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RESEARCH ARTICLE

Association of detoxification enzymes with butene-fipronil in larvae and adults of *Drosophila melanogaster*

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Abstract

Insecticide resistance is a major challenge in successful insect pest control as the insects have the ability to develop resistance to various widely used insecticides. Butene-fipronil is a novel compound with high toxicity to insects and less toxicity to the non-target organisms. In the present study, the effect of butene-fipronil alone and in combination with three enzyme inhibitors, piperonyl butoxide (PBO), diethyl maleate (DEM), and triphenyl phosphate (TPP), was carried out on larvae and adults of *Drosophilia melanogaster*. Our results indicated that the co-toxicity indices of butene-fipronil + PBO, butene-fipronil + TPP, and butene-fipronil + DEM mixtures were 437.3, 335.0, and 210.3, respectively, in the second-instar larvae, while 186.6, 256.2, and 238.5, respectively, in the adults, indicating synergistic effects. Interestingly, butene-fipronil increased the expression of CYP28A5 in the larvae; CYP9F2, CYP304A1, CYP28A5, and CYP318A1 in the female adults; and CYP303A1 and CYP28A5 in the male adults. Furthermore, high-level expression of *Est-7* was observed in the female adults compared to larvae and male adults. Our results suggest that there is no difference in butene-fipronil metabolism in larvae and male and female adults of *D. melanogaster*.

Keywords Butene-fipronil · Detoxification enzymes · *Drosophila melanogaster* · Synergists

Introduction

The use of insecticides has become an important part of modern era agriculture to protect crops from insect pest (Cooper and Dobson 2007). Therefore, the concerns regarding the exposure of these insecticides to natural enemies and other organisms in the ecosystem have been increasing with the passage of time (Liu et al. 2001). To combat this problem,

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insecticides with novel mode of action and formulation are designed with qualities like high toxicity to insect pests and be less toxic to the non-target organisms (Shakeel et al. 2017a). Butene-fipronil, a novel compound, is a GABA-gated chloride channel-blocking insecticide. It is not only effective against insect pests of Lepidoptera, Hemiptera, Coleoptera, and Orthoptera (Liu et al. 2009; Niu et al. 2008; Yuan et al. 2009), but also safe to non-target insects (Wang et al. 2013b; Yu et al. 2012), and other organisms like fish as well (Niu et al. 2008).

Insecticide resistance is also a major challenge for successful insect pest control. Metabolic resistance is based on enzymatic system and detoxification enzymes and is regarded as a common defense mechanism (Panini et al. 2016). An increase in the detoxification enzyme levels and/or reduction in target site sensitivity are assumed to be the base of insecticide resistance (Ffrench-Constant 1999). These detoxification enzymes belong to members of cytochrome P450, glutathione *S*-transferase (GST), UDP-glycosyltransferases (UGT), and esterase families and help the insect to detoxify xenobiotics into non-toxic compounds (Panini et al. 2016). Detoxification can be divided into three phases. During phase I, enzymes introduce reactive and polar groups into their substrates through oxidation, hydrolysis, or reduction. Following phase I, the activated metabolites are conjugated with compounds such as glutathione, sulfate, or glucuronate in phase II reactions. Finally, a variety of ATP-binding cassette transporters in Malpighian tubules are involved in the removal of the products generated in phase I and phase II reactions (van Leeuwen and Vermeire 2007).

To control insecticide resistance, the use of metabolic inhibitors (synergists) is considered as an important tool (Raffa and Priester 1985). The synergists are mixed with insecticide to enhance the toxicity of that particular insecticide at doses that normally are not toxic to an organism (Matsumura 2012). Piperonyl butoxide (PBO), diethyl maleate (DEM), and triphenyl phosphate (TPP) are the most widely used synergists and inhibit enzymatic system of P450, GST, and esterases, respectively (Scott 1991).

Several cases documented the metabolic resistance to fipronil, another GABA-gated chloride channel-blocking insecticide. A laboratory *Chilo suppressalis* strain showed 45.3-fold resistance to fipronil, and PBO, TPP, and DEF showed 7.55-, 1.93-, and 2.91-fold synergistic ratios to fipronil. Esterase activity in the resistant strain was 1.89-fold higher than that in susceptible strain (Li et al. 2007). In a field *Sogatella furcifera* strain 50.5-fold resistant to fipronil, TPP showed synergism on fipronil and esterase activity was greatly increased (Tang et al. 2010). Similarly, in *Apanteles plutellae*, obvious synergisms of PBO and TPP were observed on fipronil (Wu 2004). Inhibitor synergism and/or biochemical analysis in the above three cases imply that Ests confer metabolic resistance to fipronil. As for Cyps, in the western corn rootworm (*Diabrotica virgifera virgifera*, LeConte), fipronil is oxidatively converted to fipronil-sulfone by CYP (Scharf et al. 2000). In the German cockroach, *Blattella germanica* (L.), the prolonged exposure to baits containing fipronil increased physiological resistance to this compound. Fipronil exposure also increased cross-resistance to indoxacarb (Liang et al. 2017).

In the present study, the toxicity effects of three enzyme inhibitors (PBO, DEM, and TPP) and dietary butene-fipronil alone and in combination were tested to evaluate the involvement of these enzymes in butene-fipronil metabolism in *Drosophila melanogaster* larvae and adults. Furthermore, the expression of a set of CYP and *Est* genes was also measured after butene-fipronil exposure to *D. melanogaster*.

Materials and methods

Insect

The wild-type Canton-S strains of the *Drosophila melanogaster* larvae and adult flies were used in the experiments. The larvae were reared on the agar-based conventional diet having agar (0.7%), yeast 2%, cornmeal (5%), and molasses sucrose (10.5%) under managed temperature (25 ± 1 °C) and humidity (about 50%) and a photoperiod (12 h light/12 h dark) as per the method adopted by Wang et al. (2012, 2013a).

Chemicals

Butene-fipronil was purchased from Shenzhen Noposion Agrochemicals Co., Ltd. DEM, PBO, TPP, and acetone were procured from Shanghai Chemical Reagent (Shanghai, China), Sigma Chemical Co. (St. Louis, MO), Beijing Chemical Reagent (Beijing, China), and Nanjing Chemical Reagent Co., Ltd. (Nanjing, China), respectively. The purity levels of all of these were claimed to be 99%. Pureness of butane-fipronil was verified through injection into a Hewlett Packard 5988A gas chromatograph with a FID fitted, a split/splitless injector, and a fused silica capillary column (DB-35 ms, 30 m × 0.25 mm i.d., film thickness 0.25 mm, J&W Scientific, Folsom, CA), at a temperature of 200 °C in column and of 250 °C in vaporizing chamber and in detector.

All the chemicals used in the experiments were of analytical grade and were kept in a refrigerator (maintained at 4 °C) between the experimental sessions.

Bioassays

For the larvae, a method described previously (Wang et al. 2012) was used to determine toxicities of butene-fipronil and the three inhibitors (PBO, DEM, and TPP) individually and in binary combinations to the larvae. The butene-fipronil, the individual three inhibitors (DEM, TPP, and PBO), and their binary mixtures

were dissolved and further diluted in a serialized manner in order to get several solutions of variable concentrations.

An aliquot of 200 μL for test solutions (treatment) was mixed with 10 g of hot conventional artificial diet to prepare at least four to six toxic artificial diets within a mortality range of around 0–100% based on preliminary assays. The concentration range for the butene-fipronil was maintained from 0.01–10.00 mg of a.i. per 1 g of the diet. A negative control in the form of ddH_2O or acetone was maintained during the bioassay experiments.

A total of 30 4-day-old second-instar larvae were put in a vial (having length into diameter dimensions of 12 × 3 cm) having 10 g mixture of insecticide and diet or insecticide and ddH_2O controlled food, further sealed with the cotton plug. The three replicates having 90 second-instar larvae were kept at 25 °C, till the appearance of the adults, and the dead individuals (mortality) were noted after the adult emergence or eclosion.

Toxicities of the butene-fipronil and the three inhibitors (DEM, PBP, and TPP) in isolation and combination as binary mixtures were determined as described by Wang et al. (2013a).

Butene-fipronil, the three inhibitors (PBO, DEM, and TPP), and their binary mixtures were dissolved in and serially diluted twofold with acetone to obtain several concentrations. The concentrations 0.07, 0.05, and 0.03 mg/g for TPP, DEM, and PBO and 1.6000, 0.032, 0.0640, 0.0128, and 0.0025 mg/g for butene-fipronil were used in the experiments (Table 2). An aliquot of 200 μL of test solution (treatment) was mixed with 10 g of hot conventional artificial diet to prepare at least four toxic artificial diets within a mortality range of approximately 0–100% based on preliminary assays.

Negative control (acetone) was employed in all the bioassay trials. Thirty emerged adults were kept in the rearing vial (having length into diameter dimensions of 12 × 3 cm) having the diet (standard agar-based diet consisting of cornmeal-sucrose-yeast) for a period of 4 days. After the given period, these 90 adults were shifted into three vials, containing the uniform amount of the toxic diet media and the control in the form of the acetone control diet. The emerged flies were reared on standard environmental conditions and after rearing on toxic diet, mortalities were recorded for 24, 48, 72, and 96 h.

The mortalities of the control diet-fed larvae were less than 10%. The correction was done with the help of Abbott's formula (Abbott 1925). Probit analysis was utilized for calculating the 50% mortality (LC_{50}), their fiducial limits, and the slope of the line relating probit mortality to the log dose by POLO Plus logit probit software (LeOra Software Company, Petaluma, CA, USA).

For the synergistic effects of the inhibitors, DEM, TPP, and PBO were dissolved in the solvent, acetone. Due to the individual toxic effects of the enzyme inhibitors on the biology of immature and mature stages of *D. melanogaster*, the combinatorial effects could either be synergistic (greater than expected additive effect) or antagonistic (less than expected additive effect or summation).

The synergism/antagonism obtained from individual and binary mixtures was evaluated using the co-toxicity index method (Wang et al. 2015). When a summation is found in the mixture of butene-fipronil and the enzyme inhibitor, the lethal dose of the given mixture is as expected and co-toxicity index equals 100.

When the synergistic effect is found in the mixture of butene-fipronil and the enzyme inhibitor, the lethal dose is as expected with the co-toxicity index exceeding 100. Similarly, in the case of antagonistic effects, the toxicity of the mixture will be higher with the co-toxicity index being less than 100.

RNA isolation and cDNA synthesis

The immature (second-instar larvae) and the mature (adult flies) were exposed to the butene-fipronil at the levels of 0.084 and 0.105 μg/g diet, respectively, for 2 days, which equals the LC_{50} values. The samples of *Drosophila melanogaster* were collected from each treatment including control and then immediately frozen by liquid nitrogen, to prepare for RNA extraction.

Using the homogenization of the larval stage in the TrizolTM reagent (Invitrogen, Carlsbad, CA), the total RNA was extracted as per the instructions provided by the manufacturer. This total RNA was further treated with the RNase free DNase I (Ambion, Austin, TX) in order to eliminate trace amounts of the chromosomal DNA present in the sample. The amount and purity of the RNA were accessed utilizing the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). First-strand cDNA of each sample was synthesized from 1 μg of total RNA template using Moloney Murine Leukemia Virus reverse transcriptase (Takara, Bio Inc., Shiga, Japan) and an Oligo (dT) 18 primer. All the experiments were replicated three times.

Quantitative real-time polymerase chain reaction

A total of 11 genes were evaluated in *D. melanogaster* larvae. Among these 11 genes, 10 were CYP genes, namely CYP9F2, CYP4D2, CYP318a1, CYP4E2, CYP28A5, CYP303A1, CYP 4AA1, CYP305A1, CYP304A1, and CYP6W1. The remaining one was *Est-7*.

qPCR was utilized to estimate the mRNA abundance of these genes in each of the available templates, using housekeeping genes *RPL11* and *RPL32* as internal control for normalization (Shakeel et al. 2017b). Online tool of the primer designing, Primer Quest of Integrated DNA technologies Co. Ltd. (http//www.idtdna.com/Scitools/Applications/Primerquest/; Table 1), was used.

qPCR was performed using SYBR Premix Ex TaqTM (Perfect Real Time; Takara Co., Otsu, Japan) and ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City,

Table 1 The primer sequences of selected cyps and the internal control genes in *D. melanogaster* used in qPCR

Gene names	Flybase ID	Sense primers	Antisense primers
Cyp303a1	CG4163	GGGCTTCTGTTTCATGCACTTGGT	TGATAGCCTCGCAATAGGGCAACT
Cyp304a1	CG7241	TCATCCAGGAGCAGTTGAACGACA	TGGCCGAGAAGGGATTAAAGAGCA
Cyp305a1	CG8733	TGGCCGAGAAGGGATTAAAGAGCA	TGGATGAGATTGTAGCCAGTGCGA
Cyp28A5	CG8864	TCCACGACAATGAGATCGCCAAGA	TTCTCCACCCACTCGGTCAACTTT
CYP6w1	CG8345	ACATGGACGCATACATGCCGTTTG	TTTGAGGCCATAACCGGGCTAGTT
Cyp9 f2	CG11466	AGCACTCTGAGTCCGGCATTTACA	TCGAGGCGATCACATCATTGGTGA
CYP4aa1	CG8302	AAAGAGGTCAGGATGTGGCCATGA	TTTGCGTGTGAAGTCATTGAGGCG
Cyp4d2	CG3466	GGCGTGAAACGCTGGTTAACAACT	TCGTGTCATGGCCCTCGAACATAA
CYP4e2	CG2060	AGGAGCAACGCGAAGTAATGGGTA	AATCGTCCAATGAAAGGCACGCTG
CYP318a1	CG1786	GGCACTCCAACCAATTTCACCCAA	ACGAAATCCTCCAGCAATTTGGCG
α-est-7	CG17148	ACGACAAGGATGTGCTGGAGTTCT	GGGATGGGCCAAAGGCAAACATTA
RPL-32	CG7939	AAGAAGCGCACCAAGCACTTCATC	ACGCACTCTGTTGTCGATACCCTT
RPL-11	CG7726	AGGAACACATCGATCTGGGCATCA	ACTTCATGGCATCCTCCTTGGTGA

CA), according to the manufacturer's instruction. Each 20 μL qPCR reaction contained 10 μL of SYBR *Premix Ex Taq*TM (containing *TaKaRa Ex Taq*TM HS, dNTP Mixture, Mg^{2+}, SYBR Green I), 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.4 μL of Rox Reference Dye (50×), 2 μL of cDNA (0.9 ng), and 6.8 μL of double-distilled water.

The inclusion of no template control was done to evaluate the presence of any possible contamination. The PCR protocol included the denaturation at 95 °C which continued for 30 s, followed by 40 cycles of 95 and 60 °C for 5 and 31 s, respectively.

Following the amplification of DNA, heating of the sample up to 95 °C for 15 s was done to determine the melting curves, followed by cooling down to 60 °C for 15 s, and heating the samples to 95 °C for 15 s. To confirm the PCR products' purity, the amplification cycling parameters were set as 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 55 °C for 10 s with a dissociation curve generated from 65 to 95 °C (Shakeel et al. 2015). Gel electrophoresis was done to confirm the production of the specific PCR products. Primer pair efficiency was calculated by testing primer pair with logarithmic dilution of cDNA mixture and generation of the linear standard curve (crossing point plotted vs. log of template concentration). Each sample was triplicated. Analysis of data was done by utilizing $2^{-\Delta\Delta Ct}$ method as given by Pfaffl (2001), by the use of the geometric mean of two selected housekeeping genes *RPL11* and *RPL32* for normalization according to the previously described strategy (Pfaffl 2001; Vandesompele et al. 2002). The data were expressed as means ± SE. The difference of CYP activities was subjected to one-way ANOVA and followed by the Tukey–Kramer test for multiple comparisons using SPSS for Windows (SPSS, Chicago, IL). In all statistical analyses, a p value less than 0.05 was considered statistically significant.

Results

Synergistic effects of butene-fipronil on larvae of *Drosophila melanogaster*

D. melanogaster second-instar larvae were exposed to dietary butene-fipronil and the three enzyme inhibitors

Table 2 Toxicity of butene-fipronil and three enzyme inhibitors alone and in a binary mixture to *D. melanogaster* larvae exposed during second instar through adult eclosion

Insecticide	*N*	Slope (± SE)	LC_{50} (μg/g) (95%CL)	Co-toxicity index
TPP	450	2.80 (± 0.01)	70.1 (51.3–92.2)	
PBO	450	1.43 (± 0.12)	151.2 (41.1–550.0)	
DEM	450	1.89 (± 0.05)	120.4 (52.0–290.1)	
Butene-fipronil	450	1.50 (± 0.08)	0.06 (0.05–0.08)	
TPP + butene-fipronil	450	1.04 (± 0.01)	1.41 (0.41–2.74)	335.0
DEM + butene-fipronil	450	1.10 (± 0.07)	2.31 (1.03–3.15)	210.3
PBO + butene-fipronil	450	1.84 (± 0.11)	1.12 (0.34–2.43)	437.3

Triphenyl phosphate (TPP), diethylmaleate (DEM), and piperonyl butoxide (PBO) at the concentration of LC_{50} were mixed with butene-fipronil (at the concentration of LC_{50}) at a volume ratio of 100:1. The concentrations 0.07, 0.05, and 0.03 for TPP, DEM, and PBO and 1.6000, 0.032, 0.0640, 0.0128, and 0.0025 mg/g for butene-fipronil were used in experiments

(PBO, TPP, and DEM). The mortalities were recorded after adult eclosion. When used alone, the LC_{50} value for butene-fipronil was 0.06 μg/g, whereas LC_{50} values for PBO, TPP, and DEM were 151.2, 70.1, and 120.4 μg/g, respectively (Table 2). Although the toxicity of butene-fipronil was observed to be higher than that of each of the three enzyme inhibitors, the inhibitors also exhibited obvious toxic effects to the second-instar larvae.

Furthermore, co-toxicity index was used to evaluate the synergistic effects of the three enzyme inhibitors to butene-fipronil. LC_{50} values for butene-fipronil + PBO, butene-fipronil + TPP, and butene-fipronil + DEM mixtures were 1.12, 1.41, and 2.31 μg/g, respectively. The co-toxicity indices of three mixtures were 437.3, 335.0, and 210.3, respectively. All the indices were higher than 100, indicating synergistic effects (Table 2).

Synergistic effects of butene-fipronil on the adults of *Drosophila melanogaster*

The *D. melanogaster* adults were exposed to dietary butene-fipronil, PBO, TPP, and DEM, respectively. The mortalities were recorded 96 h after exposure. When used alone, the LC_{50} value for butene-fipronil was 1.05 μg/g, whereas LC_{50} values for PBO, TPP, and DEM were 368.4, 180.2, and 138.4 μg/g, respectively (Table 3). Although the toxicity of butene-fipronil was observed to be higher than that of each of the three enzyme inhibitors, the inhibitors also exhibited obvious toxic effects to the second-instar larvae.

Furthermore, co-toxicity index was used to evaluate the synergistic effects of the three enzyme inhibitors to butene-fipronil. LC_{50} values for butene-fipronil + PBO, butene-fipronil + TPP, and butene-fipronil + DEM mixtures were 8.41, 5.88, and 6.17 μg/g, respectively. The co-toxicity indices of three mixtures were 186.6, 256.2, and 238.5, respectively. All the indices were higher than 100, indicating synergistic effects (Table 3).

Butene-fipronil-inducible Cyp genes in larvae of *D. melanogaster*

A total of 10 representative CYP genes, CYP4AA1, CYP4D2, CYP4E2, CYP6W1, and CYP9F2, CYP28A5, CYP303A1, CYP304A1, CYP305A1, and CYP318A1, were selected and their expression levels in control and butene-fipronil-treated larvae were measured by qPCR (Fig. 1).

At the concentrations of 0.084 μg/g diet, butene-fipronil increased the CYP28A5 mRNA level, with the relative expression level more than 2.0. Moreover, butene-fipronil slightly enhanced (1.5–2-fold) the expression of CYP4E2, CYP303A1, CYP304A1, CYP305A1, and CYP6W1 (Fig. 1).

Butene-fipronil-inducible Cyp genes in the adults of *D. melanogaster*

The expression levels of the same 10 representative *Cyp* genes including CYP4AA1, CYP4D2, CYP4E2, CYP6W1, CYP9F2, CYP28A5, CYP303A1, CYP304A1, CYP305A1, and CYP318A1 were measured in the male and female adults of *D. melanogaster* (Fig. 2). At the concentration of 1.03 μg/g diet, butene-fipronil increased the expression level of CYP18A1 up to 8-fold. Furthermore, the expression of CYP9F2, CYP304A1, CYP28A5, and CYP318A1 was also increased up to 2-fold by butene-fipronil (Fig. 2).

For the male adults, butene-fipronil at the concentrations of 1.03 μg/g diet increased the expression level of CYP303A1 and CYP28A5 more than 2-fold. Moreover, butene-fipronil slightly enhanced (1.5–2-fold) the expression of CYP4D2, CYP9F2, CYP304A1, and CYP6W1 (Fig. 3).

Butene-fipronil induced Est-7 expression

The expression level of the representative esterase gene *Est-7* was estimated by qPCR in the second-instar larvae and male and female adults of *D. melanogaster* (Fig. 4). The results indicated that butene-fipronil slightly increased (1.3-fold) the expression level of *Est-7* at the concentration of 0.084 μg/g in

Table 3 Toxicity of butene-fipronil and three enzyme inhibitors alone and in a binary mixture to *D. melanogaster* adults

Insecticide	*N*	Slope (± SE)	LC_{50} (μg/g) (95%CL)	Co-toxicity index
TPP	450	1.41 (± 0.12)	180.2 (42.3–450.1)	
PBO	450	2.88 (± 0.09)	368.4 (77.4–387.6)	
DEM	450	2.01 (± 0.15)	138.4 (50.1–371.4)	
Butene-fipronil	450	1.50 (± 0.01)	1.05 (0.45–2.58)	
TPP + butene-fipronil	450	2.13 (± 0.21)	5.88 (2.47–9.13)	256.2
DEM + butene-fipronil	450	1.10 (± 0.01)	6.17 (2.84–9.77)	238.5
PBO + butene-fipronil	450	1.84 (± 0.01)	8.41(3.53–12.58)	186.6

Triphenyl phosphate (TPP), diethylmaleate (DEM), and piperonyl butoxide (PBO) at the concentration of LC_{50} were mixed with butene-fipronil (at the concentration of LC_{50}) at a volume ratio of 100:1

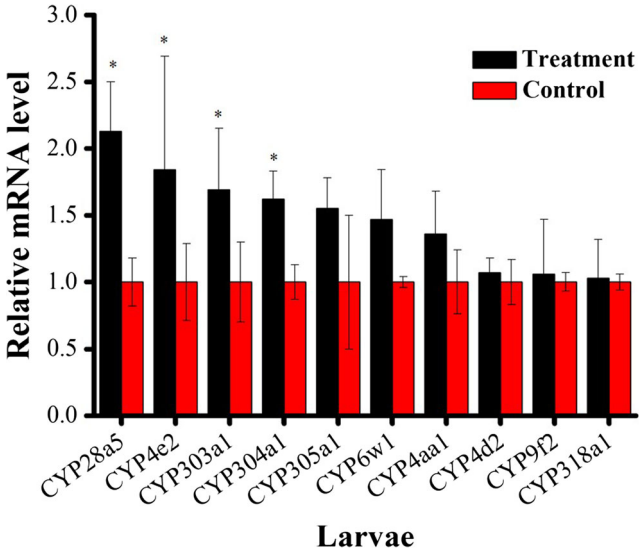

Fig. 1 The relative expression levels of representative Cyp genes induced by butene-fipronil in the second-instar larvae of *D. melanogaster*. For each sample, three independent pools of 5–30 individuals were measured in technical triplicate using qPCR. The relative copy numbers of *Cyps* were calculated according to the $2^{-\Delta\Delta Ct}$ method, using the geometric mean of the internal control genes for normalization. The relative expression levels were the ratios of relative copy numbers in individuals of butene-fipronil treated to that in control. The columns represent averages with vertical lines indicating SE (*sig at $p = 0.05$)

the second-instar larvae. Whereas, the expression of *Est-7* was enhanced up to 2.2-fold at the concentrations of 1.03 μg/g in the male adults and up to 4.8-fold in the female adults (Fig. 4).

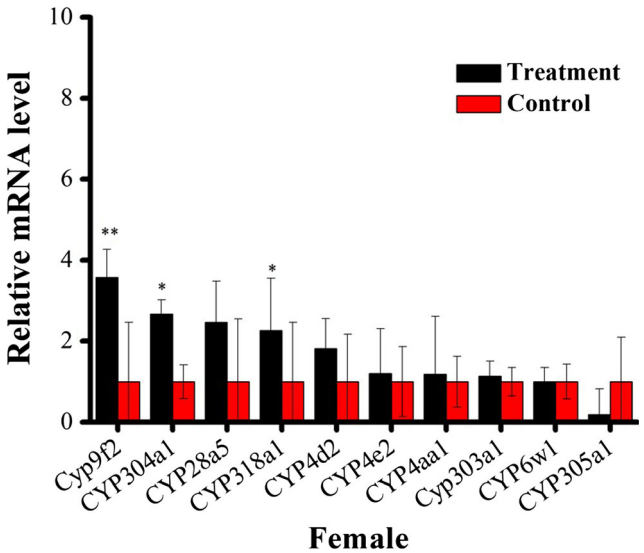

Fig. 2 The relative expression levels of representative *Cyp* genes induced by butene-fipronil in the female adults of *D. melanogaster*. For each sample, three independent pools of 5–30 individuals were measured in technical triplicate using qPCR. The relative copy numbers of *Cyps* were calculated according to the $2^{-\Delta\Delta Ct}$ method, using the geometric mean of the internal control genes for normalization. The relative expression levels were the ratios of relative copy numbers in individuals of butene-fipronil treated to that in control. The columns represent averages with vertical lines indicating SE (*sig at $p = 0.05$)

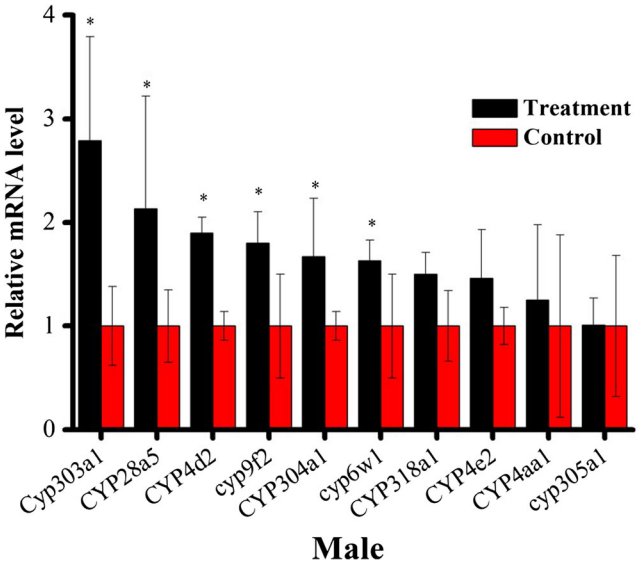

Fig. 3 The relative expression levels of representative *Cyp* genes induced by butene-fipronil in the male adults of *D. melanogaster*. For each sample, three independent pools of 5–30 individuals were measured in technical triplicate using qPCR. The relative copy numbers of *Cyps* were calculated according to the $2^{-\Delta\Delta Ct}$ method, using the geometric mean of the internal control genes for normalization. The relative expression levels were the ratios of relative copy numbers in individuals of butene-fipronil treated to that in control. The columns represent averages with vertical lines indicating SE (*sig at $p = 0.05$)

Discussion

In our previous studies, we found that butene-fipronil was more toxic to larvae than to the adults, with the ratios of

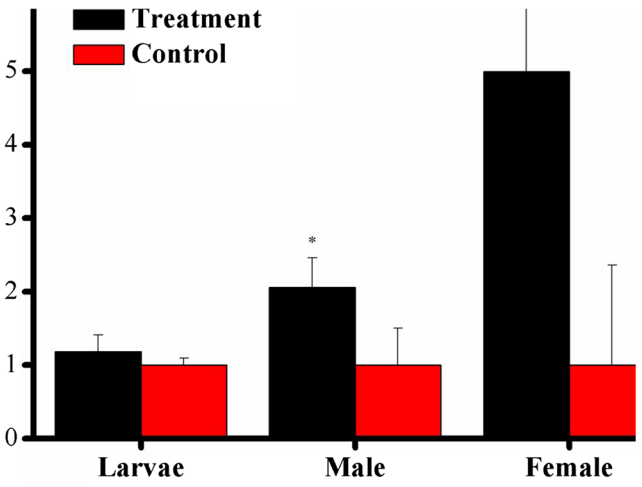

Fig. 4 The relative expression level of *Est-7* induced by butene-fipronil in the larvae and male and female adults of *D. melanogaster*. For each sample, three independent pools of 5–30 individuals were measured in technical triplicate using qPCR. The relative copy numbers of *Est-7* were calculated according to the $2^{-\Delta\Delta Ct}$ method, using the geometric mean of the internal control genes for normalization. The relative expression levels were the ratios of relative copy numbers in individuals of butene-fipronil treated to that in control. The columns represent averages with vertical lines indicating SE (*sig at $p = 0.05$)

LC_{50} values for adults to corresponding LC_{50} values for larvae being 16 and 45, respectively (Arain et al. 2014). Both larvae and adults can detoxify butene-fipronil by several types of enzymes. Among these enzymes are GSTs, esterases, and cytochrome P450 monooxygenases. Therefore, we hypothesized that there may be a difference in detoxification abilities of larvae and the adults.

In the present study, three enzyme inhibitors were selected to test synergistic effects on butene-fipronil. Among them, diethylmeleate (DEM) and triphenyl phosphate (TPP) are specific inhibitors of GST and esterases, respectively, whereas piperonylbutoxide (PBO) has the ability to inhibit both cytochrome P450 monooxygenases and esterases (Moores et al. 2009). Our results showed that LC_{50} values for PBO, TPP, and DEM were 151.2, 70.1, and 120.4 μg/, respectively, to the larvae and 368.4, 180.2, and 138.4 μg/g, respectively, to the adults. Our results are consistent with those of our previous study that revealed that the LC_{50} values for PBO, TPP, and DEM were 0.15, 0.05, and 0.16 mg/g to *D. melanogaster* larvae (Wang et al. 2012) and 0.370, 0.179, and 0.140 mg/g to *D. melanogaster* adults when exposed to methanol (Wang et al. 2013a). These results indicate that the three inhibitors exhibited obvious toxic effects to larvae and the adults of *D. melanogaster*.

Thus, the co-toxicity index was used to evaluate the synergistic effects of the three enzyme inhibitors to butene-fipronil. The co-toxicity indices of butene-fipronil + PBO, butene-fipronil + TPP, and butene-fipronil + DEM mixtures were 437.3, 335.0, and 210.3, respectively, in the second-instar larvae, and 186.6, 256.2, and 238.5, respectively, in the adults. All the indices were higher than 100, indicating synergistic effects. These data indicated that cytochrome P450 monooxygenases, esterases, and GST may be involved in the detoxification of butene-fipronil and may be a potential mechanism of resistance to butene-fipronil.

In *D. melanogaster*, 90 CYPs were identified (Feyereisen 2011). These CYPs fall into CYP2, CYP3, and CYP4 and mitochondrial clades and are known to redundantly catalyze an extremely diverse range of chemical reactions important in foreign compound detoxification (Feyereisen 2005). Cytochrome P450s form a diverse and vital gene superfamily present in almost all organisms. In both plants and mammals, some P450s are known to carry out reactions essential for processes such as hormone synthesis, while others are involved in the detoxification of environmental compounds. Moreover, in *D. melanogaster*, P450s are involved in behavioral phenotypes and CPY6A20 are associated with aggressive behavior in males (Dierick and Greenspan 2006). Compared to the plants and mammals, much less is known about the functions of the different insect P450 enzymes. One exception is the involvement of P450s in the biosynthesis of the major insect hormone 20-hydroxyecdysone (20H) from plant sterols, where in *Drosophila melanogaster* at least six P450s are involved (Gilbert 2004). Characterizing the *Drosophila melanogaster* P450 expression patterns in embryos and two stages of third-instar larvae additionally also identified numerous P450s expressed in the fat body, Malpighian (renal) tubules, and in distinct regions of the midgut, consistent with hypothesized roles in detoxification processes, and other P450s expressed in organs such as the gonads, corpora allata, oenocytes, hindgut, and brain (Chung et al. 2009). In the present study, a subset of 10 CYP genes was selected to examine their inducibility by butene-fipronil. Our results showed that several CYP genes were upregulated after ingestion of butene-fipronil by the larvae and the adults. In the larvae, butene-fipronil increased CYP28A5 expression level. In the females, butene-fipronil enhanced the expression of CYP9F2, CYP304A1, CYP28A5, and CYP318A1. In the male adults, butene-fipronil increased CYP303A1 and CYP28A5 expression levels. Interestingly, CYP28A5 was the common gene showing upregulated expression in the larvae and the male and female adults. CYP28A5 was induced by phenobarbital in both sexes and by atrazine in males of *D. melanogaster* (Le Goff et al. 2006; Willoughby et al. 2006). However, whether CYP28A5 is involved in the detoxification of butene-fipronil needs further experimental evidence to confirm.

In *D. melanogaster*, α-EST7 is one of the evolutionary ancient members, which predicts important physiological function. α-EST7 structural mutation leads to the increased hydrolase activity to organophosphate pesticides and causes the insecticide resistances in several higher dipteran species including the housefly *Musca domestica* (Claudianos et al. 1999), the blowfly *Lucilia cuprina* (Newcomb et al. 1997), and *D. melanogaster* strains (Wilson 2001). Our results indicated that the expression of *Est-7* was slightly increased in the second-instar larvae, whereas it was increased up to 2-fold in the male adults, and up to 4-fold in the female adults. Our results suggest that *Est-7* is a candidate esterase to detoxify butene-fipronil in *D. melanogaster*.

In conclusion, our results suggest that the detoxification metabolism to butene-fipronil was not much stronger in the adults than that in the larvae. Thus, detoxification by GST, esterases, UDP-glycosyltransferases (UGT), and cytochrome P450 monooxygenases was not responsible for the difference in toxicity between the larvae and the adults of *D. melanogaster*.

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