Co-up-regulation of Three P450 Genes in Response to Permethrin Exposure in Permethrin Resistant House Flies, Musca domestica

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Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, *Musca domestica*

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Abstract

**Background:** Insects may use various biochemical pathways to enable them to tolerate the lethal action of insecticides. For example, increased cytochrome P450 detoxification is known to play an important role in many insect species. Both constitutively increased expression (overexpression) and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides. However, unlike constitutively overexpressed P450 genes, whose expression association with insecticide resistance has been extensively studied, the induction of P450s is less well characterized in insecticide resistance. The current study focuses on the characterization of individual P450 genes that are induced in response to permethrin treatment in permethrin resistant house flies.

**Results:** The expression of 3 P450 genes, CYP4D4v2, CYP4G2, and CYP6A38, was co-up-regulated by permethrin treatment in permethrin resistant ALHF house flies in a time and dose-dependent manner. Comparison of the deduced protein sequences of these three P450s from resistant ALHF and susceptible aabys and CS house flies revealed identical protein sequences. Genetic linkage analysis located CYP4D4v2 and CYP6A38 on autosome 5, corresponding to the linkage of P450-mediated resistance in ALHF, whereas CYP4G2 was located on autosome 3, where the major insecticide resistance factor(s) for ALHF had been mapped but no P450 genes reported prior to this study.

**Conclusion:** Our study provides the first direct evidence that multiple P450 genes are co-up-regulated in permethrin resistant house flies through the induction mechanism, which increases overall expression levels of P450 genes in resistant house flies. Taken together with the significant induction of CYP4D4v2, CYP4G2, and CYP6A38 expression by permethrin only in permethrin resistant house flies and the correlation of the linkage of the genes with resistance and/or P450-mediated resistance in resistant ALHF house flies, this study sheds new light on the functional importance of P450 genes in response to insecticide treatment, detoxification of insecticides, the adaptation of insects to their environment, and the evolution of insecticide resistance.
Background
Cytochrome P450s constitute the largest gene superfamily and are found in the organs and tissues of many organisms, including mammals, fish, plants, arthropods, fungi, and bacteria. Cytochrome P450s have long been of particular interest because they are critical for the detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens; and for metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Basal and up-regulation of P450 gene expression can significantly affect disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their pharmacological/toxicological effects [1]. Insect cytochrome P450s are known to play an important role in detoxifying insecticides [2,3] and plant toxins [4,5], resulting in the development of resistance to insecticides [3,6-10] and facilitating the adaptation of insects to their plant hosts [11,12]. A significant characteristic of insect P450s that is associated with enhanced metabolic detoxification of insecticides in insects is the constitutively increased levels of P450 proteins and P450 activity that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects [3,6-9,13,14]. Another feature of insect P450 genes is that the expression of some P450 genes can be induced by xenogenous and endogenous compounds [3], a phenomenon known as induction. It has been suggested that the induction of P450s and their activities in insects is involved in the adaptation of insects to their environment and the development of insecticide resistance [15,16].

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 [16] and to the development of resistance. The constitutively increased expression and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides, but unlike the constitutively overexpressed P450 genes, whose association with insecticide resistance has been extensively studied, the induction of P450s by xenobiotics, especially phenobarbital, is less well linked to insecticide resistance, although it is well documented in insects [17-22]. It has been proposed that many chemical inducers act as substrates for P450s and that the induction or modulation of P450s by the substrates will, in turn, reduce the effects of the substrates by enhancing substrate metabolism [23]. It has been suggested that the modulation of gene expression reflects a compromise between the insect's need to both conserve energy and adjust to a rapidly changing environment by enhancing the activity of the detoxification system only when a chemical stimulus occurs [24].

Result
Identification of P450 genes in response to permethrin challenge
In order to identify the P450 genes raised in response to the insecticide treatment, we used the PCR technique with degenerated PCR primers [29] to amplify P450 cDNAs from house flies. We initially isolated a total of 19 P450 cDNA fragments from ALHF house flies using three primer pairs, C2/Flyh1, C2/Flyc1, and HemeR1/CYP6AD1 (Table 1). Northern blot analysis was conducted using the 19 P450 cDNA fragments as probes to compare expression levels of these cDNAs in both permethrin treated and untreated susceptible CS and resistant ALHF house flies. Three of the 19 P450 cDNAs were found to be significantly induced in the permethrin treated ALHF house flies after 24 hours treatment (Fig. 1) at the pilot dose of LD50 that caused ~50% mortality of each house fly strain.

The full length of the three putative P450 cDNAs were isolated using 5′ and/or 3′ RACE with the primers (Table 1) designed based on the 5′ and/or 3′ end sequences of the putative P450 cDNA fragments. The sequences of the 5′ and/or 3′ RACE amplified cDNAs overlapped with their corresponding putative P450 cDNA fragments. An entire
cDNA fragment for each of the putative P450 genes was subsequently amplified for both ALHF and aabys house flies by PCR using the primer pair (Table 1) synthesized based on the respective 5' and 3' end sequences of each gene. The sequences were named CYP4D4v2 [accession number: EF615001, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=EF615001.1], CYP4G2 [accession number: EF615002, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=EF615002.1], and CYP6A38 [accession number: EF615003, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=EF615003.1] by the P450 nomenclature committee (Dr. D. Nelson, personal communication). The cDNA sequences of CYP4D4v2, CYP4G2, and CYP6A38 have open reading frames of 1515, 1647, and 1500 nucleotides encoding proteins of 505, 549, and 500 residues, respectively. Comparison of the deduced protein sequences of CYP4D4v2, CYP4G2, and CYP6A38 between ALHF and aabys revealed identical protein sequences, although several nucleotide polymorphisms were found in the coding regions of these three genes between the two strains (Figs. 2, 3, 4).

Response of P450 genes to permethrin challenge in resistant and susceptible house flies

Based on this pilot experiment, we focused our study on CYP4D4v2, CYP4G2, and CYP6A38 in order to further characterize their overexpression in response to permethrin challenge in resistant and susceptible house flies using the quantitative real-time PCR (qRT-PCR) method, as described below under Materials and Methods. To minimize the possibility that these P450 genes have nothing to do with resistance but arose solely because of a strain-strain difference, the study used ALHF and two susceptible house fly strains, CS and aabys. To examine the effect of permethrin on induction of the three target P450s, we measured the expression of the genes in house flies challenged with permethrin at a corresponding dose range (LD10, LD50, and LD90) for various durations. Although no induction was detected in either the susceptible CS flies of the aabys for the dose range and time intervals tested (data not shown), our results showed that permethrin induced all three P450 genes in ALHF with varying levels in a time (24 h)- and dose (LD50 -10 μg/fly)-dependent manner (Fig. 5). Based on these data, a dose of 10 μg/fly and a time interval of 24 h were chosen for the further induction studies.

We detected no significant induction in the expression of the three P450 genes in susceptible CS and aabys house flies that had either been treated with acetone alone or with permethrin solution in acetone compared with untreated house flies (Fig. 6). Similarly, no significant induction was obtained in acetone treated ALHF house flies compared with their untreated counterparts (Fig. 6). However, these three genes were induced at a variety of levels in permethrin treated ALHF house flies compared with untreated or acetone treated ALHF flies; a marked induction of CYP4D4v2 and CYP6A38 mRNA (~4-fold) were detected in permethrin treated ALHF house flies (Fig. 6B, C), whereas a low level (~1.5-fold) of induction for CYP4G2 was detected in the permethrin treated ALHF house flies (Fig. 6A). The significant induction of the three target P450 genes only in ALHF house flies suggests their importance in response to permethrin treatment in the resistant ALHF house flies. No significant differences were observed in the basal expression of CYP4D4v2, CYP4G2,
and CYP6A38 between ALHF and both or one of the susceptible CS and aabys strains without permethrin treatment (Fig. 6). These results suggest that, unlike some P450s in which constitutive expression may play important role in insecticide resistance [30-32], CYP4D4v2, CYP6A38, and/or CYP4G2, may be uniquely featured in ALHF in response to the insecticide exposure through induction of their expression, which, in turn, enhances their capacity to detoxify the insecticide and leads to enhanced insecticide resistance.

**Chromosomal linkage and allele determination of CYP4D4v2, CYP4G2, and CYP6A38**

We previously demonstrated that permethrin resistance in ALHF house flies was linked to autosomes 1, 2, 3, and 5, with major factors on autosomes 3 and 5 [26]. We also demonstrated that the P450-mediated resistance in ALHF was predominantly linked to autosome 5 [26]. To determine whether there is a causal link between the P450 genes and insecticide resistance, we examined the genetic linkage of CYP4D4v2, CYP4G2, and CYP6A38 with 5 back-cross (BC1) house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, by allele specific single nucleotide polymorphism (SNP) determination. Sequence comparisons of the three genes between ALHF and aabys revealed several nucleotide polymorphisms in the coding regions of CYP4D4v2 and CYP4G2, while no nucleotide polymorphisms were identified in the coding region of CYP6A38. We therefore cloned a ~700 bp 5' flanking region of CYP6A38 in order to genetically map the CYP6A38 gene. Comparison of the nucleotide sequence of the 5' flanking region of CYP6A38 uncovered several nucleotide polymorphisms between ALHF and aabys (data not shown), so the nucleotide polymorphisms, C to T, C to T, and G to T, in CYP4D4v2, CYP4G2, and CYP6A38, respectively, in ALHF relative to...
aabay (Fig. 7), were used to determine the linkage of P450 genes relative to the recessive morphological markers in the aabay strain. The SNP determination reactions were conducted for each of the genes using a specific primer (Fig. 7) designed according to the sequences immediately upstream of the nucleotide polymorphism in order to distinguish the single nucleotide polymorphism for the P450 allele in each house fly strain or line. Our results showed that the BC1 lines with the genotypes of ac/ac, +/ar, +/bwb, +/ye, +/su (A2345), +/ac, ar/ar, +/bwb, +/ye, +/su (A1345), +/ac, ar/ar, bwb/bwb, +/ye, +/su (A1245), and +/ac, ar/ar, bwb/bwb, +/ye, +/su (A1235) were heterozygous for CYP4D4v2 and CYP6A38, whereas as the BC1 line with the genotype of +/ac, ar/ar, bwb/bwb, +/ye, su (A1235) was homozygous for both the CYP4D4v2 and CYP6A38 alleles from aabys (Table 2). These results strongly indicate that both CYP4D4v2 and CYP6A38 are located on autosome 3 in house flies. The BC1 lines of A2345, A1345, A1234, and A1235 were heterozygous for CYP4G2, whereas the A1245 line was homozygous for the CYP4G2 allele from aabay (Table 2), indicating that CYP4G2 is located on autosome 3 in house flies.

Discussion

The primary goal of this study was to investigate whether insecticide resistant insects may be uniquely resistant to insecticides due to their ability to mount an adequate cellular response when challenged with insecticides by up-regulating the production of P450s, which, in turn, may significantly diminish toxicological effects of the insecticides to these insects [1]. It also appears that the induction of gene expression may reflect a good compromise between energy saving (i.e., enhancing the activity of the detoxification system only when a chemical stimulus occurs) and adjustment to a rapidly changing environment [33]. Multiple P450 genes that are induced in insects...
Figure 3
Alignment of the nucleotide and deduced amino acid sequences of CYP4G2 in ALHF and aabys house flies. The nucleotide polymorphisms between ALHF and aabys are highlighted and underlined.
may indicate a dysfunction of the induction system in insects that have been highly poisoned. A lack of induction of P450s has also been reported in Drosophila melanogaster when the insects were challenged with insecticides at concentrations that exceeded LC99 [44].

It has been proposed that induction and/or constitutive overexpression of P450s is linked to the adaptation of insects to their environment [15,16]. Further, in many cases increased levels of P450 gene expression have resulted in increased levels of both total P450 and the activities of those P450s, strongly suggesting this as a major cause of insecticide resistance [3,7,10,31,42]. We recently identified two P450 genes that were constitutively overexpressed in ALHF house flies [27,28]. Our current study identified a further three 450 genes that were co-up-regulated in response to permethrin exposure in ALHF house flies. Taken together, these findings suggest both constitutive overexpression and induction mechanisms participate in increasing P450-mediated metabolic detoxification of permethrin in resistant ALHF house flies and imply the role of these genes in the evolution of insecticide resistance. It has been proposed that there could be similar regulatory mechanisms governing P450 constitutive overexpression and induction, and that both contribute to the development of insecticide resistance [15]. Accordingly, we hypothesize that both induction and constitutive overexpression of P450 genes in the resistant ALHF share an altered regulatory system, which differs from that in the susceptible strains and regulate P450 gene expression in resistant house flies. Further study of the regulation of both constitutively overexpressed [27,28] and permethrin induced P450 genes will allow us to test this hypothesis.

Early studies in our laboratory on permethrin resistance in ALHF house flies led to the identification of permethrin

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**Figure 4**

Alignment of the nucleotide and deduced amino acid sequences of CYP6A38 in ALHF and aabys house flies. The nucleotide polymorphisms between ALHF and aabys are highlighted and underlined.
resistance that could be largely suppressed by PBO, an inhibitor of cytochrome P450s [25]. Furthermore, genetic linkage studies had associated permethrin resistance in ALHF to the autosomes 3 and 5, while PBO-suppressible resistance (or P450-mediated resistance) had been mainly tied to autosome 5, with minor factors linked to autosomes 1 and 2 [26]. The genetic linkage between an overexpressed P450 gene or protein and insecticide resistance appears to be an important step in establishing a causal link between a P450 gene and its role in resistance [3,6,32,45-47]. We therefore went on to examine the linkage of the three P450 genes with 5 house fly BC1 lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using allele specific PCR determination. The results revealed that both CYP4D4v2 and CYP6A38 were located on autosome 5, whereas CYP4G2 was located on autosome 3, of the house flies. Given that CYP4D4v2 and CYP6A38 are highly induced in ALHF and specifically located on autosome 5, on which P450-mediated resistance in ALHF has been mapped, it seems likely that the induction of CYP4D4v2 and CYP6A38 plays an important role in the development of insecticide resistance in ALHF house flies. Compared to the induction of the other two P450 genes, the relatively low level (~1.5-fold) of CYP4G2 induction obtained in the ALHF house flies may suggest its relatively minor role in resistance.

CYP4G2 has been linked on autosome 3. To our knowledge, this is the first report of a P450 gene located on autosome 3 of house flies. An earlier study indicated that although factors on autosome 3 were very important in the overall level of permethrin resistance in ALHF house flies [26], the resistance in ALHF governed by them was not suppressed by PBO [26]. These conflicting results may suggest that an ~1.5-fold level of induction of a P450 gene is too small to be detected by a synergism study. Alternatively, since PBO appears not to be a perfect inhibitor for some of the P450s responsible for resistance [3,48,49], the product of CYP4G2 may not be sensitive to the inhibition of PBO and the corresponding level of resistance due to the metabolism of the gene may therefore not be suppressed by PBO.

**Conclusion**

This study provides direct evidence that multiple P450 genes, CYP4D4v2, CYP4G2, and CYP6A38, are up-regulated in insecticide resistant house flies through the induction mechanism. Taken together with the induction of CYP4D4v2, CYP4G2, and CYP6A38 only in resistant house flies and the correlation linking the genes with the development of resistance and/or P450-mediated resistance in ALHF, this study suggests the functional importance of these three P450 genes in the increased detoxification of insecticides in ALHF. Our previous studies [27,28] have also indicated that two P450 genes are constitutively overexpressed in ALHF house flies. Taken together, these studies indicate that both P450 induction and constitutive overexpression may be co-responsible for detoxification of insecticides, evolutionary insecticide
selection, and the ability of insects to adapt to changing environments.

Methods

House fly strains

Three house fly strains were used in this study. ALHF is a wild-type strain collected from a poultry farm in Alabama in 1998, selected with permethrin for 6 generations, achieving a 6,600-fold resistance, and maintained under biannual selection with permethrin [25,26]. CS is a wild type insecticide-susceptible strain. aabys is an insecticide-susceptible strain with recessive morphological markers ali-curve (ac), aristapedia (ar), brown body (bwb), yellow eyes (ye), and snipped wings (sw) on autosomes 1, 2, 3, 4, and 5, respectively. Both CS and aabys were obtained from Dr. J. G. Scott (Cornell University).

Permethrin challenge experiments

Two-day old resistant ALHF and susceptible CS house flies were treated with permethrin by topical application [25,26] with 0.5 ul permethrin solution (in acetone) and acetone alone dropped on the thoracic notum. Preliminary dose range, time course, and P450 gene induction assays were performed with a corresponding dose range of LD_{10}, LD_{50}, and LD_{90} (2 ng/fly, 10 ng/fly, and 20 ng/fly for susceptible CS and aabys; 5 μg/fly, 10 μg/fly, and 20 μg/fly for ALHF). Based on the results, in which the induction of P450s reached its peak at 24 h after ALHF were treated with permethrin at the dose of LD_{50}, the dose of 20 μg/fly that resulted in 50–60% mortality for resistant ALHF were chosen as the challenge dose. The surviving flies were collected for RNA extraction after exposure to permethrin. The control flies that had not been exposed to permethrin treatment and acetone treated flies were collected on the same day as the permethrin treated flies. The experiments were repeated three times.

RNA extraction, cDNA preparation, and the putative P450 gene isolation

Total RNAs were extracted from the house flies using the acidic guanidine thiocyanate-phenol-chloroform method [7]. mRNA was isolated with oligotex-dT suspension as described by the manufacturer (QIAGEN). The first strand cDNA was synthesized with SuperScript II reverse transcriptase (Stratagene) and an antisense 5'-anchored oligo(dT) primer (Table 1) [50]. The PCR products were amplified using three primer pairs of C2/Flyh1, C2/Flyc1 and HemeR1/CYP6AD1 (Table 1). CYP6AD1 was designed based on a conserved amino acid region found in the house fly, Drosophila, and mosquitoP450 sequences after alignment of these insect P450 genes; Flyh1 was designed based on the heme binding consensus sequence
HemeR1 was generated based on the complementary sequence of Flyh1; and Flyc1 was synthesized based on a conserved 13 amino acid region found in rat, human, and insect P450 sequences [51]. The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced. Cloning and sequence analyses of

**Table 2: The nucleotide(s) at the polymorphism site of each P450 gene in different house fly strains and lines generated by the crosses and resistant ALHF and susceptible aabys strains.**

| House Fly Strain | P450 Gene | ALHF | aabys | BC1 Lines* (ALHF ♀ x aabys ♂) | BC1 Lines* (ALHF ♂ x aabys ♀) |
|------------------|-----------|------|-------|-----------------------------|-----------------------------|
|                  | CYP4G2    | C    | T     | A2345 C/T                    | A2345 C/T                   |
|                  | CYP4D4v2  | C    | T     | A1345 C/T                    | A1345 C/T                   |
|                  | CYP6A38   | G    | T     | A1245 T                       | A1245 T                     |
|                  |           |      |       | A1235 T                       | A1235 T                     |
|                  |           |      |       | A1234 T                       | A1234 T                     |

* These lines were named according to the autosomes bearing wild-type markers from ALHF. For example, the A1234 strain had wild-type markers on autosomes 1, 2, 3, and 4 from ALHF and the recessive mutant marker on autosome 5 from aabys (see details in Methods).
P450 gene fragments were repeated at least three times with different preparations of mRNAs. Three TA clones from each replication were sequenced.

**Rapid amplification of cDNA ends (RACE) of the putative P450 gene fragments**

RACE was carried out using the Marathon™ cDNA Amplification Kit (Clontech) as described by the manufacturer and Liu and Zhang [51]. The first strand cDNAs were synthesized with AMV reverse transcriptase using ALHF mRNAs as templates. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of each double strand cDNA using T4 DNA ligase as described by the manufacturer. The 5’ and/or 3’ ends of the P450 cDNA fragments were amplified by PCR using adapter primer AP1 and gene specific primers generated based on the 5’ and/or 3’ end sequences of the putative P450 cDNA fragments. The full lengths of putative P450 cDNAs were subsequently generated by reverse transcription PCR (RT-PCR) using specific primer pairs of AP450HF5F/AP450HF5R, AP450HF20F/AP450HF20R and P450HF16F-3/AP450HF17R (Table 1) synthesized based on the 5’ and 3’ end sequences of the putative P450 genes. Cloning and sequence analyses of the P450 cDNAs were repeated at least three times and three TA clones from each replication were verified by sequencing.

**Cloning and sequencing of the 5’ flanking region of CYP6A38 from ALHF and aabys**

House fly genomic DNAs were digested with 5 different restriction enzymes using the Universal GenomeWalker™ Kit (Clontech) and generated 5 adaptor-ligated ALHF genomic DNA libraries as described by the manufacture. The adaptor ligated DNA fragments in the GenomeWalker libraries were amplified by PCR with Advantage Tth polymerase (Clontech), the antisense primer, P450HF17P (Table 1), based on the 5’ coding region of the CYP6A38, and a sense primer, AP1, based on the sequence of the adaptor. The PCR products were cloned into the TA cloning vector (Invitrogen)and sequenced. Cloning and sequence analyses of PCR products were repeated at least three times each with three TA clones from each replication. The 5’ flanking region of CYP6A38 in aabys was subsequently generated by PCR from the genomic DNA using a primer pair, P450HF17P-F/P450HF17P (Table 1) designed according to the 5’ flanking region of CYP6A38 in ALHF.

**Northern blot analysis**

Northern blot analyses were performed according to Sambrook et al. [52]. Twenty micrograms of total RNA from each sample were fractionated on 1% formaldehyde denaturing agarose gel and transferred to Nytran membranes (Schleicher and Schuell) [52]. The P450 cDNAs were labeled with [α-32P] dCTP using a Primer-It II Random Primer Labeling kit (Stratagene) and hybridized with RNA blots using QuickHyb solution (Stratagene). The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer [53]. All Northern blot analyses were repeated three times with different preparations of RNA samples.

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

Total RNA samples (0.5 μg/sample) were 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. qRT-PCR 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× SYBR Green master mix, 1 μl of cDNA, and a gene specific primer pair, RTP450HF5F/RTP450HF5R, RTP450HF20F/RTP450HF20R, or RTP450HF17F/RTP450HF17R (Table 1), at a final concentration of 3–5 μM. A ‘no-template’ negative control and all samples 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 [54]. Relative expression levels for specific genes were calculated by the 2−ΔΔC(t) method using SDS RQ software [55]. The β-actin gene, an endogenous control, was used to normalize expression of target genes [56,57]. The preliminary assay had shown that the β-actin gene remained constant in different tissues and in both permethrin treated and untreated house flies and could therefore be used for internal normalization in qRT-PCR assays. Each experiment was repeated three 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.

**Genetic linkage analysis of cytochrome P450 genes**

To determine the genetic linkage of P450 genes, a genetic cross experiment was conducted [26,58]. Briefly, reciprocal crosses between ALHF and aabys were conducted and F1 males were back-crossed to aabys females. Five lines were saved from the back-cross generation (BC1+) with the genotypes of: ac/ac, +/ar, +/bwb, +/ye, +/sw (A2345); +/ac, ar/ar, +/bwb, +/ye, +/sw (A1345); +/ac, +/ar, +/bwb, +/ye, +/sw (A1245); +/ac, +/ar, +/bwb, ye/ye, +/sw (A1235); and +/ac, +/ar, +/bwb, +/ye, sw/sw (A1234). Since crossing over does not or very rarely occurs in male flies [59], the presence of a mutant phenotype indicated that the respective
The P450 specific alleles in ALHF and aabys and five house fly BC	extsubscript{1} lines were genetically mapped by SNP determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism	extsuperscript{TM} 3100 Genetic Analyzer using Genemapper software according to the manufacture’s instructions (A&B Applied Biosystems). Briefly, the cDNA fragments, which covered the SNP sites of P450 cDNAs, were generated by PCR. The SNP determination reactions were conducted for CYP4D4v2, CYP4G2, and CYP6A38 using allele specific primers, SNP450HF5F, SNP450HF20F, and SNP450HF17F, respectively (Table 1, Fig. 7), designed according to the sequences immediately upstream of the nucleotide polymorphism to distinguish the single nucleotide polymorphism for the P450 allele in each house fly strain or line. Three replications of the SNP determination were carried out with different preparations of the PCR templates. To confirm that the PCR products used for the SNP determination were in fact the P450 gene fragments, the PCR products were sequenced at least once each.

### Abbreviations

ANOVA: a one-way analysis of variance; BC	extsubscript{1}: back-cross generation 1; PBO: piperonyl butoxide; qRT-PCR: quantitative real-time PCR; RACE: rapid amplification of cDNA ends; RT-PCR: Reverse transcription PCR; SNP: single nucleotide polymorphism.

### Authors’ contributions

FZ was involved in coordinating and carrying out the experiments described in this report and wrote the manuscript. TL was involved in the dose range and time course studies. LZ was involved in the review of data, supervising implementation of the overall process, sequencing, and editing the manuscript. NL was involved in the overall design of study, review of data, and editing the manuscript. All authors read and approved the final manuscript.

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