Introduction

Cytochrome P450s have long been of particular interest as they are critical for the detoxification and/or activation of xenobiots such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens. They are also involved in metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Basal and up-regulation of P450 gene expression can significantly affect the disposition of xenobiots or endogenous compounds in the tissues of organisms, thus altering their pharmacological/toxicological effects [1]. Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides [2–4] and plant toxins [5,6]. While all insects probably possess some capacity to detoxify insecticides and xenobiotics, the degree to which they can metabolize and detoxify these toxic chemicals is of considerable importance to their survival in a chemically unfriendly environment [7] and to the development of resistance. A significant characteristic of insect P450s is their transcriptional up-regulation, resulting in increased P450 protein levels and P450 activities, which, in turn, cause enhanced metabolic detoxification of insecticides and plant toxins in insects, leading to the development of insecticide resistance [4,8–16] and a higher tolerance to plant toxins [17,18].

Insect P450s are also known to be an important part of the biosynthesis and degradation pathways of endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH) [19–23] and thus play important roles in insect growth, development, and reproduction.

Cytochrome P450s are a superfamily that can take a number of related forms that frequently co-exist in the same cell type [24]. The rate at which a particular substrate is oxidized differs from one P450 to another, so that the overall metabolism of a specific substrate depends on the different forms present and varies between tissues, life stages, and sexes [25]. Because of the multiple cytochrome P450s expressed in each organism and the broad substrate specificity of some of these isoforms, P450s are capable of oxidizing a bewildering array of xenobiots [25]. While the importance of P450s in insect physiology and toxicology is widely recognized, it is not yet clear how many P450 genes precisely are involved in insecticide resistance in a single insect such as the mosquito.

With the availability of the whole genome sequence for the mosquito Culex quinquefasciatus [26], we are now able to
characterize the expression profiles of P450s in insecticide resistant mosquitoes and thus improve our understanding of the P450 gene interactions that play a role in the physiological and toxicological processes of insects. The current study focused on characterizing the expression profiles of these P450 genes from mosquito populations of *C. quinquefasciatus* bearing different phenotypes in response to permethrin (susceptible, intermediate and highly resistant) in order to pinpoint the key P450 genes involved in insecticide resistance.

**Materials and Methods**

**Mosquito strains**

Five strains of the mosquito *Cx. quinquefasciatus* were studied. HAmCqG8 and MAmCqG6 were field resistant strains collected from Huntsville and Mobile, respectively, from sites located >600 km apart in the state of Alabama, USA in 2002; the locations were not privately-owned or protected in any way, no specific permissions were required for these locations/activities, and the study did not involve endangered or protected species. Because *Cx. quinquefasciatus* is an important urban pest in Alabama, it has been a major target for several insecticides, including Bti, malathion, resmethrin, and permethrin, and control difficulties have been reported before the collection [27]. Both Field strains had the similar levels (10-fold compared with susceptible S-Lab) of resistance to permethrin [28] and did not exposure to insecticides after established as colonies in the laboratory. HAmCqG8 was the 8th generation of permethrin-selected HAmCqG0 offspring with a 2,700-fold level of resistance and MAmCqG6 was the 6th generation of permethrin-selected MAmCqG0 offspring with a 570-fold level of resistance [29]. The permethrin selections for both HAmCqG8 and MAmCqG6 were performed at the 4th instar larval stage [28,29]. S-Lab was an insecticide susceptible strain provided by Dr. Laura Harrington (Cornell University).

All the mosquitoes were reared at 25±2°C under a photoperiod of 12:12 (L:D) h and fed blood samples from horses (Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University).

**Quantitative real-time PCR (qRT-PCR)**

The 4th instar larvae and 2–3 day-old adults (before blood feeding) of each mosquito population had their RNA extracted for each experiment using the acidic guanidine thiocyanate-phenol-chloroform method [8]. Total RNA (0.5 μg/sample) from each mosquito sample was reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR, which was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 μl final volume) contained 1 x SYBR Green master mix, 1 μl of cDNA, and a P450 gene specific primer pair designed according to each of the P450 gene sequences (http://cquinquefasciatus.vectorbase.org/), Table S1 with accession number for each of P450 genes) at a final concentration of 3–5 μM. All samples, including the A ‘no-template’ negative control, were performed in triplicate. The reaction cycle consisted of a melting step of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction using Dissociation Curves software [30]. Relative expression levels for the P450 genes were calculated by the 2–ΔΔCT method using SDS RQ software [31]. The 18S ribosome RNA gene, an endogenous control, was used to normalize the expression of target genes [15,32,33].

Preliminary qRT-PCR experiments with the primer pair (Table S1) for the 18S ribosome RNA gene designed according to the sequences of the 18S ribosome RNA gene revealed that the 18S ribosome RNA gene expression remained constant among all 5 mosquito strains, so the 18S ribosome RNA gene was used for internal normalization in the qRT-PCR assays. Each experiment was repeated three to four times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student’s t-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons [SAS v9.1 software]; a value of P≤0.05 was considered statistically significant. Significant overexpression was determined using a cut-off value of a ≥2-fold change in expression [34].

**Results**

**Cytochrome P450 genes in *Cx. quinquefasciatus***

The *Cx. quinquefasciatus* genome sequence has revealed 204 putative P450 (CYP) genes (including 8 pseudogenes) in *Cx. quinquefasciatus* mosquitoes [26,35], (http://cquinquefasciatus.vectorbase.org/). The *Cx. quinquefasciatus* P450s fall into four major clans of CYP2, CYP3, CYP4, and mitochondrial (Fig. 1), as do those identified in other insects [36]. Of the 204 *Cx. quinquefasciatus* P450s, the majority assemble in clans 3 and 4: 89 P450s were found in the clan CYP3, with 24 in the CYP9 family, 64 in the CYP 6 family and 1 in the CYP329 family, and 82 in the clan CYP4, with 34 in the CYP4 family, 47 in the CYP325 family, and 1 in the CYP326 family. Sixteen P450s were found in clan 2, with CYP families of 303 to 307, 18 and 15. The remaining 12 P450s were found in the mitochondrial clan with 6 P450 families of CYP12, CYP49, CYP301, CYP302, CYP314 and CYP315. Comparing this distribution with those of other insect species, *Cx. quinquefasciatus* showed a clear expansion of P450s in clans 3 and 4. This expanded P450 supergene family in the *Cx. quinquefasciatus* genome may provide a clue to the mechanisms that permit Culex mosquitoes to adapt to polluted larval habitats [26].

**Dynamic changes of P450 gene expression in the mosquito populations of Culex quinquefasciatus following permethrin selection**

To understand how the P450 gene expression profile changes following permethrin selection, we compared the gene expression of 204 Pi450 genes [26], (http://cquinquefasciatus.vectorbase.org/), http://drnelson.utmem.edu/CytochromeP450.html in both larvae and adults between susceptible and resistant *Culex* mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring using qRT-PCR. The accession numbers of the P450 genes were listed in Table S1. Mosquito populations bearing 3 different resistance phenotypes in response to permethrin were used, ranging from susceptible (S-Lab), through intermediate resistant (HAmCqG8, field parental population) to highly resistant (HAmCqG0, 8th generation permethrin selected offspring of HAmCqG0). Comparing the P450 gene expression profiles in both larvae and adults of permethrin selected HAmCqG8 mosquitoes with those of their field parental population revealed that 69% of genes were expressed at a similar level in both HAmCqG8 and HAmCqG0 (Fig. 2A), 11% were up-regulated in HAmCqG8 compared to HAmCqG0, 7% were up-regulated in HAmCqG0 adults, 2 gene were up-regulated in both larvae and adults, 5% were down-regulated in HAmCqG8 larvae, and 4% were down-regulated in HAmCqG0 adults, 2% were down-regulated in both larvae and adults of HAmCqG0. Applying a
cut off level of 2 [34], among the up-regulated P450 genes in larvae and adults of HAmCqG8, the majority were expressed at 2- to 4-fold elevated levels compared with HAmCqG0 and only 32% and 12% in larvae and adults, respectively, had >5-fold overexpression (Fig. 2A).

Similar expression patterns were also identified in another permethrin selected mosquito strain. Here, MAmCqG6, the 6th generation of permethrin selected field strain of MAmCqG0, were compared with their parental strain of MAmCqG0, which was collected at a location 600 km south of the collection site for the HAmCqG0 mosquitoes (Fig. 2B). In MAmCqG0, 6% of genes were found to be up-regulated in the larvae of MAmCqG6 compared with those of MAmCqG0 and S-Lab (Fig. 2B), 4% were up-regulated in MAmCqG6 adults, 3 genes were up-regulated in both larvae and adults, 11% were down-regulated in larvae, 2% were down-regulated in adults, 2% were down-regulated in both larvae and adults, and 2% were down-regulated in larvae but up-regulated in adults. Taken together, these results revealed equally dynamic changes in abundance in both increased and decreased P450 gene expression in the two field mosquito strains of Culex quinquefasciatus following permethrin selection.

P450 genes involved in up- and down-regulation in the larvae of resistant Cx. quinquefasciatus

Twenty five P450 genes were found to be up-regulated in the larvae stage (4th larval instar) of HAmCqG8 mosquitoes. The expression levels of these P450 genes were ≥2-fold higher in HAmCqG8 than that in both S-Lab and HAmCqG0 mosquito strains (Table 1). The genes were distributed in clans CYP3, CYP4, and mitochondria with 7 genes in family 9, 7 in family 6, 5 in family 4, 3 in family 325, 2 in mitochondria, and 1 without annotation. Except the six P450 genes CYP6AG12, CYP6AA7, CYP4C38, CYP9J35, CYP6BZ2, and CYP9M10 whose expression levels in parental HAmCqG0 mosquitoes were 2.2-, 2.8-, 2.1-, 11-, 2.0- and 5-fold higher than in susceptible S-Lab mosquitoes, the expression levels of other genes were similar or lower in HAmCqG8 compared with the susceptible S-Lab strain (Table 1). Similar patterns were observed when comparing the changes in P450 expression in the larvae of MAmCqG6 with those of both the S-Lab and MAmCqG0 mosquito strains. Fifteen P450 genes were found to be up-regulated in the larvae of MAmCqG6 mosquitoes. The expression levels of these P450 genes in MAmCqG6 were ≥2-fold higher than those in both the S-Lab and MAmCqG0 mosquito strains (Table 1). These genes were distributed in clans CYP2, CYP3, and CYP4 with 7 genes in family 9, 5 in family 6, and 1 in each of families 4, 306, and 307. The expression of these genes was similar or lower in MAmCqG0 compared with the susceptible S-Lab strain (Table 1) except for CYP9M10 and CYP6AA7, whose expression levels in the parental MAmCqG0 mosquitoes were 8.9- and 4.5-fold higher, respectively.

Beside the up-regulation of P450 genes identified in the larvae of Cx. quinquefasciatus following permethrin selection, a number of P450 genes were found to be down-regulated in larvae of permethrin selected Cx. quinquefasciatus. Sixteen P450 genes were down-regulated in the larvae (4th larval instar) of HAmCqG8 mosquitoes. The expression levels of these P450 genes in HAmCqG8 were ≤2-fold lower than that in HAmCqG0 mosqui-
toes (Table 2). These down-regulated genes were distributed in clans CYP3 and CYP4, with 2 genes in family 9, 10 in family 6, and 2 in each of families 4 and 325. The expression of the majority of these genes in HAmCqG8 was at similar or lower levels compared with that in susceptible S-Lab mosquitoes, even though most were expressed at higher levels in HAmCqG0 than in S-Lab (Table 2). Although the similar P450 down-regulation patterns were also found in the larvae of MAmCqG6 compared with both S-Lab and MAmCqG0, we noticed extended numbers and distribution of these genes in the CYP clans compared with HAmCq mosquitoes. Thirty P450 genes were down-regulated in the larvae (4th larval instar) of MAmCqG6 compared with HAmCq mosquitoes. The expression levels of these P450 genes in MAmCqG6 were ≥2-fold lower than that in both S-Lab and MAmCqG0 mosquito strains (Table 2). The overexpression levels of the up-regulated P450 genes in all the mosquito populations tested were closely correlated with their levels of resistance and were higher in permethrin-selected mosquitoes than in their parent field strain. These genes were mainly distributed in clans CYP3 and CYP4, with 3 genes in family 9, 5 in family 6, 5 in family 4, and 3 in family 325. One gene was in mitochondria clan, family 12. The expression of all these genes in MAmCqG0 was similar or lower than in susceptible S-Lab mosquitoes (Table 3). The expression of these genes was similar or lower in MAmCqG0 compared with susceptible S-Lab mosquitoes except for CYP325BF1v2 and CYP325K3v1, which were 2.4- and 3-fold higher, respectively, in MAmCqG0 (Table 4).

P450 genes involved in up- and down-regulation in resistant Cx. quinquefasciatus adults

The expression of 204 Culex P450 genes in the adults of the 5 mosquito populations was examined using qRT-PCR. Seventeen P450 genes were found to be up-regulated in the adult stage (2–3 day old) of HAmCqG8 mosquitoes. The expression levels of these P450 genes in HAmCqG8 were ≥2-fold higher than in both S-Lab and HAmCqG0 mosquito strains (Table 3). The overexpression levels of the up-regulated P450 genes in all the mosquito populations tested were closely correlated with their levels of resistance and were higher in permethrin-selected mosquitoes than in their parent field strain. These genes were mainly distributed in clans CYP3 and CYP4, with 3 genes in family 9, 5 in family 6, 5 in family 4, and 3 in family 325. One gene was in mitochondria clan, family 12. The expression of all these genes in HAmCqG0 was similar or lower than in susceptible S-Lab mosquitoes (Table 3). Similar changes in the P450 gene expression were also found in MAmCqG6 adults compared with their S-Lab and MAmCqG0 counterparts. Fifteen P450 genes were up-regulated in adult MAmCqG6 mosquitoes. The expression levels of these P450 genes were ≥2-fold higher than those in both S-Lab and MAmCqG0 adults (Table 3). As in the HAmCqG8 mosquitoes, the genes whose expression changed in MAmCqG6 mosquitoes following permethrin selection were also distributed in clans CYP3 and CYP4, with 1 gene in family 9, 2 in family 6, 1 in family 4, and 3 in family 325. The expression of these genes was similar or lower in MAmCqG0 compared with susceptible S-Lab mosquitoes except for CYP925BF1v2 and CYP925K3v1, which were 2.4- and 3-fold higher, respectively, in MAmCqG0 (Table 4).
Table 1. Up-regulation of P450 genes in larvae of permethrin selected offspring of the field populations of *Culex quinquefasciatus*.

| Mosquitoes | Transcript IDa | Gene | Parental strainb | Resistant strainb | Ratioe |
|------------|---------------|------|------------------|-------------------|--------|
| HAmCq (25) | CPI002538  | CYP6AG12 | 2.2±0.3 | 4.7±1.8 | 2.1 |
|            | CPI005959*  | CYP6A7 | 2.8±0.9 | 5.9±0.6 | 2.1 |
|            | CPI003082  | CYP9J42 | 1.3±0.2 | 2.9±1.5 | 2.2 |
|            | CPI001810  | CYP4C38 | 2.1±0.6 | 5.1±0.3 | 2.4 |
|            | CPI015957  | CYP325G4 | 1.2±0.2 | 3.3±0.3 | 2.8 |
|            | CPI005957  | CYP6A9 | 0.9±0.04 | 2.5±0.6 | 2.8 |
|            | CPI007091  | CYP325Y6 | 1.1±0.3 | 3.1±1.2 | 2.8 |
|            | CPI010546* | CYP9J34 | 0.8±0.3 | 2.3±0.3 | 2.9 |
|            | CPI000926* | - | 0.7±0.1 | 2.2±0.2 | 3.1 |
|            | CPI016847  | CYP6CQ1 | 0.9±0.2 | 2.8±0.9 | 3.1 |
|            | CPI009478  | CYP4D42v1 | 1.3±0.1 | 4.1±0.9 | 3.2 |
|            | CPI010540* | CYP9J15 | 11.2±0.0 | 39±10 | 3.5 |
|            | CPI005956  | CYP6Z2 | 2.0±0.6 | 7.3±1.1 | 3.7 |
|            | CPI010537* | CYP9J45 | 0.6±0.3 | 2.3±1.3 | 3.8 |
|            | CPI012470* | CYP9A1 | 0.6±0.2 | 2.3±0.4 | 3.8 |
|            | CPI014218* | CYP9M10 | 5.0±1.2 | 21±4.0 | 4.2 |
|            | CPI005954  | CYP6CC2 | 0.6±0.004 | 2.5±0.6 | 4.2 |
|            | CPI010543  | CYP12F7 | 0.5±0.06 | 2.6±0.5 | 5.2 |
|            | CPI010227  | CYP12F13 | 0.5±0.06 | 2.6±0.5 | 5.2 |
|            | CPI010543  | CYP9J40 | 0.6±0.2 | 3.6±1.3 | 6.0 |
|            | CPI018943* | CYP4C52v1 | 0.5±0.04 | 3.1±1.8 | 6.2 |
|            | CPI005955* | CYP6P14 | 0.6±0.1 | 3.8±0.6 | 6.3 |
|            | CPI001759  | CYP4H40 | 0.3±0.09 | 2.1±0.6 | 7.0 |
|            | CPI020229  | CYP4D42v2 | 0.4±0.1 | 2.8±2.4 | 7.0 |
|            | CPI017021  | CYP325K3v1 | 0.2±0.02 | 2.1±0.2 | 11 |
| MAmCq (15) | CPI014218* | CYP9M10 | 8.9±1.6 | 14±3.9 | 1.6 |
|            | CPI010548* | CYP9J39 | 0.9±0.4 | 2.0±0.3 | 2.2 |
|            | CPI005958  | CYP6A8 | 0.7±0.001 | 1.8±0.8 | 2.6 |
|            | CPI001039  | CYP306A1 | 1.0±0.1 | 2.6±0.06 | 2.6 |
|            | CPI005959* | CYP6A7 | 4.5±1.0 | 12±4.2 | 2.7 |
|            | CPI000332  | CYP9J43 | 0.9±0.08 | 2.5±0.02 | 2.8 |
|            | CPI004411  | CYP6Z12 | 1.4±0.2 | 4.0±1.9 | 2.9 |
|            | CPI005955* | CYP6P14 | 1.6±0.1 | 4.6±0.5 | 2.9 |
|            | CPI008566  | CYP6Z15 | 0.7±0.06 | 2.1±0.7 | 3.0 |
|            | CPI010546* | CYP9J34 | 1.1±0.4 | 3.4±1.1 | 3.1 |
|            | CPI010537* | CYP9J45 | 1.0±0.3 | 3.1±1.3 | 3.1 |
|            | CPI012470* | CYP9A1 | 0.9±0.2 | 3.4±0.2 | 3.8 |
|            | CPI010544  | CYP9J33 | 0.6±0.1 | 2.9±1.0 | 4.8 |
|            | CPI000989  | CYP307B1 | 0.5±0.2 | 2.5±1.2 | 5.0 |
|            | CPI018943* | CYP4C52v1 | 0.2±0.02 | 2.7±1.7 | 14 |

*The transcript ID number from the vectorbase of the *Cx. quinquefasciatus* genome sequence (http://cquinquefasciatus.vectorbase.org/).

bThe relative level of gene expression represents the ratio of the gene expression in each permethrin selected strain compared with that in the susceptible S-Lab strain. The relative level of gene expression for S-Lab is 1.

cParental strain for HAmCq population is HAmCqG0 with a 10-fold level of resistance to permethrin compared with S-Lab and for MAmCq is MAmCqG0 with a 10-fold level of resistance to permethrin [28].

dPermethrin selected strain for HAmCq population is HAmCqG8 with a 2700-fold level of resistance to permethrin and for MAmCq is MAmCqG6 with a 570-fold level of resistance to permethrin [28].

eThe ratio of the relative gene expression in each permethrin selected strain compared its parental strain.

fThe genes that are up regulated in both larvae of HAmCqG8 and MAmCqG6.

gThe genes that are up regulated in both larvae and adults of HAmCqG8 and/or MAmCqG6.

hNo annotation in Dr. Nelson’s P450 homepage http://drnelson.utmem.edu/CytochromeP450.html.

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Table 2. Down-regulation of P450 genes in larvae of permethrin selected offspring of the field populations of Culex quinquefasciatus.

| Mosquitoes | Transcript ID | Gene       | Relative Gene expression ± SEb |
|------------|---------------|------------|--------------------------------|
|            |               | Parental strain | Resistant strain | Ratioa |
| HAmCq (16) | CPU09085      | CYP6AG13   | 1.3±0.07 | 0.6±0.04 | −2.2 |
|            | CPU019580     | CYP6Z13P   | 2.8±2.2  | 1.3±0.3  | −2.2 |
|            | CPU006950     | CYP325BG1  | 6.7±1.9  | 3.0±0.7  | −2.2 |
|            | CPU016852     | CYP6N19    | 2.9±0.9  | 1.0±0.3  | −2.9 |
|            | CPU005683     | CYP325Y10  | 5.3±2.7  | 1.8±0.5  | −2.9 |
|            | CPU008972     | CYP6FS5P   | 2.9±0.7  | 0.9±0.1  | −3.2 |
|            | CPU018716     | CYP4C38    | 1.0±0.06 | 0.3±0.1  | −3.3 |
|            | CPU014219     | CYP9M10-de1b | 2.6±0.5 | 0.8±0.2  | −3.3 |
|            | CPU009473     | CYP4D1     | 2.1±0.8  | 0.6±0.01 | −3.5 |
|            | CPU017462     | CYP6E1     | 0.7±0.02 | 0.2±0.0  | −3.5 |
|            | CPU011129     | CYP6N25    | 3.5±1.5  | 0.9±0.1  | −3.9 |
|            | CPU000299     | CYP6AH3    | 4.8±0.7  | 1.1±0.2  | −4.4 |
|            | CPU010547     | CYP9J47    | 1.6±0.3  | 0.3±0.08 | −5.3 |
|            | CPU003377     | CYP9B5Y    | 2.9±0.008| 0.5±0.08 | −5.8 |
|            | CPU003361     | CYP6B2Y    | 1.9±0.3  | 0.2±0.005| −9.5 |
|            | CPU003375     | CYP6B3Y    | 1.7±0.2  | 0.05±0.02| −34 |
| MAmCq (30) | CPU017351     | CYP4C50v1  | 1.4±0.3  | 0.7±0.07 | −2.0 |
|            | CPU018854     | CYP4C50v2  | 1.4±0.3  | 0.7±0.07 | −2.0 |
|            | CPU010542     | CYP9J38    | 1.2±0.2  | 0.6±0.2  | −2.0 |
|            | CPU017198     | CYP325BF1-de1b | 1.4±0.2 | 0.7±0.06 | −2.0 |
|            | CPU010228     | CYP12F12   | 1.2±0.2  | 0.6±0.3  | −2.0 |
|            | CPU017243     | CYP304B4   | 2.3±0.4  | 1.1±0.2  | −2.1 |
|            | CPU007090     | CYP325Y5   | 2.2±0.07 | 1.0±0.07 | −2.2 |
|            | CPU014579     | CYP4AR3    | 2.1±1.2  | 0.9±0.08 | −2.3 |
|            | CPU010231     | CYP12F9    | 1.4±0.2  | 0.6±0.3  | −2.3 |
|            | CPU019765     | CYP9M14    | 0.7±0.8  | 0.3±0.1  | −2.3 |
|            | CPU007091     | CYP325Y6   | 1.5±0.1  | 0.6±0.2  | −2.5 |
|            | CPU015953     | CYP325BF1v2| 1.5±0.2  | 0.6±0.004| −2.5 |
|            | CPU015318     | CYP325V5v2 | 3.3±0.5  | 1.3±0.09 | −2.5 |
|            | CPU018944     | CYP4C51v1  | 8.9±0.6  | 3.4±0.2  | −2.6 |
|            | CPU003377     | CYP6B5Y    | 2.7±0.6  | 1.0±0.3  | −2.7 |
|            | CPU011843     | CYP325BH1  | 9.8±2.5  | 3.6±2.5  | −2.7 |
|            | CPU001038     | CYP18A1    | 5.2±1.6  | 1.9±1.4  | −2.7 |
|            | CPU009569     | CYP326BK1  | 3.1±1.4  | 1.1±0.2  | −2.8 |
|            | CPU001757     | CYP4H39    | 3.6±1.2  | 1.2±0.03 | −3.0 |
|            | CPU001754     | CYP4I6     | 4.5±0.4  | 1.5±0.6  | −3.0 |
|            | CPU001755     | CYP4J9     | 0.9±0.1  | 0.3±0.0  | −3.0 |
|            | CPU009477     | CYP4D19    | 4.3±0.7  | 1.4±0.6  | −3.1 |
|            | CPU018716     | CYP4C38    | 1.9±0.8  | 0.6±0.05 | −3.2 |
|            | CPU009475     | CYP4D43    | 1.7±0.4  | 0.5±0.3  | −3.4 |
|            | CPU014220     | CYP9M12    | 1.4±0.1  | 0.4±0.1  | −3.5 |
|            | CPU015961     | CYP325BE1  | 2.8±0.6  | 0.7±0.09 | −4.0 |
|            | CPU009471     | -          | 1.2±0.07 | 0.2±0.04 | −6.0 |
|            | CPU003375     | CYP6B5Y    | 2.8±0.06 | 0.3±0.01 | −9.3 |
|            | CPU001810     | CYP4C38    | 16±2.5   | 1.4±0.3  | −11 |
|            | CPU017200     | CYP325N3v2 | 6.0±1.5  | 0.5±0.1  | −12 |
As in the mosquito larvae, a number of P450 genes were down-regulated in adult Cx. quinquefasciatus following permethrin selection. Fourteen P450 genes were down-regulated in adult HAmCqG0 mosquitoes. The expression levels of these P450 genes in HAmCqG0 were ≤2-fold lower than in HAmCqG8 strain (Table 4). These genes were distributed in clans CYP3 and CYP4, with 3 genes in family 9, 4 in family 6, 3 in family 4, and 4 in family 325. Apart from CYP6MI2, whose expression was ~2-fold higher in HAmCqG8 than in the susceptible S-Lab strain, all were expressed at lower levels in HAmCqG8 than in S-Lab adults even though most of the P450 genes in HAmCqG0 were expressed at higher levels than in S-Lab mosquitoes (Table 4). Similar down-regulation patterns for P450 were also found in MAmCqG0 adults compared with both S-Lab and MAmCqG0 adults. Nine P450 genes were down-regulated in MAmCqG0 mosquitoes, the expression levels of these 9 P450 genes were ≤2-fold lower in MAmCqG0 than that in MAmCqG6 mosquitoes (Table 4). The genes were distributed in clans CYP2, CYP3, and CYP4, with 2 genes in family 304, 3 in family 9, 1 in family 6, and 5 in family 4. All these genes had lower expression levels in MAmCqG0 than in S-Lab adults; the expression of these genes in the MAmCqG6 mosquitoes was similar to that in the S-Lab strain except for CYP5F46, whose expression was much lower (Table 4).

Discussion

Two hundred and four putative P450 (CYP) genes in the genome of Cx. quinquefasciatus mosquitoes [26,35], (http://cequinquefasciatus.vectorbase.org/) have put them in the largest P450 repertoire for any insect genome that has been reported so far; it is larger than that of Anopheles gambiae (90 P450s [38]), Aedes aegypti (160 P450s [34]), Drosophila melanogaster (90 P450s [38]), Nasonia vitripennis (115 P450s, [39]), Bombyx mori (silk moth, 86 P450s [40]), honeybee Apis mellifera (46 P450s [41]), Tribolium castaneum (red flour beetle, 134 P450s [42], [43]) were reported by Dr. Nelson, (http://drnelson.utmem.edu/CytochromeP450.html).

Our previous studies have indicated that P450s may be one of the primary enzymes involved in detoxifying permethrin and conferring permethrin resistance in Culex mosquitoes [45]. In order to examine the possible role of P450 genes, as a whole, in the development of insecticide resistance in Culex quinquefasciatus mosquitoes, we, for the first time, examined the expression profiles of a total of 204 P450 genes in both larvae and adults of Cx. quinquefasciatus by comparing the profiles for susceptible and resistant mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring. Insecticide resistance is generally assumed to be a pre-adaptive phenomenon, where prior to insecticide exposure rare individuals carrying an altered (varied) genome already exist, thus allowing the survival of those carrying the genetic variance after insecticide selection [46]. We therefore expected that the number of individuals carrying the resistance P450 genes or alleles should increase in a population following selection and become predominant under severe selection pressure. The approach adopted for this study, which compared P450 gene expression among different mosquito populations and between two parental field populations, HAmCqG0 and MAmCqG0, and their permethrin selected offspring, HAmCqG8 and MAmCqG6, for different levels of insecticide resistance highlighted the importance of P450 genes in resistance by detecting the changes in their expression within each population following permethrin selection. Our results showed a dynamic change in the P450 genes expressed in both of the field mosquito strains of Cx. quinquefasciatus following permethrin selection. Interestingly, most of these up- and down-regulated P450 genes in Cx. quinquefasciatus were found to be developmentally regulated following selection: changes in the level of expression (either increasing [up-regulation] or decreasing [down-regulation]) in the larval stage of mosquitoes following the selection were not found in the adult stage and vice versa. However, several genes were identified that had up- or down-regulation patterns that not only reflected the permethrin selection but were also consistent in both the larval and adult stages of the mosquitoes, suggesting the importance of these genes in response to insecticide resistance over the mosquitoes’ whole life span. Comparison of the P450 gene expression between two different field mosquito populations following permethrin selection revealed that although both mosquito populations had a similar number of the P450 genes that were up- and down-regulated, the two populations for the most part regulated a different gene set in response to the insecticide selection. However, several genes were identified as being up- or down-regulated in either the larvae or adults for both HAmCqG0 and MAmCqG8; of these, CYP6A17 and CYP6B13 were up- and down-regulated, respectively, across all the life stages and populations of mosquitoes, suggesting that these genes are indeed related to insecticide selection. These results further propose that different mechanisms and/or P450 genes may be involved in the response to insecticide pressure for different developmental stages of mosquitoes and in different populations of mosquitoes [28]; some are specific to certain development stages and others provide protection throughout the insect’s life cycle.

Basal up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their
creased P450-mediated detoxification has been found to be pharmacological/toxicological effects [1]. In many cases, increased P450-mediated detoxification has been found to be associated with enhanced metabolic detoxification of insecticides, as evidenced by the increased levels of P450 proteins and P450 activity that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects [4,9,10,13–16,47–50]. In addition, multiple P450 genes have been identified as being up-regulated in several individual resistant organisms, including house flies and mosquitoes [12–14,16,49], thus increasing the overall expression levels of P450 genes. These findings suggest that overexpression of multiple P450 genes is likely to be a key factor governing increased levels of detoxification of insecticides and insecticide resistance. Nevertheless, although their importance in insect physiology and toxicology is widely recognized, there are gaps in our knowledge of insect P450s. One crucial piece of information that has been missing up until now is the issue of how many P450 genes are involved in insecticide resistance in a single organism, in this case the mosquito. The availability of the whole genome sequence of mosquito "Culex quinquefasciatus" [26] has enabled us to address this question by characterizing the expression profiles of P450s in insecticide resistant mosquitoes at a genome-wide level.

Our comparison of P450 gene expression profiles between two field mosquito populations following permethrin selection has revealed that although both mosquito populations have similar numbers of P450 genes that are up-regulated, for the most part the mosquito populations regulate an array of P450 genes that differ from each other. However, several P450 genes are up- and down-regulated across the two different field mosquito populations of HAmCq and MAmCq in the same way and these are distributed in families 9, 6, 4, and 325. This finding is in agreement with previous studies on the expression levels of P450 transcripts, which have often reported up-regulated expression of the P450 genes in insecticide resistant strains in CIP families 4, 6, and 9 [2–4,9,10,13,14,16,51–54] and suggested this to be a factor in the detoxification of insecticides. Unlike the previous studies, however, our study has for the first time uncovered abundant genes in CYP family 325 that are up-regulated in resistant mosquitoes in the same way as those in families 4, 6, and 9. In addition, a few of genes from clans 2 and mitochondria were up-regulated. This discovery brings new information to bear on the issue of which P450 genes and families might be involved in insecticide resistance. A previous study by our group [16] has indicated that four P450 genes, CYP6AI7, CYP74J4, CYP73J4, and CYP9M10, from mosquitoes Cx. quinquefasciatus are up-regulated and the overexpression levels of these four P450 genes are closely correlated to their levels of resistance, being markedly higher in HA MAmCq than in the parental HAmCq strain. The overexpression of CYP9M10 has also been reported in a resistant Culex mosquito strain in Japan and has been tentatively linked with pyrethroid resistance in Culex mosquito [49,50,55]. These four P450 genes have, again, been identified as being overexpressed in resistant mosquitoes across two different field populations, strongly suggesting a common feature of these P450 genes in pyrethroid resistance in Culex quinquefasciatus. The significant change in the expression of these P450 genes between field parental and permethrin selected highly resistant mosquito offspring, along with the sound correlation with the levels of P450 gene expression following permethrin selection, provides a strong case further supporting the importance of these P450 genes, particularly in families 9, 6, 4, and 325, in the response to permethrin selection of resistant mosquitoes and in the development of insecticide resistance.

Our study has also revealed a down-regulation characteristic of P450 gene expression following permethrin selection in Culex mosquitoes. The number of down-regulated P450 genes. The clans and CYP families over which these genes were found to be distributed were similar to the up-regulated P450 genes, mainly in

| Table 3. Up-regulation of P450 genes in adults of permethrin selected offspring of the field populations of Culex quinquefasciatus. |
|---|---|---|---|---|---|---|
| Mosquitoes | Transcript ID | Gene | Relative Gene expression ± SE | Parental strain | Resistant strain | Ratio |
| HAmCq (17) | CPIJ017199 | CYP325B1v1 | 0.9 ± 0.1 | 1.8 ± 0.3 | 2.0 |
| | CPIJ019587 | CYP6Z14 | 1.1 ± 0.3 | 2.3 ± 0.7 | 2.1 |
| | CPIJ010536 | CYP9J44 | 0.9 ± 0.06 | 1.9 ± 0.2 | 2.1 |
| | CPIJ005559* | CYP6A7 | 1.4 ± 0.2 | 3.2 ± 1.0 | 2.3 |
| | CPIJ015959 | CYP325B1J1 | 1.9 ± 0.1 | 4.3 ± 0.2 | 2.3 |
| | CPIJ015318 | CYP325Sv2 | 0.9 ± 0.02 | 2.2 ± 0.5 | 2.4 |
| | CPIJ010548* | CYP9J9 | 1.0 ± 0.1 | 2.5 ± 0.1 | 2.5 |
| | CPIJ016284 | CYP4J4 | 0.8 ± 0.05 | 2.0 ± 0.2 | 2.5 |
| | CPIJ011127 | CYP4H34 | 0.9 ± 0.08 | 2.4 ± 0.3 | 2.7 |
| | CPIJ012030 | CYP9AM1 | 0.9 ± 0.1 | 2.7 ± 0.6 | 3.0 |
| | CPIJ001758 | CYP4H38 | 0.7 ± 0.4 | 2.2 ± 0.7 | 3.1 |
| | CPIJ009085 | CYP6AG13 | 0.6 ± 0.2 | 1.9 ± 0.2 | 3.2 |
| | CPIJ012640 | CYP6CP1 | 0.9 ± 0.08 | 3.3 ± 1.0 | 3.7 |
| | CPIJ01943* | CYP4C52v1 | 0.8 ± 0.02 | 3.6 ± 2.0 | 4.5 |
| | CPIJ006721 | CYP4H37v1 | 0.6 ± 0.05 | 2.9 ± 0.8 | 4.8 |
| | CPIJ003389 | CYP6B7Y | 0.5 ± 0.2 | 2.7 ± 0.8 | 5.4 |
| | CPIJ010230 | CYP12F10 | 1.8 ± 0.1 | 10 ± 2.0 | 5.6 |
| MAmCq (15) | CPIJ005957 | CYP6A9 | 0.9 ± 0.09 | 1.9 ± 0.4 | 2.1 |
| | CPIJ015953 | CYP325B1v2 | 2.4 ± 0.8 | 5.3 ± 1.8 | 2.2 |
| | CPIJ007092 | CYP325Y7 | 0.9 ± 0.01 | 2.1 ± 0.4 | 2.3 |
| | CPIJ015961 | CYP325BE1 | 1.1 ± 0.2 | 2.5 ± 1.3 | 2.3 |
| | CPIJ010548* | CYP9J9 | 0.9 ± 0.04 | 2.2 ± 0.2 | 2.4 |
| | CPIJ007091 | CYP325Y6 | 1.3 ± 0.3 | 3.2 ± 1.7 | 2.5 |
| | CPIJ007090 | CYP325Y5 | 0.8 ± 0.03 | 2.0 ± 1.1 | 2.5 |
| | CPIJ005559* | CYP6A7 | 1.3 ± 0.5 | 3.4 ± 1.0 | 2.6 |
| | CPIJ015954 | CYP3253Nv1 | 0.7 ± 0.2 | 1.9 ± 0.5 | 2.7 |
| | CPIJ006952 | CYP325BG3 | 1.7 ± 0.7 | 5.9 ± 0.8 | 3.5 |
| | CPIJ010272 | CYP325B2 | 0.9 ± 0.07 | 4.1 ± 1.0 | 4.6 |
| | CPIJ014730 | CYP325AA2 | 0.3 ± 0.2 | 1.9 ± 0.6 | 6.3 |
| | CPIJ017021 | CYP325Kv1 | 3.0 ± 0.09 | 20 ± 1.6 | 6.7 |
| | CPIJ005685 | CYP325B2 | 0.9 ± 0.1 | 6.1 ± 2.0 | 6.8 |
| | CPIJ018943* | CYP4C52v1 | 0.2 ± 0.02 | 2.8 ± 0.7 | 14 |

*The transcript ID number from the vectorbase of the Cx. quinquefasciatus genome sequence (http://cquinquefasciatus.vectorbase.org/).

1. The relative level of gene expression represents the ratio of the gene expression in each resistant strain compared with that in the susceptible S-Lab strain. The relative level of gene expression represents for S-Lab is 1.
2. Parental strain for HM Cq population is HAm Cq and for MAmCq population is MAmCq.
3. Permethrin selected strain for HAmCq population is HAmCq and for MAmCq population is MAmCq.
4. The ratio of the relative gene expression in each permethrin selected strain compared its parental strain.
5. The genes that are up regulated in both adult of HAmCq and MAmCq.
6. The genes that are up regulated in both larvae and adults of each of HAmCq and MAmCq, or both.
7. doi:10.1371/journal.pone.0029418.t003
regulation could, for example, be linked to the homeostatic response that insects need to protect the cell from the toxic effects of extra P450 derived oxidizing species and metabolites from the up-regulated P450s and thus balance the usage of energy, O2, and other components needed for the synthesis of other processes [62]; and/or 3) a need for the tissue to utilize its transcriptional machinery and energy for the synthesis of other components involved in the inflammatory response [63]. These hypotheses all offer reasonable explanations for our observation of both up- and down-regulation of multiple P450 genes in the resistant mosquitoes following permethrin selection. P450 down-regulation could, for example, be linked to the homeostatic response that insects need to protect the cell from the toxic effects of extra P450 derived oxidizing species and metabolites from the up-regulated P450s and thus balance the usage of energy, O2, and the other components needed for the synthesis of proteins (including up-regulated P450s) that play important roles in insecticide resistance. It has been previously reported that some organophosphate insecticides require an oxidative biotransformation into more toxic structures that inhibit acetylcholinesterase, a process that is mediated by some P450 enzymes [2]. In such cases, a decrease in the expression levels of these CYP genes would be an advantage in the presence of an organophosphate insecticide by preventing its bioactivation by P450 enzymes. However, this argument may not apply to the permethrin used here for the selection of resistant mosquitoes [28,29].

Conclusions

The expression profiles of a total of 204 P450 genes in both larvae and adults of Cx. quinquefasciatus were compared between families 9, 6, 4 and 325. It has been pointed out that expression of many P450s is suppressed in response to various endogenous and exogenous compounds and this is also true for P450 suppression in vertebrates in response to pathophysiological signals [56–61]. Compared with our knowledge of P450 up-regulation involved in resistance, however, the mechanisms involved in P450 down-regulation and its relevance relating to resistance are poorly understood. It has been suggested that decreases in CYP gene expression could be an adaptive or homeostatic response [62,63]. A number of mechanisms have been proposed for P450 down-regulation, including: 1) an adaptive homeostatic response to protect the cell from the deleterious effects of P450 derived oxidizing species, nitric oxide, or arachidonic acid metabolites [63,64]; 2) a homeostatic or pathological response to inflammatory processes [62]; and/or 3) a need for the tissue to utilize its transcriptional machinery and energy for the synthesis of other components involved in the inflammatory response [65]. These hypotheses all offer reasonable explanations for our observation of both up- and down-regulation of multiple P450 genes in the resistant mosquitoes following permethrin selection. P450 down-regulation could, for example, be linked to the homeostatic response that insects need to protect the cell from the toxic effects of extra P450 derived oxidizing species and metabolites from the up-regulated P450s and thus balance the usage of energy, O2, and the other components needed for the synthesis of proteins (including up-regulated P450s) that play important roles in insecticide resistance. It has been previously reported that some organophosphate insecticides require an oxidative biotransformation into more toxic structures that inhibit acetylcholinesterase, a process that is mediated by some P450 enzymes [2]. In such cases, a decrease in the expression levels of these CYP genes would be an advantage in the presence of an organophosphate insecticide by preventing its bioactivation by P450 enzymes. However, this argument may not apply to the permethrin used here for the selection of resistant mosquitoes [28,29].

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susceptible and resistant mosquito populations, two different field populations of mosquitoes, and field parental mosquitoes and their permethrin selected offspring. The results provide direct evidence that up- and down-regulation of multiple P450 genes co-occur in the genome of Culex quinquefasciatus following permethrin selection. These genes are mainly distributed in class CYP3 and CYP4. These findings have important implications as they demonstrate that not only are multiple genes involved in insecticide resistance, but also multiple mechanisms are involved in P450 gene regulation. Both up- and down regulation of P450 genes may be co-responsible for the detoxification of insecticides, evolutionary insecticide selection, and the homeostatic response of mosquitoes to changing cell environments.

Supporting Information

Table S1: Oligonucleotide primers used for amplifying the P450 qRT-PCR reactions. 3The transcript ID number from the vectorbase of the Cx. quinquefasciatus genome sequence (http://cquinquefasciatus.vectorbase.org/). 3The annotation of the Culex quinquefasciatus genome (http://cquinquefasciatus.vectorbase.org). 3Specific primer pair designed according to each of the P450 gene sequences of the Cx. quinquefasciatus in vectorbase (http://cquinquefasciatus.vectorbase.org).

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Author Contributions

Conceived and designed the experiments: NL. Performed the experiments: TY. Analyzed the data: NL TY. Contributed reagents/materials/analysis tools: NL. Wrote the paper: NL.

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