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Use of isogenic strains indicates *CYP9M10* is linked to permethrin resistance in *Culex pipiens quinquefasciatus*

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Abstract

Previous studies on a strain of *Culex pipiens quinquefasciatus* from Saudi Arabia indicated permethrin resistance was a result of cytochrome P450 mediated detoxification and *kdr*. The P450 detoxification was found to be larval specific and associated with a fitness cost in certain environments. The P450 responsible for resistance (and the fitness cost) has not been identified, but recently two candidate P450s (*CYP4H34* and *CYP9M10*) have been found. We measured cytochrome P450 and cytochrome b_5 content as well as the expression levels of *CYP4H34* and *CYP9M10* in a susceptible (SLAB) and two isogenic strains (isolated by repeated backcrossing and selection) of mosquito (ISOP450 and ISOJPAL) resistant to permethrin. Cytochrome P450 protein levels of the resistant strains were significantly higher (1.5-fold) than SLAB, but were not significantly different from one another. Expression of *CYP4H34* in the larvae and adults of the resistant (ISOP450 and ISOJPAL) and susceptible (SLAB) strains were not statistically different. *CYP9M10* was found to be significantly over-expressed in larvae of both permethrin-resistant isogenic strains (1800-fold in ISOP450 and 870-fold in ISOJPAL) when compared to SLAB. Partial sequence analysis of *CYP9M10* revealed eight polymorphic sites that distinguished the susceptible allele from the resistant allele. We conclude that *CYP9M10* is linked to permethrin resistance in these strains of *C. p. quinquefasciatus*, and is likely to be the P450 gene responsible for resistance in these strains.

Keywords: cytochrome P450, insecticide resistance, Insecta, mosquito.

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Introduction

Cytochrome P450 monooxygenases are one of the primary enzymatic systems involved in conferring resistance to pyrethroid insecticides. P450-mediated resistance occurs as a result of the over-expression of one or more P450 genes, principally because of increased transcription (Liu & Scott, 1998) as opposed to gene amplification [which is common for esterase-based resistance to organophosphates (Tomita *et al*., 1995; Paton *et al*., 2000; Nikou *et al*., 2003; Wheelock *et al*., 2005)] or mRNA stabilization (Liu & Scott, 1998). Mutations in a P450 gene that result in changes in the P450 amino acid sequence (leading to a P450 with greater metabolism of the insecticide) may also be involved in resistance (Lindberg & Negishi, 1989; Amichot *et al*., 2004).

Owing to the enormous consequences of mosquito borne diseases, insecticides have frequently been used to control these pests. This has frequently led to the evolution of insecticide resistance. Studies aimed at identification of the P450s that confer resistance to insecticides in mosquitoes are relatively recent. In *Anopheles gambiae*, *CYP6Z1* was found to be over-expressed (Nikou *et al*., 2003) and able to metabolize dichlorodiphenyltrichloroethane (DDT; Chiu *et al*., 2008) in a permethrin-resistant strain from Kenya. Also in *An. gambiae*, *CYP6P3* was found to be over-expressed in bioassay survivors and shown to metabolize permethrin and deltamethrin (Muller *et al*., 2008). In *Anopheles funestus*, *CYP6P4* and *CYP6P9* have been found to be over-expressed in resistant strains (Amenya *et al*., 2008; Wondji *et al*., 2009). *CYP6M2* is over-expressed in field collected *An. gambiae* (Djouaka *et al*., 2008a), and three CYP9 P450s are over-expressed in field collected *Aedes aegypti* (Strode *et al*., 2008), but the role of these P450s in resistance remains unclear.

In the JPal-per (JPAL) strain of *Culex pipiens quinquefasciatus* (Amin & Hemingway, 1989), permethrin resistance is a result of *kdr* and P450 mediated detoxification (Kasai *et al*., 2000). The P450 mechanism is larval-specific, monofactorially inherited, autosomally linked and incompletely dominant (Hardstone *et al*., 2007). In addition, this mechanism has an environmentally variable fitness cost (Hardstone *et al*., 2009) associated with slower female emergence time and lower energy reserves (glycogen and lipids; Hardstone *et al*., 2010). Throughout these previous studies, the P450 responsible for the permethrin resistance has not been known, and therefore has not been directly measured. Expression of *CYP6F1* was assessed by Northern blotting and found to be over-expressed in JPAL compared to the susceptible strain. *CYP6F1* and *CYP6E1* were subsequently cloned from JPAL, but further analysis did not implicate them as candidate genes conferring pyrethroid resistance (Kasai *et al*., 2000). Using a microarray analysis of 46 *Culex* P450 genes, *CYP9M10* and *CYP4H34* were found to be over-expressed in the permethrin-resistant JPAL strain (Komagata *et al*., 2010).

In the present study, two candidate P450 genes previously found to be over-expressed in the highly permethrin-resistant JPAL strain (when compared to an unrelated susceptible strain, Ogasawara; Komagata *et al*., 2010) were analysed by quantitative real-time PCR (qPCR) to determine the level of over-expression in two resistant strains, ISOP450 and ISOJPAL when compared to an isogenic susceptible strain, SLAB. Owing to the repeated backcrossing (and selection) used to produce these isogenic strains, over-expression of a specific P450 (or identification of a P450 allele found only in the resistant strains) would indicate this trait was at the resistance locus. The two resistant strains (ISOP450 and ISOJPAL) were also measured for total cytochrome P450 and cytochrome b_5 content relative to the susceptible strain (SLAB). We examined the expression pattern of *CYP4H34* and *CYP9M10* in the larvae and adults of the isogenic permethrin-resistant strains to determine which gene conforms to the patterns observed in previous studies [larval-specific expression (Hardstone *et al*., 2007) and fitness associated genotype frequencies (Hardstone *et al*., 2009)]. Herein, our results (qPCR analysis and genotyping) indicate that *CYP9M10* is genetically linked to permethrin resistance, as a result of both the high levels of expression in larvae (of both the parental and isogenic strains) and the correlation of the resistance allele frequency to the fitness genotype frequencies previously observed using bioassays (Hardstone *et al*., 2009).

Results

Construction of ISOJPAL

The JPAL and SLAB strains were crossed and after five generations of backcrosses and permethrin selection, followed by an additional four generations of permethrin selection, we isolated a resistant strain of *C. p. quinquefasciatus* called ISOJPAL (Table 1). Larvae of ISOJPAL treated with permethrin were 13 000-fold resistant relative to SLAB and when treated with permethrin +

Table 1. Construction and selection of the permethrin resistant ISOJPAL strain of *Culex pipiens quinquefasciatus*

Backcrossing scheme – each generation treated with 0.02 μg/ml permethrin				
Cross (female × male)	Generation (% introgression)	% mortality	*n**	*Vssc* allele frequencies†
SLAB × JPAL	F_1 (50)			
SLAB × F_1	BC1 (75)			
SLAB × BC1	BC2 (87.5)	78	3240	TTT 0/9, TTT/A 7/9, TTA 2/9
SLAB × BC2	BC3 (93.8)	50	2560	TTT 0/8, TTT/A 3/8, TTA 5/8
SLAB × BC3	BC4 (96.9)	57	639	TTT 0/10, TTT/A 2/10, TTA 8/10
SLAB × BC4	BC5 (98.4)	50	2780	TTT 0/10, TTT/A 3/10, TTA 7/10
Selection scheme				
Generation	Concentration of permethrin (μg/ml)	% mortality	*n**	*Vssc* allele frequencies†
BC5 selection no. 1	0.1	52	3960	TTT 0/10, TTT/A 2/10, TTA 8/10
	1.0	94	1480	
BC5 selection no. 2	1.0	59	1780	TTT 3/8, TTT/A 2/8, TTA 3/8
BC5 selection no. 3	17	85	2453	TTT 10/10, TTT/A 0/10, TTA 0/10
BC5 selection no. 4	30	68	3980	TTT 10/10, TTT/A 0/10, TTA 0/10

*Number of fourth instar larvae treated in bioassay.
†*Vssc* genotypes; TTT, homozygous resistant (*kdr*); TTT/A, heterozygous; TTA, homozygous susceptible.

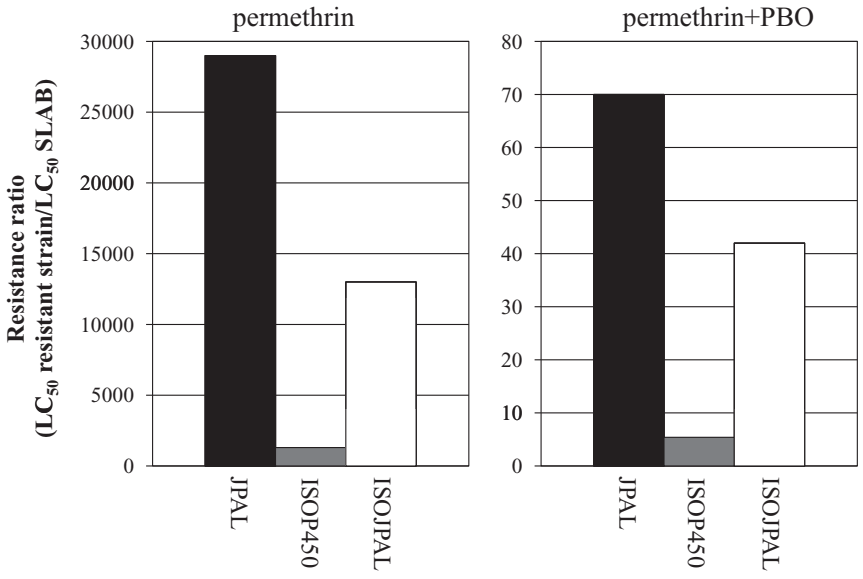

Figure 1. Resistance to permethrin and permethrin + piperonyl butoxide (PBO) in fourth instar larvae of permethrin resistant laboratory strains (JPAL, ISOP450 and ISOJPAL) relative to the laboratory susceptible (SLAB) strain of *Culex pipiens quinquefasciatus*. LC_{50}, concentration lethal to 50% of the population.

piperonyl butoxide (PBO), resistance decreased to 42-fold (Fig. 1). Thus, ISOJPAL contained both PBO suppressible and non-PBO suppressible resistance mechanisms. *Vssc* sequence data obtained from genomic DNA (gDNA) indicated that the ISOJPAL strain was homozygous for *kdr* (*n* = 10/10). Therefore, ISOJPAL is highly related to SLAB (98.4%), but contains the resistance mechanisms found in the parental JPAL strain (*kdr* and P450 detoxification).

Cytochrome P450 and cytochrome b_5 protein content in SLAB, ISOP450 and ISOJPAL

Total P450 levels were significantly higher (1.5-fold) in ISOP450 and ISOJPAL as compared to SLAB ($F = 6.52$; df = 2,6; $P = 0.03$) (Table 2). This is consistent with P450-mediated detoxification being a mechanism of resistance in these strains (Scott, 1999). Cytochrome b_5 levels were not statistically different amongst the three strains ($F = 0.742$; df = 2,6; $P = 0.52$).

qPCR

qPCR revealed that the expression of *CYP9M10* was significantly increased by 1 800 ± 320-fold and 870 ± 140-fold in fourth instar larvae of ISOP450 and ISOJPAL, respectively, compared with SLAB ($F = 29.8$; df = 2,16; $P < 0.0001$) (Fig. 2). ISOP450 (12 ± 1.3-fold) and ISOJPAL (10 ± 4.1-fold) 3-day-old adults had slightly, but significantly ($F = 11.2$; df = 2,16; $P = 0.001$) higher expression of *CYP9M10* versus SLAB 3-day-old adults (2.35 ± 0.44-fold) (Fig. 2). Thus, over-expression of *CYP9M10* is linked to permethrin resistance in *C. p. quinquefasciatus*.

No significant differences in expression of *CYP4H34* between SLAB and the resistant strains in fourth instar larvae ($F = 3.24$; df = 2,13; $P = 0.07$) or 3-day-old adult ($F = 1.12$; df = 2,13; $P = 0.35$) were observed (Fig. 3). Therefore, over-expression of *CYP4H34* is not linked to permethrin resistance in ISOP450 or ISOJPAL. Relative to the susceptible inter-plate calibrator sample, *CYP4H34* expression levels of SLAB, ISOP450 and ISOJPAL fourth instar larvae were 0.77 ± 0.12-fold, 0.95 ± 0.16-fold and 1.33 ± 0.10-fold, respectively, and 3-day-old adults were 1.33 ± 0.14, 1.63 ± 0.18 and 1.62 ± 0.25, respectively. Results obtained for expression levels of *CYP9M10* and *CYP4H34* match those found by semi-quantitative reverse transcription-PCR (Hardstone, 2009).

Table 2. Cytochrome P450 and cytochrome b_5 content in susceptible (SLAB) and resistant (ISOP450 and ISOJPAL) strains of *Culex pipiens quinquefasciatus*. Values are the mean ± SEM of three replicates (200 fourth instar larvae midguts/replicate). Different letters indicate significant differences ($P < 0.05$) between the strains within each type of protein

Strain	Cytochrome P450 (nmol/mg protein)	P450 content relative to SLAB	Cytochrome b_5 (nmol/mg protein)	b_5 content relative to SLAB
SLAB	0.500 ± 0.046 a	1.0	0.378 ± 0.019 A	1.0
ISOP450	0.753 ± 0.081 b	1.5	0.315 ± 0.030 A	0.8
ISOJPAL	0.745 ± 0.029 b	1.5	0.312 ± 0.066 A	0.8

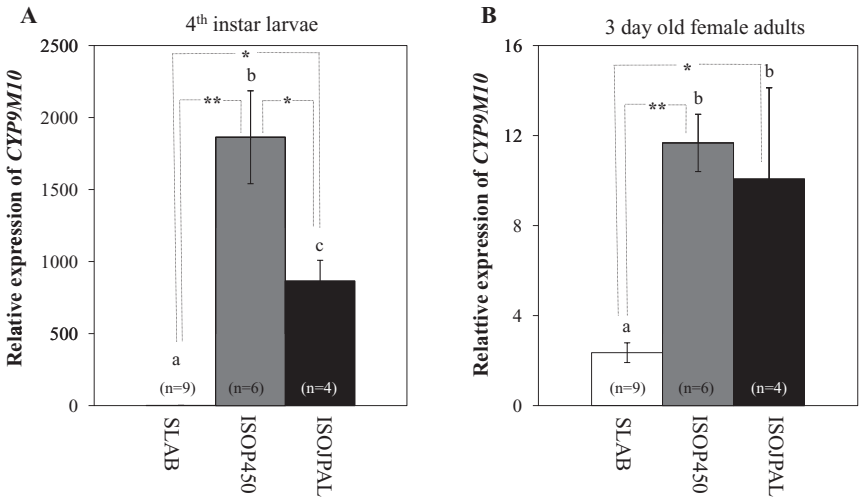

Figure 2. Expression of *CYP9M10* mRNA in (A) fourth instar larvae and (B) 3-day-old female adults relative to calibrator samples in susceptible (SLAB) and resistant (ISOP450 and ISOJPAL) strains of *Culex pipiens quinquefasciatus*. Values are the mean ± SEM ($n = 4$ to 9 biological samples as indicated) and significant differences between strains within life stages were determined using Tukey's honestly significant difference test (*$P \leq 0.05$; **$P \leq 0.01$). CYP, cytochrome P450.

Sequences of CYP9M10 *and* CYP4H34

The partial gDNA sequences of *CYP9M10* revealed two alleles that differed at eight sites. One allele was found exclusively in the susceptible strain (*CYP9M10v1*, accession no. GU974336) and the other allele was found exclusively in the resistant strains (*CYP9M10v2*: ISOP450, accession no. GU974337; and ISOJPAL accession no. GU974338) (Fig. 4). Single nucleotide polymorphisms (SNPs) between susceptible and isogenic resistant strains were detected at positions 226, 241 and 262 within the intron. Synonymous SNPs were present at positions 33 (T to C), 288 (C to T) and 390 (A to G). Non-synonymous SNPs were at positions 96 (T to A) and 134 (C to T). To examine if the frequency of the *CYP9M10v2* allele mirrored the frequency of resistant phenotypes measured (with an insecticide bioassay) in previous fitness costs experiments (Hardstone *et al*., 2009), we genotyped mosquitoes (stored from previous experiments in the freezer) and compared them to the previous results (Hardstone *et al*., 2009). Individual larvae from two different treatment groups (standard laboratory conditions and temephos treated environment) and generations were evaluated (Table 3). No significant differences were observed (standard laboratory environment $\chi^2 = 0.120$, $P = 0.94$; temephos exposed environment $\chi^2 = 0.031$, $P = 0.98$) between

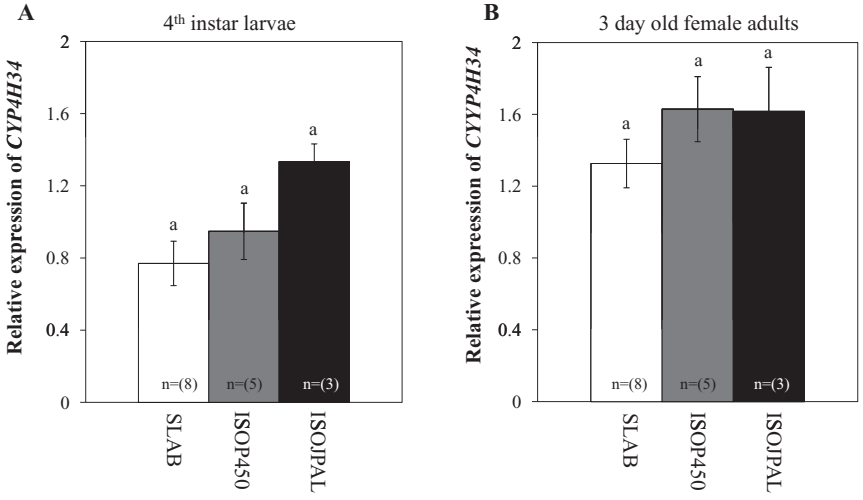

Figure 3. Expression of *CYP4H34* in (A) fourth instar larvae and (B) 3-day-old female adults relative to calibrator samples in susceptible (SLAB) and resistant (ISOP450 and ISOJPAL) strains of *Culex pipiens quinquefasciatus*. Values are the mean ± SEM ($n = 3$ to 8 biological samples as indicated) and significant differences between strains within life stages were determined using Tukey's honestly significant difference test (*$P \leq 0.05$; **$P \leq 0.01$). CYP, cytochrome P450.

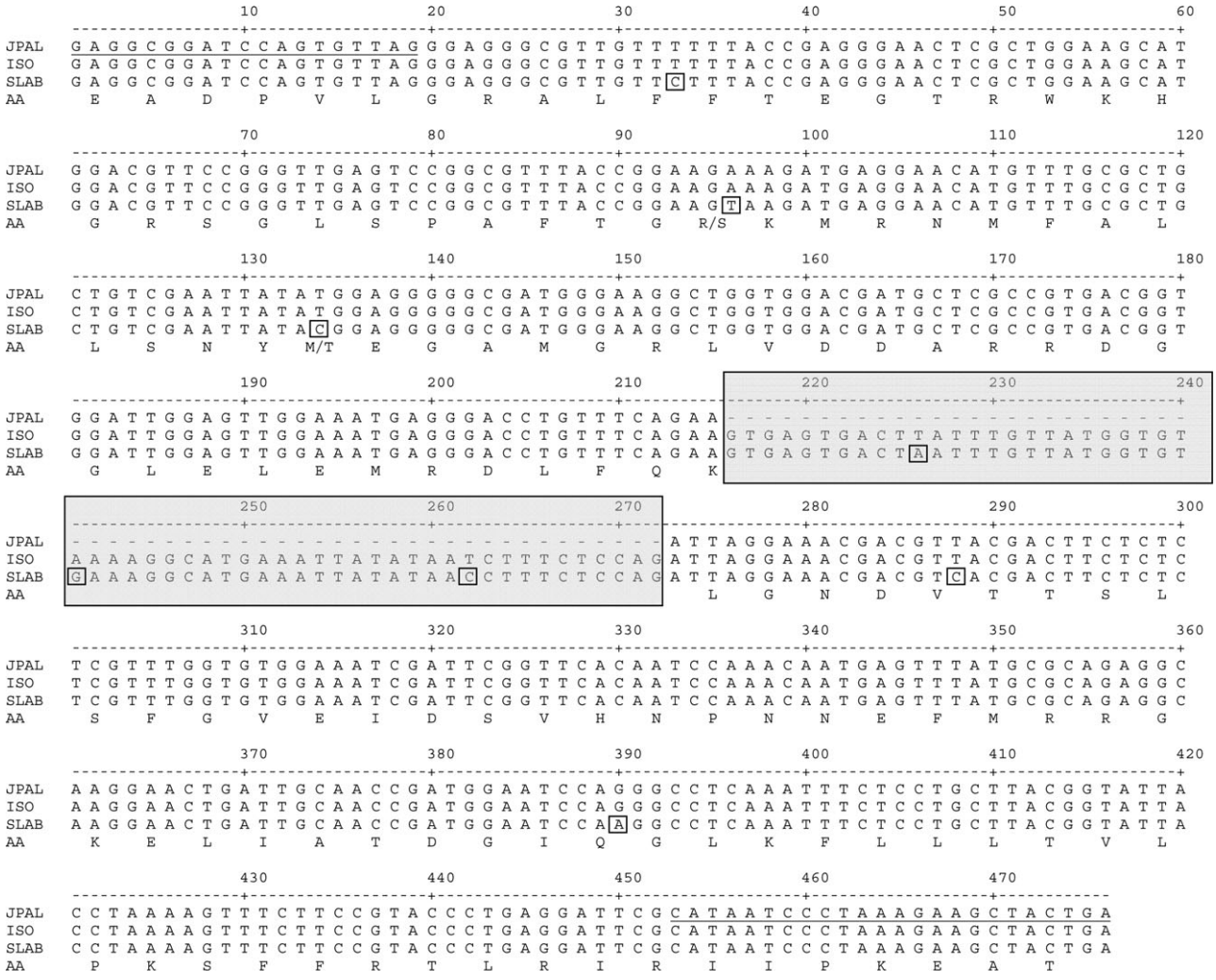

Figure 4. Partial *CYP9M10* sequence alignment of susceptible (SLAB) and resistant (ISOP450 and ISOJPAL were identical and are labelled as ISO) strains of *Culex pipiens quinquefasciatus* compared to the JPAL reference cDNA sequence (Komagata *et al*., 2010). The deduced amino acid sequence (AA) is shown in the bottom line. Primer sequences are underlined, boxes indicate single nucleotide polymorphisms between the susceptible and resistant alleles, and the shaded region indicates an intron. Nonsynonymous polymorphisms are indicated by the deduced amino acids of the resistant strain/susceptible strain. CYP, cytochrome P450.

Table 3. Comparison of bioassay method and sequencing of *CYP9M10* fragment using primers 9M-F and 9M-R to obtain P450-mediated permethrin resistance genotype frequency data

				Genotype frequency			Allele frequency	
Environment	Generation	Method	*n*	SS	RS	RR	S	R
Standard laboratory	F2A#1	Bioassay*	ND†	0.25	0.50	0.25	0.50	0.50
Standard laboratory	F2A#1	Sequencing	20	0.40	0.40	0.20	0.60	0.40
Temephos exposed	F8B#1	Bioassay*	1060	0.66	0.31	0.03	0.82	0.18
Temephos exposed	F8B#1	Sequencing	20	0.68	0.32	0.00	0.84	0.16

*Data from Hardstone *et al*. (2009).
†Not determined experimentally. Frequencies based on expected Mendelian ratios.
CYP, cytochrome P450.

the bioassays with permethrin and genotyping for *CYP9M10*. These results support the hypothesis that *CYP9M10v2* is linked to the P450 mediated fitness costs in the ISOP450 strain. It is not known if the P450 coded for by the resistance allele has a different ability to metabolize permethrin or not. Given that the *CYP9M10v2* allele is linked to permethrin resistance, this could be a valuable way to determine the resistance status of an individual *C. p. quinquefasciatus*.

No polymorphisms were present between the partial genomic sequences of *CYP4H34* from the susceptible (SLAB, accession no. GU974339) and resistant strains [(ISOP450 and ISOJPAL; accession nos. GU97434 and GU974340, respectively) and JPAL].

Discussion

Although a genome project has been started for *C. p. quinquefasciatus*, the number of P450 genes in this species is not known. A previous study (microarray and qPCR) on the JPAL strain of *C. p. quinquefasciatus* examined the expression of 62 P450s, and found that *CYP9M10* and *CYP4H34* were over-expressed in the resistant strain (Komagata *et al*., 2010). In our current study we used isogenic strains to determine if expression of these two P450s was linked to resistance. Using qPCR we found that *CYP9M10* was 1800- to 870-fold over-expressed in fourth instar larvae of the resistant strains (relative to SLAB). Thus, over-expression of *CYP9M10* is linked to permethrin resistance. Furthermore, we found that the resistant strains contained a specific allele *CYP9M10v2* not found in the susceptible strain. Given that this allele (and the over-expression of the gene, see above) is specific to the resistant strains we examined (and considering the repeated backcrossing used to produce the isogenic strains) we conclude that the *CYPM10v2* allele is linked to resistance. These results suggest it is likely that *CYP9M10* is the P450 responsible for conferring permethrin resistance in these strains of *C. p. quinquefasciatus*. Future experiments, such as heterologous expression or RNA interference, would be excellent approaches to test this hypothesis.

qPCR analysis indicates that the over-expression of *CYP9M10* in the larvae of the resistant strains is consistent between biological and technical replicates. The reason for the small difference in *CYP9M10* levels between ISOP450 and ISOJPAL is not known, although these strains were not isolated with the exact same methodology and therefore other linked loci could modify the expression. Although JPAL has 18-fold greater expression of *CYP4H34* relative to a susceptible strain (Komagata *et al*., 2010), the lack of increased expression of *CYP4H34* in ISOP450 and ISOJPAL indicates this trait is not associated with the major resistance locus. Although it is possible that CYP4H34 could have a minor role in resistance in JPAL, this seems unlikely because the levels of permethrin resistance in JPAL and ISOJPAL were similar. In contrast, *CYP9M10* is overexpressed in JPAL, ISOP450 and ISOJPAL, indicating this trait maps to the resistance locus. Although the level of expression of *CYP9M10* in JPAL previously reported (Komagata *et al*., 2010) is less than found in the ISOP450 and ISOJPAL strains, this may be because of the different susceptible strains used in these studies. The levels of *CYP9M10* over-expression in JPAL, ISOP450 and ISOJPAL are larger than most previously published values associated with insecticide resistance, which range from 2 to 50-fold (Wheelock & Scott, 1990; Waters *et al*., 1992; Carino *et al*., 1994; Liu & Scott, 1996, 1997; Kasai & Scott, 2000; Kasai *et al*., 2000; Daborn *et al*., 2001, 2007; Kamiya *et al*., 2001; Nikou *et al*., 2003; Zhu & Snodgrass, 2003; Sasabe *et al*., 2004; Bogwitz *et al*., 2005; David *et al*., 2005; Gong *et al*., 2005; Ghanim & Kontsedalov, 2007; Chiu *et al*., 2008; Karunker *et al*., 2008; Djouaka *et al*., 2008b; Muller *et al*., 2008; Zhu *et al*., 2008). However, two previous reports have found P450 mRNA over-expression in resistant strains to be as large as the values observed in this study (Daborn *et al*., 2007; Zhu & Liu, 2008). In addition, increases in P450 transcript levels of >1000-fold have been observed between control and treatment groups in some cases (Wang *et al*., 2002; Chang *et al*., 2003; Lowe *et al*., 2003; Rees & Li, 2004).

The total P450 content values obtained from fourth instar microsomes indicated that there is more 1.5-fold P450 protein present in the resistant strains versus SLAB and that the amounts present amongst the resistant strains were not statistically different (including JPAL, data not shown). This result was expected because JPAL had previously been reported to have a greater amount of total P450s than a susceptible strain (Kasai *et al*., 1998) and both resistant strains analysed here were derived from the JPAL strain. Previous studies examining P450-mediated resistance in house flies found that total P450 content and P450 gene expression are positively and linearly correlated (Scott & Lee, 1993; Tomita & Scott, 1995; Liu & Scott, 1996). However, in this study we found that the total P450 protein content and transcript levels of *CYP9M10* in both resistant strains, ISOP450 and ISOJPAL, were correlated in a positive and exponential fashion. The mechanism causing the extremely high over-expression of *CYP9M10* in larvae of the resistant strains could be caused by various molecular mechanisms such as mRNA stabilization, increased transcription or gene duplication. How the 1800-fold change in *CYP9M10* transcript results in only a 1.5-fold change in total P450s (protein) is unclear. It is possible that all *CYP9M10* transcript is not translated into protein, and/or that the amount of *CYP9M10* transcript is so small in susceptible strains that

a large increase in this transcript produces only a small change in total P450s (protein; Scott, 1990). To help understand this it would be valuable to know the level of CYP9M10 protein present in the resistant versus susceptible strains.

Experimental procedures

Mosquito strains

Four strains of *C. p. quinquefasciatus* (Say) were used and reared as previously described (Hardstone *et al*., 2007). SLAB is a laboratory susceptible strain (Georghiou *et al*., 1966). JPAL is a permethrin resistant strain (29 000-fold) containing *kdr* and P450 detoxification (Kasai *et al*., 1998; Hardstone *et al*., 2007). ISOP450 is a permethrin resistant strain (1300-fold) related to SLAB containing the P450 resistance mechanism present in JPAL, but lacking *kdr* (Hardstone *et al*., 2007). The fourth strain, ISOJPAL, was isolated using modified backcrossing methods (Berticat *et al*., 2002). As *kdr* had been lost during the isolation of ISOP450 (Hardstone *et al*., 2007), the frequency of *kdr* was assessed every generation during the isolation of ISOJPAL and the selection regime altered to assure that this allele remained in the population. The *Vssc* susceptible allele increased during the backcrossing scheme; therefore, backcrosses were halted at generation 5 in order to maintain heterozygotes in the population. Additionally, once the *kdr* resistance allele became fixed during the selection scheme, the selection concentration was increased to obtain a population that was also homozygous resistant for the P450-detoxification mechanism. Table 1 details the backcross and selection process. ISOJPAL is a permethrin resistant strain (13 000-fold) related to SLAB containing the P450 resistance mechanism present in JPAL and *kdr*.

Bioassays

Larval bioassays were conducted as previously described (Hardstone *et al*., 2007). Briefly, for larval bioassays, batches of 20 fourth instar larvae were placed in 4 oz. waxed paper cups with 99 ml water and 1 ml of insecticide (dissolved in acetone) solution. Acetone only controls were conducted alongside insecticide treatments. Larvae were considered dead if after 24 h they were ataxic. Bioassays were also performed using PBO (1 μg/ml). Permethrin (98%) was from ChemService (Westchester, PA, USA) and PBO (90%) was from Sigma-Aldrich (St. Louis, MO, USA).

gDNA extraction and genotyping of Vssc

gDNA was extracted and PCR (for the region of *Vssc* containing the *kdr* mutation) amplified as previously described from single adult male mosquitoes using primers CulexkdrF and CulexkdrR (Hardstone *et al*., 2007).

Microsome preparation

Microsomes were prepared by dissecting the midguts of 200 fourth instar larvae kept on ice as previously described by Kasai *et al*. (1998). Midguts plus 2 ml homogenization buffer (Lee & Scott, 1989) were placed in a Teflon pestle homogenizer (Kimble-Kontes, Vineland, NJ, USA). A motorized homogenizer (Caframo Wiarton, ON, USA) at 125 rpm was used to completely pulverize the tissue. The tissue extract was rinsed with homogenization buffer and centrifuged at 4 °C at 10 000 *g* for 20 min. The supernatant from the first centrifugation was then spun at 100 000 *g* for 1 h. The pellet was suspended in 2.4 ml resuspension buffer (Lee & Scott, 1989). Reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA). Microsomes were stored at –80 °C and measured within 24 h of preparation.

Total protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) used as a standard. Cytochrome P450 and cytochrome b_5 content were determined on undiluted microsomes by the method described by Omura & Sato (1964) using sodium dithionite as a reducing agent and saturating the sample with carbon monoxide. Means were statistically compared using Tukey's honestly significant difference (HSD) test.

mRNA extraction and cDNA synthesis

Each mRNA sample (referred to as a biological replicate) consisted of 15 3-day-old virgin female adults that had fed on 20% sugar water or 15 fourth instar larvae. Samples were processed using the illustra QuickPrep *Micro* mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK) and treated with DNA-*free* (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Concentrations of mRNA were determined on a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). First strand cDNA was synthesized from 5 μg mRNA with SuperScript III First Strand Synthesis System for RT PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Negative controls were prepared by excluding reverse transcriptase during cDNA synthesis.

qPCR

qPCR was performed in a 10 μl volume with the Fast SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA, USA) and a 7900 HT Sequence Detection System (Applied Biosystems Inc.). Primers (Table 4) for *CYP4H34* and *CYP9M10* were determined from published sequences (Komagata *et al*.,

Table 4. Primers for target genes *CYP4H34* and *CYP9M10* and endogenous control (*actin*) used in quantitative real-time PCR

Gene	Product size	Primer name	Primer sequence (5′ to 3′)
CYP4H34	76 bp	4H-RT-F	CATCCAGCTGGCAAAGCACC
		4H-RT-R	GACTTCTGCGCCGAGTACG
CYP9M10	77 bp	9M-RT-F	GCCTAACCAACCGAGCCTG
		9M-RT-R	GCCGACCTGGATGGAGACT
actin	97 bp	act-RT-F	GGCGTCGAGGATCTGGACTT
		act-RT-R	AGTCCTCGACCAGCCCG

CYP, cytochrome P450.

Table 5. Primers used for amplification of *CYP4H34* and *CYP9M10* alleles

Gene	Product size	Primer name	Primer sequence (5′ to 3′)
CYP4H34	451 bp	4H-F	CCGACGTGCTGGTGAAC
		4H-R	ATGTCGGAATCGGAGAGG
CYP9M10	419 bp (includes 56 bp intron)	9M-F	GAGGCGGATCCAGTGTTAG
		9M-R	TCAGTAGCTTCTTTAGGGATTATG

CYP, cytochrome P450.

2010) and the *actin* sequence was obtained from GenBank (accession no.: XM_001866844). A minimum of three biological replicates were prepared for each strain at each life stage. Measurements of gene expression were obtained in triplicate for each biological sample. The qPCR reaction cycle consisted of a melting step at 50 °C for 2 min which was increased to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (SDS 2.0 software; Livak & Schmittgen, 2001). SLAB fourth instar larvae cDNA preparation no. 1 and SLAB 3-day-old female adult cDNA preparation no. 1 served as inter-plate calibrators. Expression levels were determined by averaging multiple replicates of the same sample and then averaging samples of a particular strain at a particular life stage across all technical replicates (ie plates). Relative gene expression levels within life stages between strains were statistically compared using Tukey's HSD test.

Sequencing of candidate P450 genes,
CYP9M10 *and* CYP4H34

Partial genomic sequences of *CYP9M10* and *CYP4H34* were analysed for polymorphisms to differentiate the susceptible and resistant alleles. The primer pairs used are listed in Table 5. Thermal cycler conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min with a final extension of 72 °C for 10 min. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced at the Cornell University Biotechnology Resource Center.

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