Broflanilide prolongs the development of fall armyworm *Spodoptera frugiperda* by regulating biosynthesis of juvenile hormone

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Abstract: Broflanilide, a novel meta-diamide insecticide, has been registered worldwide to control agricultural pests, and cause negative influence to insect under not only lethal but also sublethal level in the field. Fall armyworm (FAW) *Spodoptera frugiperda* is a worldwide distributed insect frequently controlled by using insecticides, and the potential regulating mechanism of broflanilide on this key insect prompt careful characterization. In the present study, we exposed FAW larvae to sublethal dose (LD_5), low lethal doses (LD_{10} and LD_{30}) and median lethal dose (LD_{50}) of broflanilide and measured various subsequent physiological sublethal effects. FAW larvae body length became shorter (LD_5-LD_{50}), the larvae and pupae duration were increased by 0.96-4.63 days (LD_5-LD_{50}), and the juvenile hormone (JH) titer significantly increased up to 134.46% (LD_{10}-LD_{30}). Meanwhile, the JH acid methyltransferase gene (*JHAMT*) and farnesyl diphosphate synthase 1 (*FPPS*1), which are critical enzymes of JH biosynthesis, increased by 2.07- and 2.18- fold in LD_{10} broflanilide-treated group, and by 2.22- and 1.78- fold in LD_{30} broflanilide-treated group in 3rd day larvae of FAW, respectively. In the 12-hour-old adults, *SfrFPPS*1 increased to 1.92-fold in LD_{30} broflanilide-treated group. Broflanilide induced multiple physiological sublethal effects on the biosynthesis of JH by regulating the expression of *SfrFPPS* and *SfrJHAMT* genes in FAW and likely in other insects (both pests and non-target organisms). Therefore, its potential for Integrated Pest Management should be further assessed.

Keywords: sublethal effect; hormone titer; juvenile hormone acid methyltransferase gene; farnesyl diphosphate synthase gene; mRNA levels

1 Introduction

Broflanilide is a novel meta-diamide insecticide that shows high insecticidal activity on insect pests, e.g. on cotton bollworm *Helicoverpa armigera* (Hübner), rice stem borer *Chilo suppressalis* Walker, common cutworm *Spodoptera litura* Fabricius, beet armyworm *Spodoptera exigua* (Hübner) and cotton leafworm *Spodoptera littoralis* (Boisduval). In 2021, it has been used in China for control of the diamondback moth *Plutella xylostella* (Linnaeus) and the flea beetle *Phyllotreta vittuta* Fabr. (AgroNews, 2021; Katsuta et al., 2019; Qi et al., 2017). The activity of broflanilide depends on its demethylation product acting on insect γ-aminobutyric acid (GABA) receptor (GABAR) with unique mode of action (Nakao & Banba, 2016).

To date, application of insecticides is still the critical measure to prevent the outbreak of agricultural pests. However, large-scale usage of insecticides usually caused unpredictable disadvantages in the field, such as insecticidal resistance, population growth, community structure, environmental pollution and sublethal effects on organisms (Desneux et al., 2007; Lira et al., 2020; Mansouri et al., 2017; Meng et al., 2020; Qu et al., 2020; Shen et al., 2021; Xu et al., 2017). Lethal, sublethal and transgenerational hormesis effects are commonly used insecticides. Insects are exposed to low concentrations in the field after initial application, i.e. the pesticides degrade daily after field application (da Silva et al., 2021). Insecticide doses (or concentrations) targeting pests may have sublethal effect to beneficial insects due to environmental factors and metabolic process (Desneux et al., 2007). Exposure to lethal or sublethal doses of insecticides could influence the whole lifecycle of insects, including growth, development, reproduction and lifespan, *etc.* (Han et al., 2012; Lai & Su, 2011; Shen et al., 2021; Xu et al., 2017). For example, in *Frankliniella occidentalis* (Pergande), the egg stages was decreased and the preadult duration, adult longevity, pre-oviposition and total pre-oviposition period was shorted at sublethal concentrations of spirotetramat (Liang et al.,

2021). The sublethal concentrations of chlorantraniliprole prolonged larval duration and reduced hatching rate of eggs in *S. exigua* (Lai & Su, 2011), decreased survival rate and reproduction in *P. xylostella* (Han et al., 2012). The mean generation time of F_1 melon aphid *Aphis gossypii* Glover was extended after F_0 exposure to the low lethal and sublethal concentration of imidacloprid (Ullah et al., 2019). They can also drive habitat changes, induce hormesis effects in key pests, resistance development, modulate both direct and indirect interactions among species within food webs, and lead to secondary pest outbreaks (Paula et al., 2021; Desneux et al., 2007; Liang et al., 2021; Qu et al., 2020; Ullah et al., 2019; Ullah et al., 2021).

The fall armyworm (FAW), *Spodoptera frugiperda* Smith & Abbot (Lepidoptera: Noctuidae) is one of the most destructive crop pests worldwide due to its polyphagous nature and voracity of feeding (Day et al., 2017; Kebede & Shimalis, 2019; Montezano et al., 2018). Since broflanilide will be used worldwide and may cause potential negative effect to environment and organisms, the sublethal effects on growth and development of FAW, juvenile hormone (JH) titer, and the change in expression of JH biosynthesis related genes, JH acid methyltransferase (*JHAMT*) and farnesyl diphosphate synthase (*FPPS*), were evaluated after FAW were treated with sublethal and lethal doses of broflanilide to explore the regulation mechanism of broflanilide to insect *in vivo*.

2. Materials and methods

2.1 FAW and broflanilide

FAW was collected from Guangdong province (113°E, 23°N) in April 2019, and reared with a standard artificial food at temperature of 26 ± 2 °C, relative humidity (RH) of 60-70 % and photoperiod of 16:8 h (light: dark). Broflanilide with technical grade (purity > 98.67 %) was obtained from BASF Corporation (Florham Park, NJ, USA).

2.2 Treatment of FAW with sublethal and lethal doses of broflanilide

Third instar FAW larvae were treated with sublethal dose 5% (LD_5), low lethal doses (LD_{10} and LD_{30}) and median lethal dose (LD_{50}) of broflanilide, respectively as described previously (Jia et al., 2020; Meng et al., 2020). Briefly, broflanilide was dissolved in acetone, subsequently diluted in 0.1% Tween-80 solution (1:1, v/v), and then mixed with 100-fold volume of fresh artificial food (v/v) with final concentrations of 0.056 (LD_5), 0.074 (LD_{10}), 0.132 (LD_{30}) and 0.199 (LD_{50}) mg kg^{-1} (Zhan et al., 2021). The artificial food containing broflanilide was cut into slices and put into 12-well-plates. Three hundred FAW larvae were used for bioassay, and sixty FAW larvae were divided into three technical repetitions for each concentration. FAW larvae treated with artificial food containing only acetone and 0.1% Tween-80 was used as control. Three days later, the body length of survival FAW was measured, and then the FAWs were transferred into another 12-well-plate containing a new artificial food without broflanilide, acetone or Tween-80. The duration of larvae and pupae of broflanilide-treated larvae was also counted.

For biochemical and molecular assays, the 3rd instar FAW larvae were individually transferred into 12-well-plates containing artificial food with LD_{10} or LD_{30} broflanilide, and the artificial food containing only acetone and 0.1% Tween-80 was used as control. Two hundred 3rd instar larvae were used for each treatment. Three days later, survival FAW larvae were transferred into another 12-well-plate containing a fresh artificial food without broflanilide, acetone or Tween-80. Six survival larvae at 3rd day (3rd day larvae), pre-pupae, female pupae at 1st day (1st day pupae), 3rd day (3rd day pupae) and 5th day (5th day pupae), and female adults at 0th hour (newly emerged adults), 12 hours (12-hour-old adults) and 1st day (1st day adults) were collected, immediately frozen in liquid nitrogen, and stored in the -80 ^{o}C refrigerator. Six individuals from each group were collected in triplicate.

2.3 Measurement of JH titer

FAW samples were ground into powder using pre-cooled pestles and mortars, and the titers of JH was measured with the Insect JH ELISA Kit (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, CHN) (Liu, 2019). Briefly, 100 mg ground powder was mixed with 900 μL ice-cold phosphate buffered solution (PBS, 0.01M, pH=7.4), and centrifuged for 5 min at 5,000 g to obtain the supernatant. Firstly, in the reaction well of microtiter plate, the supernatant, standard substance and 100 μL enzyme conjugate were mixed and incubated for 60 minutes at 37 °C. Secondly, the reaction wells were treated with washing solution for 5 times. Thirdly, 50 μL of substrate A and 50 μL substrate B were added into each reaction well, gently mixed and incubated for 15 min at 37 °C without light. Fourthly, 50 μL stop solution was added into each reaction well and the optical density (OD) was read at 450 nm using a Microtiter Plate Reader Rayto, RT-6100 (Rayto Life and Analytical Sciences Co., Ltd, Shenzhen, Guangdong province, CHN) within 15 minutes. Finally, the standard curve is generated by plotting the average OD_{450nm} obtained for each of six standard concentrations on the vertical (X) axis versus the corresponding concentration on the horizontal (Y) axis, and the JH titers were calculated according to the standard curve.

2.4 Cloning and analysis of JHAMT and FPPS genes

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) and purified using the RNA Clean Kit (TianGen Biotech (Beijing) Co., Ltd, Beijing, CHN). The complementary DNA (cDNA) was reverse - transcribed by the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology Co. Ltd, Beijing) (Jia et al., 2020). Three putative genes related to JH synthesis were identified from a published FAW transcriptome (GenBank: GESP00000000.1) using the BLAST with amino acid query sequences from

Bombyx mori Moore [GenBank accession number: NP_001036901.1 (*BmJHAMT*), BAF62113.1 (*BmFPPS1*) and NP_001093301.1 (*BmFPPS2*)]. The open reading frames (ORFs) of these genes were amplified using the specific primers (Table S1) and 2 × Phanta® Max Master Mix (Vazyme Biotech Co., Ltd, Nanjing, CHN). The amplified DNA products were purified using the *EasyPure*® PCR Purification Kit (TransGen Biotech Co., Ltd, Beijing), ligated into the *pEASY*®-Blunt 3 Cloning Vector (TransGen Biotech Co., Ltd), transferred into *Trans*1-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech Co., Ltd) and sequenced using the Sanger sequencing (BGI Tech Solutions Co., Limited, Beijing).

The sequenced nucleotides were analyzed using DNAMAN 7 software (Lynnon Corporation, San Ramon, CA) and conserved domains were predicted by alignment to other published orthologs. The confirmed amino acid sequences were also aligned with those from other insects to construct phylogenetic trees with 1000 bootstrap replications using MEGA 7 (Kumar et al., 2016), and the neighbor-joining method was used to evaluate the branch strength of each tree. The phylogenetic trees were annotated using the EvolView (https://www.evolgenius.info) (He et al., 2016).

2.5 Detection of mRNA relative expression

The relative mRNA expression levels of the putative JH synthesis genes in eight developmental stages of FAW and in broflanilide-treated FAW were measured by real-time quantitative polymerase chain reaction (RT-qPCR). For RT-qPCR reaction, 20 μL reactions were performed on a Quant Studio™ 6 Flex Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA) using TB Green ® *Premix Ex Taq* ™ (Tli RNaseH Plus) (Takara Biomedical Technology Co., Ltd) (Jia et al., 2020). The relative mRNA expression levels of the putative genes were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak & Schmittgen, 2001).

Primers for putative genes and reference gene (Table S1) were designed by Becon Designer 8.13 (Premier Biosoft International, Palo Alto, CA).

2.6 Statistical analysis

Values for insect body length, larvae and pupae duration, ~~relative~~ JH titer and relative mRNA levels were shown as mean ± standard error (SE), and the statistical figures were generated by GraphPad 5 (GraphPad Software, Inc., La Jolla, CA). Significant differences were analyzed using IBM SPSS Statistics 22 (International Business Machines Corporation, Armonk, NY) by one-way ANOVA with Tukey tests, and values were considered statistically significant when $P < 0.05$.

3 Results

3.1 Broflanilide reduced larvae body length and prolonged development duration of FAW

The 3rd instar FAW larvae were treated with sublethal, low lethal and median lethal doses of broflanilide, and the body length of FAW was measured at 3rd day after treatment. Compared to the control group, the body length was significantly shorter in broflanilide-treated group (Fig. S1). The average body length of FAW were decreased to 12.15 (LD_5), 11.37 (LD_{10}), 9.86 (LD_{30}) and 7.11 (LD_{50}) mm from 16.21 mm (control) ($F = 321.908$, df = 4, 249, $P < 0.0001$; Fig. 1).

Compared with the control group, the duration of larvae was significantly prolonged for 1.02, 1.13, 2.34 and 4.63 days in LD_5, LD_{10}, LD_{30} and LD_{50} broflanilide-treated group, respectively ($F = 115.028$, df = 4, 189, $P < 0.0001$; Fig. 2A), and the duration of pupae was prolonged for 0.96, 1.38, 2.31 and 3.63 days, respectively ($F = 63.734$, df = 4, 177, $P <$

0.0001; Fig. 2B).

3.2 Broflanilide induced the JH titer in FAW

The JH titer in LD_{10} and LD_{30} broflanilide-treated FAW were determined (Fig. 3). Compared with the control group, the JH titer in all tested stages including 3rd larvae (F = 406.984, df = 2, 6 $P < 0.0001$), prepupae (F = 56.880, df = 2, 6, $P < 0.0001$), 1st day pupae (F = 246.415, df =2, 6, $P < 0.0001$), 3rd day pupae (F = 63.770, df = 2, 6, $P < 0.0001$), 5th day pupae (F = 341.477, df = 2, 6, $P < 0.0001$), newly emerged adults (F = 271.597, df = 2, 6, $P <$ 0.0001), 12-hour-old adults (F = 237.408, df = 2, 6, $P < 0.0001$) and 1st day adults (F = 80.789, df = 2, 6, $P < 0.0001$) were significantly increased to 22.32%-116.76% in LD_{10} group, and 37.94%-134.46% in LD_{30} group, respectively. Compared with the LD_{30} broflanilide-treated group, the JH titer in LD_{10} broflanilide-treated group was lower at 3rd instar larvae and higher at 5th day pupae, newly emerged adults, 12-hour-old adults and 1st day adults.

3.3 Isolation and characterizations of *JHAMT* and *FPPS* from FAW

The full-length of cDNA of *SfrFPPS1*, *SfrFPPS2* and *SfrJHAMT* were cloned by RT-PCR. The ORF of *SfrJHAMT* contains 816 base pair (bp) encoding 271 amino acid residues with a conserved SAM binding motif (motif I) hh(D/E)hGxGxG (at position 38-46, Fig. S2A), where of "h" represents a hydrophobic amino acid residue. The ORFs of *SfrFPPS1* and *SfrFPPS2* contain 1,287 and 1,056 bp, respectively, encoding 428 and 351 amino acid residues that contain the first-aspartate rich motif (FARM) (NDIME, N178-E182 for *Sfr*FPPS1; and DMIMD, D102-D106 for *Sfr*FPPS2) and second-aspartate rich motif (SARM) (DDFLD, D318-D322 for *Sfr*FPPS1; and DDYID, D241-D245 for *Sfr*FPPS2) (Fig. S2B).

*Sfr*JHAMT shares the highest amino acid identity with *Bm*JHAMT (52.52%) and 50.29%

and 48.25% amino acid identities in pairwise comparisons with *Dp*JHAMT and *Ha*JHAMT, respectively (Fig. S3A). The deduced amino acid of *Sfr*FPPS1 showed 91.59%, 85.28% and 83.88% identities with *Ha*FPPS1, *Px*FPPS1 and *Bm*FPPS1, respectively, while of *Sfr*FPPS2 shared 48.12% and 49.10% identities with *Cf*FPPS2 and *Bm*FPPS3, respectively (Fig. S3B).

3.4 Change of *SfrJHAMT* and *SfrFPPS* during development

The relative mRNA levels of *JHAMT*, *FPPS1*, and *FPPS2* in eight developmental stages of FAW were determined (Fig. 4). The mRNA levels of *SfrFPPS2* and *SfrJHAMT* were highest at the 5th day pupae, and lowest at the 1st day pupae or 3rd day larvae (*SfrFPPS2*: $F = 35.574$, df = 7, 58, $P < 0.0001$; *SfrJHAMT*: $F = 50.114$, df = 7, 59, $P < 0.0001$). In contrast, except for the 1st day pupae, the mRNA level of *SfrFPPS1* was not significantly different at the 3rd day larvae, pre-pupae, 3rd day pupae, newly emerged adults and 12-hour-old adults stages ($F = 19.177$, df = 7, 58, $P < 0.0001$).

3.5 Effect of broflanilide on *SfrJHAMT* and *SfrFPPS* mRNA

Sublethal effects of broflanilide to *SfrJHAMT* and *SfrFPPS* were examined in three representative stages (Fig. 5). Compared with the control group, the mRNA levels of *SfrJHAMT* and *SfrFPPS1* were significantly increased to 2.07- and 2.18- fold in 3rd day larvae in LD_{10} broflanilide-treated group, 2.22- and 1.78- fold in LD_{30} broflanilide-treated group (*SfrJHAMT*: $F = 45.417$, df = 2, 24, $P < 0.0001$; *SfrFPPS1*: $F = 11.647$, df = 2, 24, $P < 0.0001$; Fig. 5A and 5B), whereas the mRNA level of *SfrFPPS2* was significantly decreased ($F = 51.747$, df = 2, 24, $P < 0.0001$; Fig. 5C). In the 5th day pupae, the mRNA levels of *SfrFPPS1* and *SfrFPPS2* in LD_{30} broflanilide-treated group significantly down-regulated (*SfrFPPS1*: $F = 72.912$, df = 2, 24, $P < 0.0001$; *SfrFPPS2*: $F = 30.230$, df = 2, 24, $P < 0.0001$; Fig. 5B and 5C), while the mRNA level of *SfrFPPS1* in LD_{10} broflanilide-treated group up-regulated (Fig.

5B). For the 12-hour-old adults, the mRNA level of *SfrFPPS*1 was significantly increased by 1.92-fold in LD_{30} broflanilide-treated group ($F = 28.320$, df = 2, 24, $P < 0.0001$; Fig. 5B).

4 Discussion

Broflanilide, as a newly registered meta-diamide insecticide, has excellent insecticidal activity against lepidopteran pests including *P. xylostella*, FAW and the rice leaf folder *Cnaphalocrocis medinalis* (Guenée) (Xu et al., 2019). In addition, broflanilide at sublethal or lethal doses can interrupt the normal physiological processes of FAW, such as significantly shortening the body length and prolonging the development phase (Fig. 1 and 2).

As one important hormone in insects, the JH participates in various biological processes by regulating the insect ecdysis and development (Jindra et al., 2013; Qu et al., 2018). Consist with the shorten body and delayed durations, JH titers in the broflanilide-treated groups were significantly higher than those in the control group (Fig. 3). In fact, the change of JH titer in insect treated with sublethal doses of insecticides and toxins has been reported (Pérez-Hedo et al., 2011; Yu et al., 2010; Yu et al., 2007). For example, sublethal concentrations of chlorantraniliprole up-regulated JH titer for up to 41.38% during the lifespan of *C. suppressalis*, with prolonged duration of larvae for 2.34 days (Xu et al., 2017). Sublethal concentration of imidacloprid led to a significant up-regulation of JH titer combined with change of fecundity in both of peach aphid *Myzus persicae* (Sulzer) (Yu et al., 2010) and *C. suppressalis* (Yu et al., 2007). In the corn borer *Sesamia nonagrioides* Lefebvre, sublethal dose of *Bt* protein also altered the JH titer (Pérez-Hedo et al., 2011).

As is well established, JH biosynthesis is regulated by the mevalonate pathway and JH branch (Rivera-Perez et al., 2014). Both JHAMT and FPPS play important roles in the biosynthesis of JH. JHAMT is a critical enzyme that converts JH acids or inactive precursors of JHs to active JHs at the final step of JH biosynthesis pathway in insects (Shinoda &

Itoyama, 2003). FPPS, a crucial enzyme of biosynthesis of JHs, could catalyze the form of farnesyl diphosphate (FPP), which is the precursor of JH (Alam et al., 2022). To explore whether broflanilide affects these pathways, we firstly isolated the *JHAMT* and *FPPS* from FAW, and detected their expression levels (Jindra et al., 2013; Shinoda & Itoyama, 2003). In this study, one *JHAMT* and two *FPPS* genes were cloned from FAW. We found that only one *JHAMT* and at least two *FPPS* appear ubiquitous to lepidopteran insects, such as *Pieris rapae* L., *Papilio xuthus* L.*,* and *C. suppressalis, etc*. However, there are three and six *FPPS*s in *B. mori* and *H. armigera*, respectively (Bomtorin et al., 2014; Kinjoh et al., 2007; Li et al., 2013; Shinoda & Itoyama, 2003; Xu et al., 2017).

To make clear the effect of broflanilide to JHAMT and FPPS, the LD_{10} and LD_{30} were selected because the duration between LD_5 and LD_{10} broflanilide-treated groups are same and the LD_{50} is not a sublethal dose. As shown in Fig. 5, the mRNA levels of *SfrJHAMT* were significantly up-regulated in 3^{rd} day larvae in LD_{10} and LD_{30} broflanilide-treated groups. These findings are similar to those reported in *P. xylostella* (Duan, 2016). When *M. persicae* nymphs were treated with sublethal concentration of precocene, the mRNA levels of *MpFPPS* decreased up to 31-fold in adults (Ayyanath et al., 2015). When *C. suppressalis* was treated with LC_{30} chlorantraniliprole, the mRNA levels of *CsJHAMT* in larvae, and of *CsFPPS*2 in 5^{th} day female pupae as well as in older female adults were significantly higher than those in the control group (Xu et al., 2017). Therefore, we speculated that sublethal and low lethal doses of broflanilide could induce the up-regulation of JH biosynthesis related genes resulting in the increase of JH titer, which might delay the development of FAW.

In fact, people always paid more attention to the sublethal effects of pesticides on no-targeted insect, especially beneficial arthropods, e.g. *Coccinella septempunctata* L. (He et al., 2019) , *Harmonia axyridis* (Pallas) (Oliveira et al., 2019), *Trichogramma brassicae* Bezdenko (Parsaeyan et al., 2020), *Apis mellifera* (L.) (Williams et al., 2020) and African

honey bee (*A. mellifera intermissa*) (Menail et al., 2020). In this study, we used the FAW to explore the potential sublethal effect of broflanilide to insect, because there are similar regulation pathways or mechanism among target and non-target insect. In addition, exploring the sublethal effect of novel insecticides to pest insects can provide useful information on pest resurgence and a more definitive conclusions on suitability for Integrated Pest Management (Desneux et al. 2007; Shah et al., 2020) .

In conclusion, broflanilide has been approved for crop protection in China and will likely be widely used, its sublethal effects on organisms including insect should be studied. Based on the results of this study, we concluded that broflanilide could shorten the body size and prolong developmental phase of FAW larvae and pupae. In addition, the broflanilide probably affect the JH titers by regulating JH biosynthesis genes, including *JHAMT* and *FPPS*.

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453

454 **Figure Legends**

455 **Fig. 1** The body length of FAW after three days' treatment of sublethal dose
456 broflanilide. Histograms bearing different letters are significantly different ($P < 0.05$,
457 ANOVA followed by a Tukey's post hoc test).

458 **Fig. 2** Duration of larvae (A) and pupae (B) after FAW larvae treated with low
459 lethal doses (LD_{10} and LD_{30}) of broflanilide (CK=control). Histograms bearing
460 different letters are significantly different ($P < 0.05$, ANOVA followed by a Tukey's
461 post hoc test).

462 **Fig. 3** Change of JH titers in FAW after exposure to LD_{10} and LD_{30} of broflanilide
463 (CK=control). Histograms bearing different letters, per FAW development step, are
464 significantly different ($P < 0.05$, ANOVA followed by a Tukey's post hoc test).

465 **Fig. 4** Relative mRNA levels of *SfrJHAMT*, *SfrFPPS1* and *SfrFPPS2* genes in FAW.
466 Histograms bearing different letters are significantly different ($P < 0.05$, ANOVA
467 followed by a Tukey's post hoc test).

468 **Fig. 5** Change of mRNA levels after exposure of FAW to low lethal doses (LD_{10}
469 and LD_{30}) of broflanilide (CK=control). Histograms bearing different letters, per FAW
470 development step, are significantly different ($P < 0.05$, ANOVA followed by a
471 Tukey's post hoc test).

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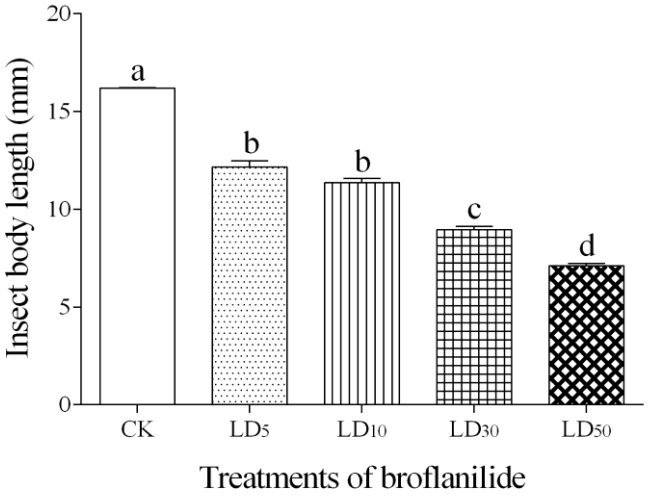

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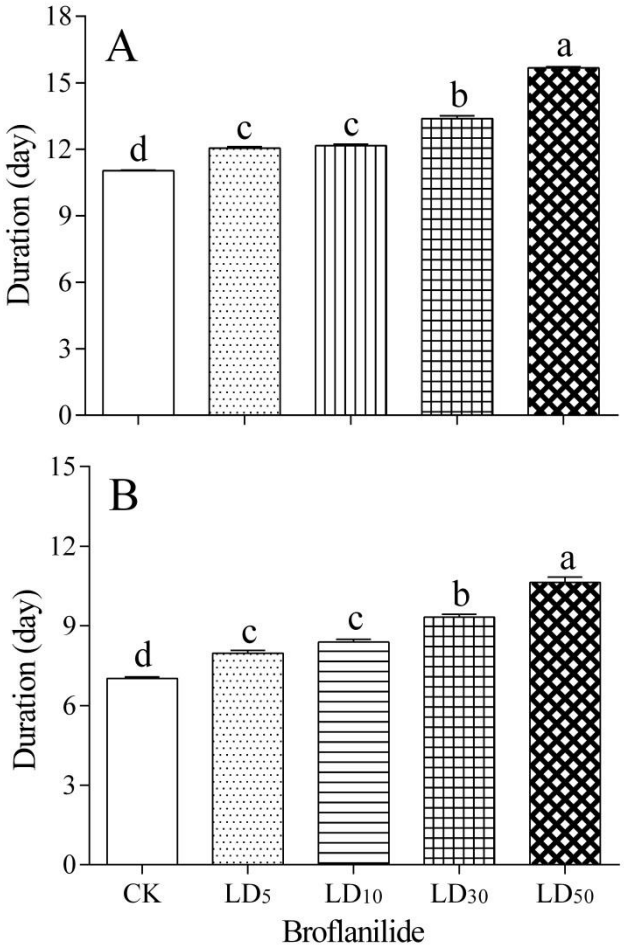

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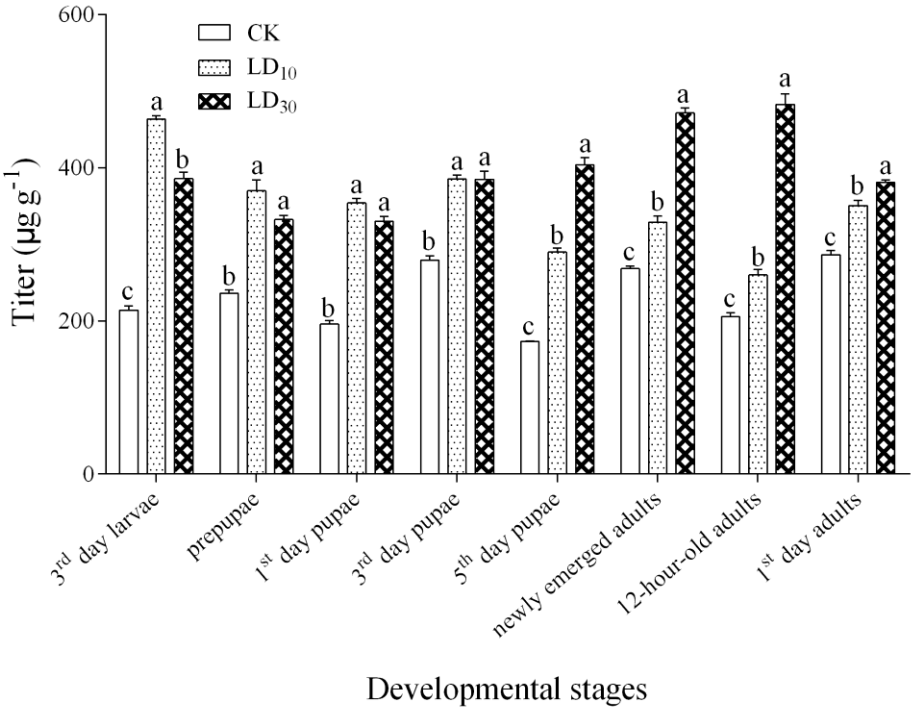

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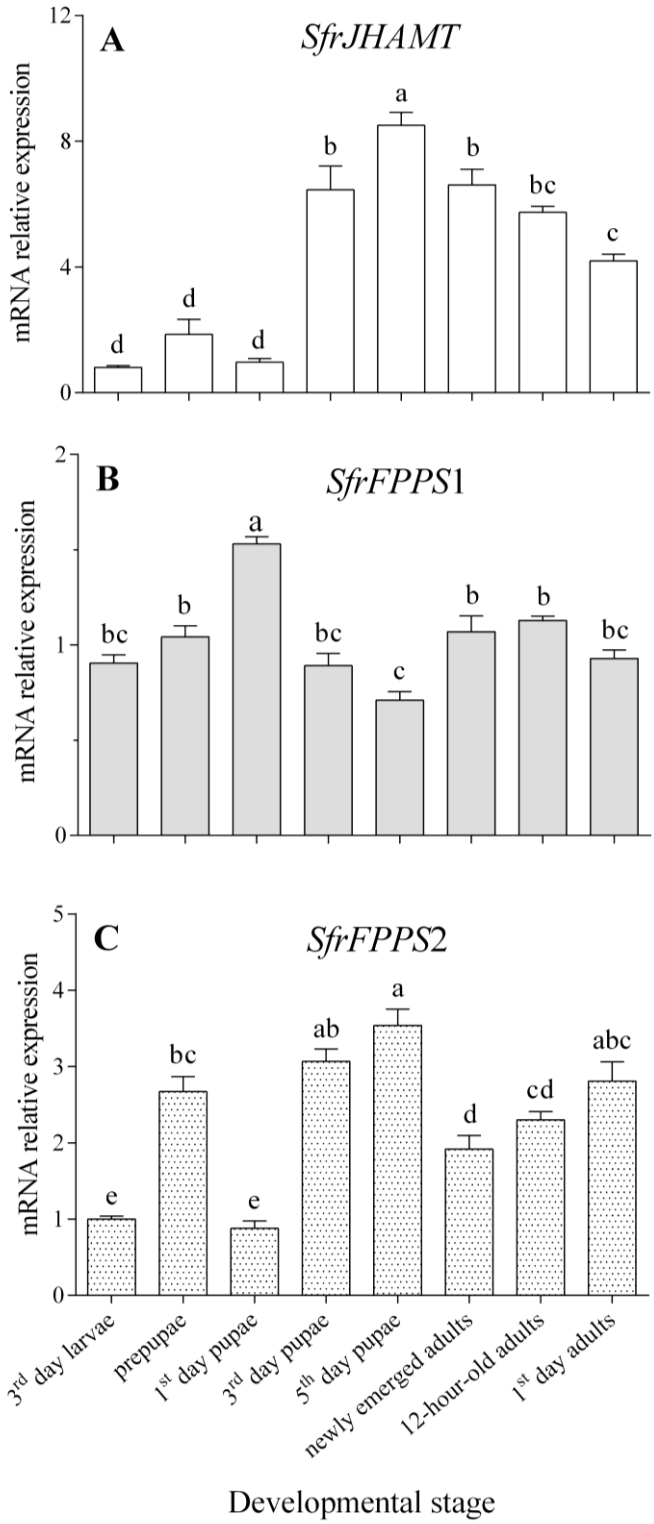

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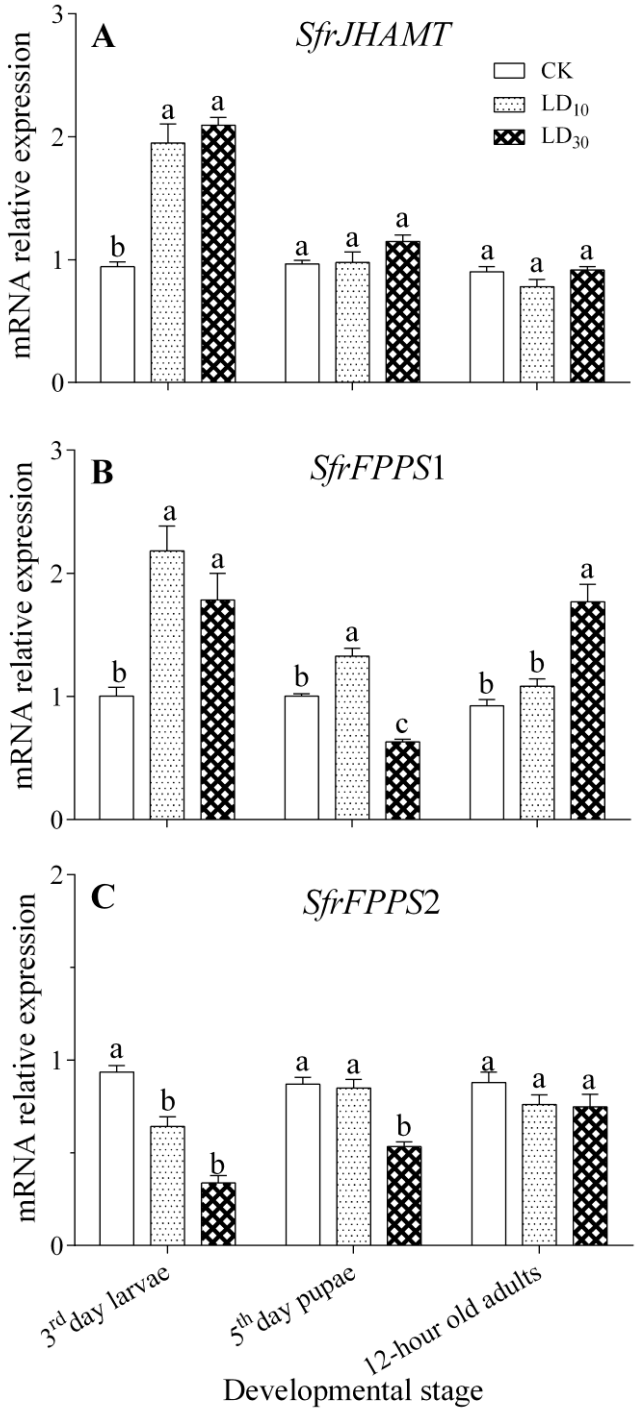

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