-1 theta	*Lucilia cuprina*	2E-76	6	0	0	15	BPA_11599
hiGSTD5	204	GST-3	*M. domestica*	6E-90	3	0	0	0	BPA_13668
hiGSTD6	**208**	glutathione S-transferase 2	*M. domestica*	2E-88	52	27	319[e]	92	BPA_3726
hiGSTD7	**211**	glutathione S-transferase 2	*M. domestica*	8E-82	12[f]	2	258[e]	188	BPA_4691
hiGSTO1	**254**	glutathione S-transferase omega 1	*Ceratitis capitata*	3E-120	202	82	163	544	STEP1_C4107
hiGSTT1	**236**	PREDICTED: glutathione S-transferase theta-1	*M. domestica*	1E-106	226	78	475[e]	592	BPA_2484
hiGSTT2	**226**	glutathione S-transferase theta 1	*Delia antiqua*	6E-105	69	41	158	293	STEP1_C69
hiGSTS1	**241**	glutathione S-transferase	*M. domestica*	2E-112	17,843	8490	8839	8594	MALE_454_SRA_171
hiGSTS2[h]	41	glutathione S-transferase sigma 2	*Chilo suppressalis*	1E-11	0	0	0	0	BPA_26_HORNFLY_RNASEQ_628877
hiMAPEG	**150**	PRED. microsomal glutathione S-transferase 1	*M. domestica*	7E-48	219	131	541[e]	589	STEP1_REP_C481

[a] Length in amino acids, entries in bold are putative full-length ORFs.
[b] Total number of raw reads aligning to the assembled transcript in the DSN-treated sequenced library, normalized during the NCGR analysis.
[c] Newly eclosed adult male flies surviving exposure to LD_{25} dose of permethrin for 2 h.
[d] Newly eclosed adult male flies killed by exposure to LD_{25} dose of permethrin + 1% PBO for 2 h.
[e] Contigs with > 4-fold higher expression counts in Permethrin treated Alive Male sample vs. Control Untreated Male sample.
[f] Contigs with > 4-fold higher expression counts in Control Untreated Female sample vs. Control Untreated Male sample.
[g] Contigs with > 4-fold higher expression counts in Control Untreated Male sample vs. Control Untreated Female sample.
[h] This contig was not found in the DSN-treated library read data, but it was present in non-DSN-Treated library sequence data assembled in Supplementary File 2.

Male sample. Both the Georgia and Kerrville Resistant populations showed higher phenotypic pyrethroid resistance in bioassays than the St. Gabriel population and perhaps the enhanced cytochrome P450 6a14-like gene contributes to the enhanced resistance. Because the CYP9 family was overexpressed in pyrethroid resistant mosquitos (Rahman et al., 2021; Mackenzie-Impoinvil et al., 2019), we selected the CYP9F30 transcript for qRT-PCR expression analysis (Fig. 3), as the DSN expression counts showed approximately 15-fold overexpression in the Permethrin-treated males and females (Table 2). Fig. 3 depicting the qRT-PCR showed overexpression of over 20-fold and over 5-fold in the St. Gabriel Permethrin exposed males (Perm Male) and females (Perm Female) compared to the untreated St. Gabriel males (Con Male) and females (Con Female). The overexpression of CYP9F30 in the Kerrville Super Resistant males and females are approximately 4-fold above their corresponding untreated St. Gabriel males and females.

We investigated gene expression in response to feeding in cytochrome P450 6a14-like transcript, comparing the Kerrville Susceptible Reference strain to the Kerrville Super Resistant strain (Fig. 4). Expression in the Super Resistant strain was higher than in the susceptible strain and responsive to feeding in both strains. In the susceptible strain, male and females overexpress the transcript ~10-fold and ~3-fold, respectively, after feeding. In the Super Resistant strain, males and females overexpress 6a14-like transcript ~4-fold and ~3-fold, respectively, after feeding. The unfed flies were less than 24 h emerged from the pupal case and likely required nutrients from the blood meal to fully express their adult complement of transcripts. Fed flies were a sample of mixed post-emergence ages. Since the fed Super Resistant samples showed the strongest expression of the 6a14-like transcript in Fig. 4, we used the fed Super Resistant flies to examine sex- and tissue-specific expression of the 6a14-like transcript, comparing head, thorax, and abdomen expression (Fig. 5). We also included samples from the Con Male and Perm Male samples in this qRT-PCR experiment to test for consistency between the experiment reported in Fig. 2 and this experiment. In our first experiment using 6a14-like transcript (Fig. 2), the Perm Male expression of the 6a14-like transcript was ~7-fold over the Con Male expression. In the tissue-specific expression experiment (Fig. 5), Perm Male was ~9-fold higher than Con Male, documenting reproducibility between the two qRT-PCR experiments. The Super Resistant Female Head showed the highest expression of the cytochrome P450 6a14-like transcript, followed by the Male Thorax, the Male Head, and the Female Thorax. Expression in the male and female abdomens was similar.

Table 3
Esterases expressed in the horn fly transcriptome.

Contig ID	Length[a]	BlastX Hit Information			Expression Counts[b]			
		Description	Species	E-value	Untreated		Permethrin Males	
					Female	Male	Alive[c]	+PBO Dead[d]
BPA_11494	**300**	Palmitoyl-protein thioesterase 1	*Lucilia cuprina*	2E-122	86[e]	0	84[f]	60
BPA_16904	**593**	PREDICTED: esterase B1	*M. domestica*	2E-134	158	177	165	503
STEP1_C3553	**550**	Alpha Esterase-3, Isoform A	*D. melanogaster*	0	206	151	155	495
STEP1_REP_C1115	**351**	PREDICTED: phosphotriesterase-related protein	*M. domestica*	1E-147	218	64	408[f]	434
STEP1_REP_C985	**262**	Putative ubiquitin thioesterase otubain	*L. cuprina*	3E-128	127	39	71	156
BPA_4302	**341**	PRED.: glycerophosphodiester phosphodiesterase 1	*M. domestica*	2E-151	86	39	55	105
BPA_833	**593**	alpha-esterase 5	*Drosophila borborema*	1E-153	151	97	363	303
GI=164673849|GB=FD457649.1| FD457649	**159**	PRED. probable cGMP 3′5′-cyclic phosphodiesterase subunit delta	*M. domestica*	9E-80	176[e]	34	67	120
BPA_446	**573**	PREDICTED: esterase B1	*M. domestica*	0	214	109	1875[f]	1108
BPA_34_HORNFLY_RNASEQ_488007	**400**	PRED. protein phosphate methylesterase 1	*M. domestica*	0	281	186	328	356

[a] Length in amino acids, entries in bold are putative full-length ORFs.
[b] Total number of raw reads aligning to the assembled transcript in the DSN-treated sequenced library, normalized during the NCGR analysis.
[c] Newly eclosed adult male flies surviving exposure to LD_{25} dose of permethrin for 2 h.
[d] Newly eclosed adult male flies killed by exposure to LD_{25} dose of permethrin + 1% PBO for 2 h.
[e] Contigs with > 4-fold higher expression counts in Control Untreated Female sample vs. Control Untreated Male sample.
[f] Contigs with > 4-fold higher expression counts in Permethrin treated Alive Male sample vs. Control Untreated Male sample.

Table 4
St. Gabriel adult transcripts selected for qRT-PCR.

Contig ID[a]	Assigned Name[b]	BlastX Hit Information	Expression Counts[c]				qRT-PCR Approach
			Untreated		Permethrin Males		
			Female	Male	Alive[d]	+PBO Dead[e]	
STEP1_REP_C2171	CYP9F30	Cytochrome P450 9f2 in adult *Drosophila yakuba*	234	266	3802	4078	SYBR
BPA_19806[f]	–	Cytochrome P450 in *M. domestica*	0	0	0	0	SYBR
BPA_896	CYP6GW2 ortholog	Cytochrome P450 in *M. domestica*	355	44	726	261	SYBR
STEP1_REP_C3186	–	NADPH cytochrome P450 reductase in *M. domestica*	4081	1907	9973	9328	SYBR
BPA_735	–	Progesterone receptor membrane component 1 of *Glossina morsitans*	490	205	1249	1434	TaqMan
BPA_1033	CYP3073H1	Cytochrome P450 CYP313A2 in *Drosophila ananassae*	260	89	3305	2447	TaqMan
BPA_14230	CYP6EK4 ortholog	Cytochrome P450 *G. morsitans*	12	21	320	415	TaqMan
STEP1_REP_C589	–	Cytochrome P450 CYP6A37 in *M. domestica*	440	499	3009	3603	TaqMan
STEP1_C4604	–	Cytochrome P450 CYP4G2v1 in *M. domestica*	0	28	0	0	TaqMan
STEP1_REP_C5674	–	Cytochrome P450 6a9 *Drosophila willistoni* GK21808	174	81	330	599	TaqMan
STEP1_REP_C3807	hiGSTE5	GST 6A*M. domestica*	493	467	2647	2718	TaqMan
STEP1_REP_C4816	hiGSTD3	GST 1–1 *Lucilia cuprina*	2	16	10	95	TaqMan
BPA_16883	–	Cytochrome P450 6d1 *M. domestica*	17	0	0	0	TaqMan

[a] Contig identifier in St. Gabriel Pooled Transcriptome of Supplementary File 2.
[b] Name (when available) of translated P450 or GST protein as determined by Dr. Nelson (P450s) or sequence alignments described in Methods section (GSTs).
[c] Total number of raw reads aligning to the assembled transcript in the DSN-treated sequenced library, normalized during the NCGR analysis.
[d] Newly eclosed adult male flies surviving exposure to LD_{25} dose of permethrin for 2 h.
[e] Newly eclosed adult male flies killed by exposure to LD_{25} dose of permethrin +1% PBO for 2 h.
[f] This contig was not found in the DSN-treated library read data, but it was present in non-DSN-Treated library sequence data assembled in Supplementary File 2.

3.3. Comparative metabolic gene expression analysis in susceptible vs. highly resistant Georgia flies

To focus our study of gene expression to highly pyrethroid resistant horn flies, we used TaqMan-based qRT-PCR to compare gene expression in the Kerrville Susceptible Reference population with the highly pyrethroid resistant Georgia population, looking at 6 cytochrome P450s, 2 GSTs, and a non-metabolic protein, the Progesterone Receptor Membrane Component 1-like transcript (PGRMC1). The optimal set of reference genes in the TaqMan analyses was identified by NormFinder to include the horn fly 18S, beta-actin, and GAPDH for the CYP3073H1 and PGRMC1 experiments depicted in Supplementary Fig. 1, while β-actin and PGK were the optimal reference genes for the other targets depicted in Figs. 6 and 7. The efficiencies of the reactions were not all within our acceptable range of 90–100% and results from the less efficient reactions should be interpreted with that caveat. For the target transcripts P450 6a9-like, CYP6EK4, P450 4g1-like, P450 6a21-like, P450 6d1-like, GSTD3, GSTE5, CYP3073H1, and PGRMC1, the efficiencies were 87%, 97%, 85%, 89%, 81%, 91%, 78%, 100%, and 97%, respectively. Expression profiles of CYP3073H1 and PGRMC1 are consistent across the 5 samples which include the pyrethroid susceptible Kerrville Susceptible Reference (Sus), highly pyrethroid resistant Georgia and

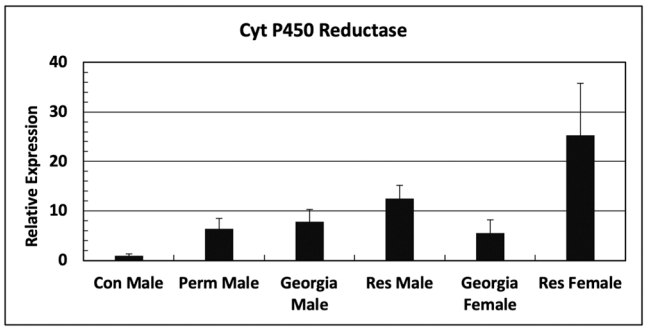

Fig. 1. qRT-PCR SYBR analysis of P450 reductase transcript in horn fly populations with varying degrees of pyrethroid resistance. Total RNA was extracted from 1 g samples of adult flies from the St. Gabriel Control Untreated Males (Con Male) and Permethrin-Treated Alive Males (Perm Male), Georgia males and females, and Kerrville pyrethroid resistant colony males (Res Male) and females (Res Female) with target transcript of cytochrome P450 reductase (Contig STEP1_REP_C3186 in Supplementary File 2). The horn fly 18S RNA was used as reference for normalization. Results are plotted as relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 5.

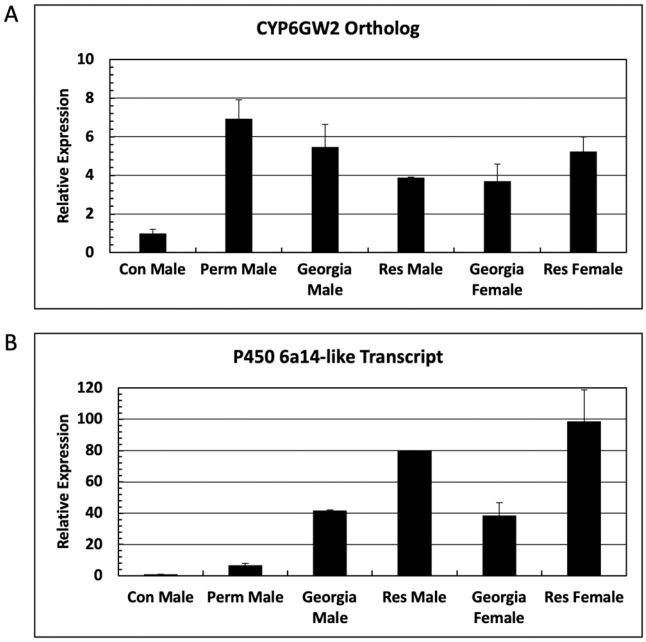

Fig. 2. qRT-PCR SYBR analysis of CYP6GW2 ortholog and P450 6a-14-like transcripts in horn fly populations with varying degrees of pyrethroid resistance. Total RNA was extracted from 1 g samples of adult flies from the St. Gabriel Control Untreated Males (Con Male) and Permethrin-Treated Alive Males (Perm Male), Georgia males and females, and Kerrville pyrethroid resistant colony males (Res Male) and females (Res Female) with target transcript of A: CYP6GW2 (Contig BPA_896 in Supplementary File 2); and B: cytochrome P450 6a-14-like (BPA_19806 in Supplementary File 2). The horn fly 18S RNA was used as reference for normalization. Results are plotted as relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 5.

Kerrville Super Resistant (SuperRes), and the organophosphate resistant LSU Rosepine samples (Supplementary Fig. 1).

The cytochrome P450 4g1-like transcript is involved in cuticular hydrocarbon biosynthesis in a coexpression complex that includes cytochrome P450 reductase (Qiu et al., 2012). The 4g1-like transcript is expressed at a similar level in both the Susceptible and Georgia flies (Fig. 6B). Expression counts of 4g1-like transcript was also quite low in

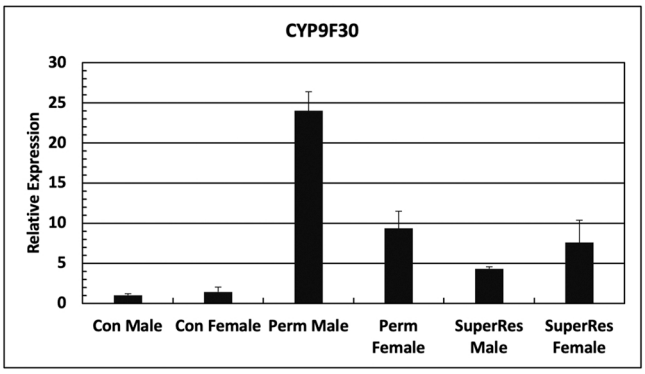

Fig. 3. qRT-PCR SYBR analysis of CYP9F30 transcript in horn fly populations with varying degrees of pyrethroid resistance. Total RNA was extracted from 1 g samples of adult flies from the St. Gabriel Control Untreated Males (Con Male) and females (Con Female), Permethrin-Treated Alive Males (Perm Male) and females (Perm Female), and Kerrville pyrethroid Super Resistant colony males (SuperRes Male) and females (SuperRes Female) with target transcript of CYP9F30 (STEP1_REP_C2171 in Supplementary File 2). The horn fly 18S RNA was used as reference for normalization. Results are plotted as relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 5.

the Control Male and Control Female DSN (Table 4) and non-DSN adult fly transcriptomes (Contig STEP1_C4604 in Supplementary File 4). In contrast to the other cytochrome P450s in our gene expression analysis, the cytochrome P450 6d1-like transcript expression is 7-fold upregulated in the Kerrville Susceptible compared to the Georgia population (Fig. 6B).

We found 18 transcripts encoding putative GSTs (Table 2, Supplementary File 4). We selected GSTD3 and GSTE5 for gene expression analysis in the Kerrville Susceptible and Georgia populations (Fig. 7). While the GSTE5 result showed higher expression in the resistant Georgia flies, the poor qRT-PCR efficiency (78%, Supplementary File 6) precludes definitive assignment of a role in resistance. In the expression counts of the 4 St. Gabriel transcriptomes (Table 2), GSTE5 showed 5.7-fold overexpression in the permethrin-treated males compared to the untreated males. The GSTD3 expression was similar in both the Kerrville Susceptible and Georgia fly populations.

4. Discussion

With our goal of discovering a comprehensive set of horn fly metabolic enzyme transcripts, we choose to use the St. Gabriel horn fly population for this study due to Domingues et al. (2019) documentation of it having low target site-mediated pyrethroid resistance alleles for *super-kdr* and *kdr*. The PBO synergist bioassays verified a moderate level of pyrethroid resistance that was due almost entirely to PBO-suppressible metabolic resistance. We identified multiple P450 sequences (Table 2), which are members of the CYP 4, 6, and 9 families. Scharf et al. (2001) found three members of the CYP4 family were inducible and overexpressed in a carbamate and organophosphate resistant population of the western corn rootworm. The CYP6 family in *Anopheles gambiae* has been found to contain members that are able to metabolize pyrethroids (David et al., 2013), and these insecticides are a primary control method used to reduce adult horn fly infestations of cattle. However, the role of cytochrome P450s in resistant insect populations is complex, as Yang and Liu (2011) discovered both up- and down-regulated P450s in permethrin resistant *Culex quinquefasciatus* larvae and adults that were exposed to permethrin.

We performed metabolic gene expression analysis comparison between our St. Gabriel adult fly transcriptomes with the objective to identify putative male- or female-specific P450s, GSTs, or metabolic esterases and to identify P450s, GSTs, or esterases that were inducible by

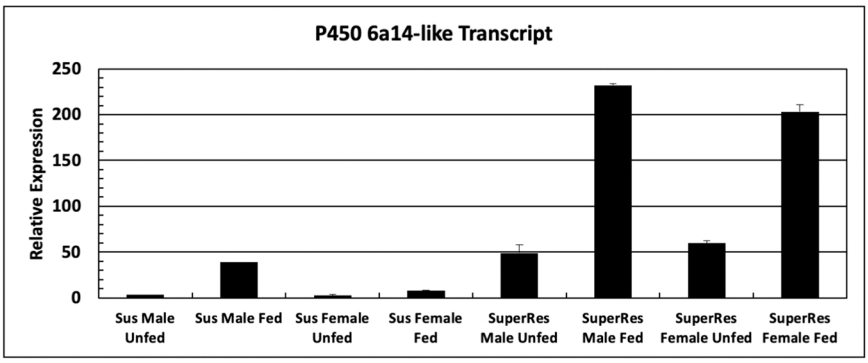

Fig. 4. qRT-PCR SYBR analysis of cytochrome P450 6a-14-like transcript expression in unfed vs. fed adult horn fly. Total RNA was extracted from 1 g samples of adult flies from Kerrville susceptible reference unfed males (Sus Male Unfed) and unfed females (Sus Female Unfed), Kerrville susceptible reference fed males (Sus Male Fed) and fed females (Sus Female Fed), Kerrville pyrethroid Super Resistant colony unfed males (SuperRes Male Unfed) and unfed females (SuperRes Female Unfed), and Kerrville pyrethroid Super Resistant colony fed males (SuperRes Male Fed) and fed females (SuperRes Female Fed), targeting the cytochrome P450 6a-14-like transcript (BPA_19806 in Supplementary File 2). The horn fly 18S RNA was used as reference for normalization. Results are plotted as relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 5.

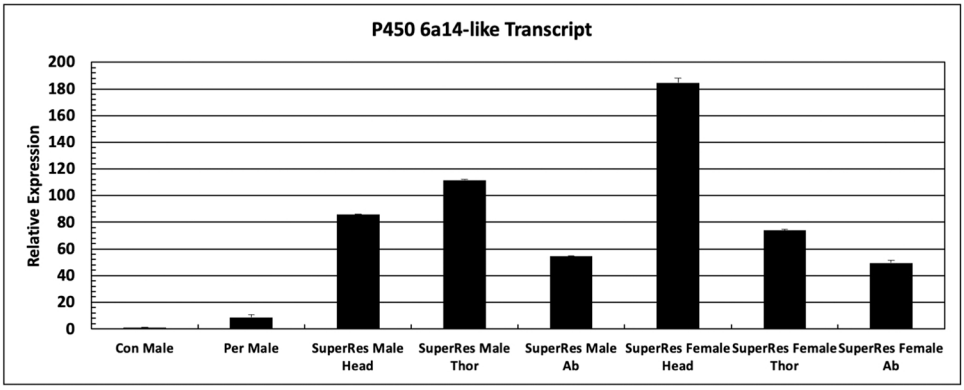

Fig. 5. qRT-PCR SYBR analysis of cytochrome P450 6a-14-like transcript expression in various tissues of adult horn fly. Total RNA was extracted from 1 g samples of adult flies from the St. Gabriel Control Untreated Males (Con Male), Permethrin-Treated Alive Males (Per Male), Kerrville pyrethroid Super Resistant colony dissected male heads (SuperRes Male Head), thoraxes (SuperRes Male Thor), and abdomens (SuperRes Male Ab) and dissected female heads (SuperRes Female Head), thoraxes (SuperRes Female Thor), and abdomens (SuperRes Female Ab), targeting the cytochrome P450 6a-14-like transcript (BPA_19806 in Supplementary File 2). The horn fly 18S RNA was used as reference for normalization. Results are plotted as relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 5.

pyrethroid treatment and might play a role in surviving pyrethroid exposure (Control Untreated Male vs. Permethrin-Treated Alive Males). Additionally, transcripts that were upregulated by pyrethroid exposure in the Permethrin-Treated Alive Male sample but not upregulated in the Permethrin + PBO Dead Males would also be of interest. PBO acts as an inhibitor of P450 enzymatic activity and PBO suppression of pyrethroid resistance is indicative of metabolic P450-based resistance. We must acknowledge and reiterate the lack of biological replicates in our RNA-Seq design limits the power of our gene expression study data for quantitative analysis. Resource limitations necessitated that research direction choice. However, we judiciously used the expression analysis to guide our selection of transcripts for further study by qRT-PCR.

Our results were consistent with those of Shi et al. (2015), cytochrome P450 reductase is important in the regulation activities of cytochrome P450s and resistance to fenopropsthrin; additionally, Adesanya et al. (2020) showed that cytochrome P450 reductase was over-expressed in pyrethroid resistant two-spotted spider mites, and inhibition of cytochrome P450 reductase expression by RNAi restored pyrethroid sensitivity to these resistant populations. Although, varying expression levels of cytochrome P450 reductase could also reflect temporal and spatial differences in the type of pyrethroid and selection pressure experienced by the different populations, horn fly cytochrome P450 reductase might be a fruitful target for further study to develop novel pesticides.

The focus of our gene expression study of highly pyrethroid resistant horn flies used qRT-PCR to compare gene expression in the Susceptible Reference population from Kerrville with a highly pyrethroid resistant population from Georgia by looking at 6 cytochrome P450s, 2 GSTs, and a non-metabolic protein, the Progesterone Receptor Membrane Component 1-like transcript (PGRMC1). Interestingly, adult flies emerging from manure treated with an LD25 dose of the organophosphate diazinon (Rosepine Diazinon Alive) expressed the highest mean level of CYP3073H1 transcript. The presence of metabolic resistance to organophosphates in Rosepine flies has not been shown, although target site organophosphate resistance in the Rosepine horn fly population was reported by Foil et al. (2010). In this study, we did not quantify Rosepine fly gene expression of other P450s, but further expression analyses for Rosepine P450s might clarify if cytochrome P450s are involved in the Rosepine organophosphate resistance mechanism. Fig. 6 shows that the expression level of cytochrome P450 6a9-like transcript was much higher than the other transcripts. By comparison to the Kerrville Susceptible population (Fig. 6A), the ~32-fold higher expression of 6a9-like transcript in the highly pyrethroid resistant Georgia population implies a role in the P450-based resistance component of this population. However, the efficiency of this qRT-PCR experiment (87%) was outside of our 90–100% acceptable range and the standard error was high. CYP6EK4 and cytochrome P450 6a21-like transcripts were also expressed at higher levels in the highly pyrethroid resistant Georgia sample compared to the Kerrville Susceptible sample (Fig. 6B), though the expression levels in the Georgia sample of both transcripts was much lower than the 6a9-like transcript expression in Fig. 6A. RNAi or more statistically powerful P450 gene expression studies could provide more conclusive evidence of the role of the 6a9-like transcript in pyrethroid resistance.

A cytochrome P450 4g1-like transcript was identified and has been shown to be involved in cuticular hydrocarbon biosynthesis in a coexpression complex that includes cytochrome P450 reductase (Qiu et al., 2012). The *Anopheles gambiae* homologue to *D. melanogaster* CYP4G1 is also involved in the mechanism of pyrethroid resistance among West Africa mosquito populations by synthesizing a less penetrative cuticular

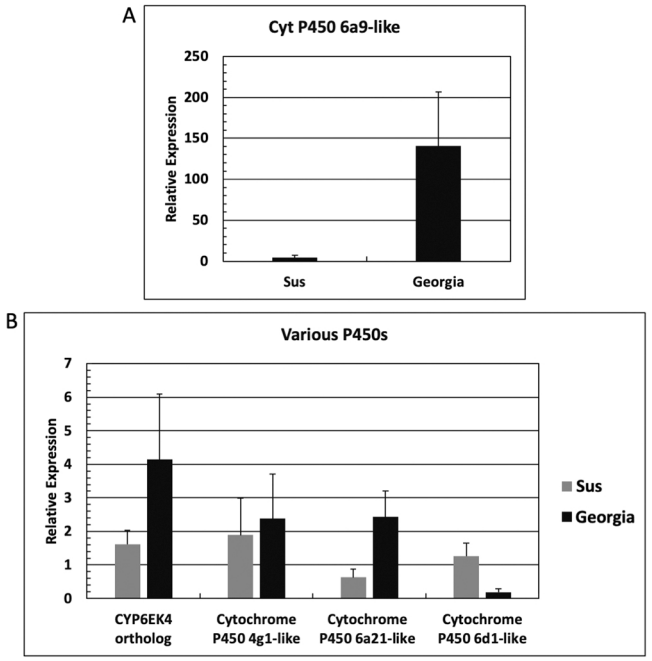

Fig. 6. qRT-PCR TaqMan analyses of expression of various putative cytochrome P450 transcripts in pyrethroid susceptible and resistant adult horn flies. Total RNA was isolated from a pool of 10 male and 10 female flies from the Kerrville susceptible reference colony (Sus, in light gray) and the Georgia (in black) population, targeting A: cytochrome P450 6a9-like (STEP1_REP_C589 in Supplementary File 2); and B: CYP6EK4 ortholog (BPA_14230 in Supplementary File 2), cytochrome P450 4g1-like (STEP1_C4604 in Supplementary File 2), cytochrome P450 6a21-like (STEP1_REP_C5674 in Supplementary File 2), and cytochrome P450 6d1-like (BPA_16883 in Supplementary File 2) transcripts. Beta-actin and phosphoglycerate kinase were used as references for normalization in these experiments and results are plotted as the relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 6.

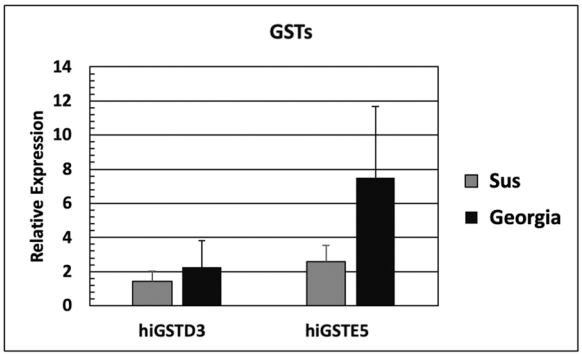

Fig. 7. qRT-PCR TaqMan analyses of expression of two GSTs in pyrethroid susceptible and resistant adult horn flies. Total RNA was isolated from a pool of 10 male and 10 female flies from the Kerrville susceptible reference colony (Sus, in light gray) and the Georgia (in black) population, targeting hiGSTD3 (STEP1_REP_C4816 in Supplementary File 2), and hiGSTE5 (STEP1_REP_C3807 in Supplementary File 2) transcripts. Beta-actin and phosphoglycerate kinase were used as references for normalization in these experiments and results are plotted as the relative expression means with error bars indicating standard error of the mean. Raw data are in Supplementary File 6.

barrier (Balabanidou et al., 2016). The 4g1-like transcript was expressed at a similar level in both the susceptible (Kerrville) and resistant (Georgia) populations (Fig. 6B), perhaps reflecting that high expression is not needed at the adult fly life stage of the horn fly. While thickening of the cuticle via cytochrome P450 4g1 activity might contribute to resistance by diminishing translocation into the hemocele, the qRT-PCR data does not support this transcript as having a major role in the resistance mechanism of the Georgia population. In contrast to the other cytochrome P450s in our gene expression analysis, the cytochrome P450 6d1-like transcript expression is 7-fold upregulated in the Kerrville Susceptible compared to the Georgia population (Fig. 6B). Although unexpected, this cytochrome P450 may play a role in the Kerrville laboratory-reared population that the Georgia field population does not possess. For example, the Kerrville population is reared in cages, not on cattle, and feed upon citrated bovine blood. The added citrate has been shown to have an optimum concentration above which colony productivity and fly survival is affected (Guerrero et al., 1993). A cytochrome P450 such as 6d1-like transcript might be required to reduce toxicity in citrated blood.

While our study focused upon acquiring the adult fly transcriptome of a natural population, it is useful to examine putative metabolic enzyme-encoding gene loci predicted from the horn fly genome (Konganti et al., 2018). This genome is from the Kerrville Susceptible laboratory-reared population described above. Table 5 compares the number of gene loci with annotation from cytochrome P450, esterase, or GST families to the number of corresponding unique annotated transcripts from our study. Annotations to cytochrome P450 families 4 and 6 and carboxylesterase B-1 similarly predominate both data sets. However, the GSTs from our adult fly transcriptome contain more sequences from GST families D and E and less from family T compared to the annotated gene loci data set derived from the Kerrville Susceptible egg DNA genome. Differences might be due to different tissue source (adult fly vs. egg) used in the protocols, differing food source (bovine host on a ranch vs. citrated blood-soaked cotton pad), or different selection pressures upon the population (natural vs. laboratory environment), Guerrero and Kunz (2000) reported on gene expression differences between Kerrville Susceptible laboratory colony adult flies and adult flies in natural conditions.

Li et al. (2009) published synergist studies that indicated a role for GSTs in pyrethroid resistant horn flies from a field population in Texas. However, they found significant components of target site- and P450-mediated pyrethroid resistance and the GST role in this population's pyrethroid resistance was deemed of lesser importance. The expression counts of the St. Gabriel transcriptomes shown in Table 2, GSTE5 showed 5.7-fold overexpression in the permethrin-treated males compared to the untreated males, which is further indication that this GST may have a role in horn fly pyrethroid resistance while GSTD3 had similar expression across populations, indicating little if any role in pyrethroid resistance.

While we presented evidence from transcriptome expression counts (Table 2, Supplementary File 4) and qRT-PCR (Figs. 1–7, Supplementary

Table 5
Tally of predicted cytochrome P450, esterase, and GST gene loci in the horn fly genome with our adult fly transcriptome.

Metabolic Enzyme Type	This Study	Genome
Cytochrome P450 Family		
4	27	48
6	52	69
9	6	6
Carboxylesterase		
B-1	7	20
E3	1	0
E4	1	1
E5	1	4
E7	1	0
GST Family		
D	7	1
E	5	1
O	1	2
S	2	0
T	2	14

Files 5 and 6) of enhanced cytochrome P450, cytochrome P450 reductase, and GST transcript expression, proof that overexpression directly leads to pyrethroid resistance will require further studies. Our expression counts for the 4 St. Gabriel transcriptomes are based upon a single replication of the population sampling, RNA purification, and transcriptome sequencing. In addition, the BUSCO results showed 32.4% missing from our pooled St. Gabriel adult fly transcriptome. Domingues et al. (2019) reported BUSCO results from the 4 individual St. Gabriel adult fly transcriptomes that we sourced and pooled for our research. Their reported BUSCOs from the St. Gabriel population untreated Control Male, untreated Control Female, Permethrin treated alive Male, and Permethrin + PBO treated killed Male samples showed 59.9%, 40.9%, 94.9%, and 79.1% missing, respectively. While our pooled transcriptome is still missing about a third of the BUSCOs, our pooling strategy produced an improved BUSCO compared to the individual transcriptomes. The flies that were used in each St. Gabriel sample were newly emerged and unfed, and both feeding and development have been reported to changes in translatable RNA of the horn fly (Guerrero, 1995). Further transcriptome sequencing studies are underway utilizing multiple sample sources and biological replicates from each sampled population to allow proper statistical analysis of expression counting algorithm outcomes. Additional pyrethroid resistant populations included in this forthcoming and expanded horn fly gene expression analysis will help ascertain if transcript expression of each P450 and GST are specific to one or a few populations or more general to all or most. Of the 136, 21, and 18 sequences annotated as putative cytochrome P450s, metabolic esterases, and GSTs, respectively (Supplementary File 4), we only tested 6 cytochrome P450s, 2 GSTs, and cytochrome P450 reductase by qRT-PCR. Despite considerable effort, some of our TaqMan PCR efficiencies were not within acceptable range (90–100%) and many of the reactions had wide standard errors. Nevertheless, the qRT-PCR results indicated cytochrome P450 reductase, CYP6GW2, CYP9F30, cytochrome P450 6a14-like, 6a9-like, and 6a21-like transcripts were overexpressed in horn flies sampled from various pyrethroid resistant population samples compared to pyrethroid susceptible populations.

Mixed-function oxidase-based resistance was reported to be the most important mechanism in pyrethroid resistant horn flies in Brazil (Barros et al., 2012). Previously, metabolic enzyme-based insecticide resistance was identified with synergist bioassays, usually using PBO, a chemical known to inhibit cytochrome P450 activity and often an added ingredient to insecticide formulations. The activity of PBO is not restricted to cytochrome P450s, but populations with pyrethroid resistance that is partially or completely eliminated by PBO are often classified as mixed function oxidase- or cytochrome P450-based resistant. Sheppard (1995) used PBO synergist bioassay results as evidence to propose the very high pyrethroid resistance in the Georgia horn fly population (600–12, 000-fold above susceptible flies) was based on a mixed function oxidase mechanism. We used archived samples of those Georgia horn flies in our qRT-PCRs (Figs. 1, 2, 6, and 7) and found various elevated cytochrome P450 transcript expression compared to expression in pyrethroid susceptible horn flies. Li et al. (2007, 2009) used PBO in synergist studies to identify mixed function oxidase-based mechanisms in both Texas and Oklahoma populations of horn flies. Our studies were designed to identify specific cytochrome P450s that might have a role in mixed function oxidase-based pyrethroid resistance. We have discovered individual P450 transcripts that are overexpressed in pyrethroid resistant horn flies. While acknowledging that cytochrome P450 enzymes are responsible for the oxidase activity and not the transcript and that transcript concentration is not indicative of protein concentration, several of these P450 transcripts are worthy of further study to better understand pyrethroid resistance in horn flies. Gene expression can also be influenced by a number of factors, including environment, circadian timing, development, and feeding. The effects of these factors must be teased out by proper experimental design to establish the roles of specific transcripts in resistance to pyrethroids in the horn fly.

Ethics approval

Not applicable.

CRedit author contribution statement

Kylie Bendele: Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Deanna Bodine:** Validation, Formal analysis, Investigation, Writing – review & editing. **Qiang Xu:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Lane Foil:** Conceptualization, Resources, Writing – review & editing. **Connor Cameron:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Andrew Farmer:** Methodology, Resources, Writing – review & editing. **Ernie Retzel:** Conceptualization, Methodology, Resources. **Victoria Moore:** Investigation. **Kimberly Lohmeyer:** Writing – review & editing, Funding acquisition. **Adalberto Perez de Leon:** Writing – review & editing, Funding acquisition. **Felix Guerrero:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2022.109699.

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