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Sublethal effects of fenoxycarb on female reproduction in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest that causes severe damage to economical crops in most parts of the world. The juvenile hormone analogues are a group of insect growth regulators that increase larval ages and disrupt larval development. Moreover, they affect reproductive system in adult insects. In this study, the effects of sublethal doses of fenoxycarb on fecundity, fertility, and longevity parameters, as well as on oocyte size, ecdysteroid titer in ovaries, and expression level of the ultraspiracle (*USP*), ecdysone receptor (*EcR*), hormone receptor 3 (*HR3*), and vitellogenin (*Vg*) genes in adult female *H. armigera*, were examined. Feeding on artificial diet containing fenoxycarb (LC10 and LC25) significantly decreased the percentage of larval survival rate, pupation rate, pupal weight, fecundity, fertility, and oviposition period. It also clearly shortened female longevity. Fenoxycarb treatment suppressed the expression of the *USP*, *EcR*, and *Vg* genes in *H. armigera* but had no effect on the *HR3* gene expression level in either pupae or adults. Overall, our results show that using fenoxycarb as a juvenile hormone analogue can reduce reproduction in *H. armigera* and may work as an alternative to the use of insecticides for controlling this important agricultural pest.

Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae), or cotton bollworm, is one of the important pests of many plants that are important in agriculture. This insect larva is a polyphagous pest and feeds on more than 200 types of agricultural products, including soybean, cotton, chickpea, peas, tobacco, corn, and a number of other crops and vegetables. The larvae show a high tendency to feed on the reproductive organs of these plants, which has a significant effect on reducing their production. The use of chemical pesticides is the simplest and least costly method of control in cotton bollworm, but especially in recent years, the use of pesticides has caused many problems, including environmental pollution, human health threats, and pesticide resistance. Alternative bollworm-control methods that reduce the use of chemical pesticides in agricultural lands and protect natural enemies are of interest to researchers (Lawo *et al.* 2008).

Hormones play an important role in controlling insect reproduction. Juvenile hormone and ecdysteroids play an important role in their physiology (Franssens *et al.* 2006). These hormones are involved in regulating the physiological processes throughout the life cycle of insects, especially the reproductive system, which produces vitellogenin and oocyte maturation. Ecdysteroid regulates the reproductive system, including oocyte maturation and vitellogenesis. (Khalid *et al.* 2021). Insect growth regulators that mimic the activity of the juvenile hormone increase larval age. In addition, Sorge *et al.* (2000) found that pseudo-hormones sterilise insects and disrupt reproductive behaviour. The application of pyriproxyfen, a juvenile hormone analogue, on adult

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female *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) could decrease reproduction. It reduced oviposition, shortened life span, and affected the expression of vitellogenin and ecdysteroid receptors in *S. litura* (Xu *et al.* 2015). Based on this evidence, the use of hormone analogues can interfere with the reproduction of insects (Xu *et al.* 2015). The insect growth regulators, pyriproxyfen, flufenoxuron, and chlorfluazuron, were evaluated on various reproductive aspects of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Xu *et al.* 2015). Toxicity and morphological deformities induced by the insect growth regulators were seen. These insect growth regulators have potential for controlling *S. littoralis* (Shaurub *et al.* 2020). Moreover, in a study by Martínez *et al.* (2021), fenoxycarb, methoxyfenozide, and tebufenozide were found to be effective against *Euprosterina elaeasa* (Dyar) (Lepidoptera: Limacodidae), inhibiting feeding in larvae, with less leaf area consumed, thereby indicating potential for controlling this pest.

Mahmoudvand and Moharramipour (2015) showed that fenoxycarb, a juvenile hormone analogue, decreased fertility of *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae).

In the present study, the effects of fenoxycarb on fecundity, fertility, ecdysteroid level, oocyte size, and expression levels of the *USP*, *EcR*, *HR3*, and *Vg* genes in adult female *H. armigera* were examined.

Materials and methods

Insect rearing

Cotton bollworm larvae were collected in cotton field in Gorgan, Iran (36° 50' 19" N, 54° 26' 05" E) and were reared in the laboratory of the entomology department of Tarbiat Modares University, Tehran, Iran, using artificial diet (800 mL distilled water, 40 g yeast powder, 260 g chickpea powder, 4 g ascorbic acid, 1.5 g sorbic acid, 1 mL formaldehyde (40%), 2.5 g Nipagin, 0.115 g cholesterol, and 12 g agar), per Shorey and Hale (1965). The larvae were maintained at 25 ± 2 °C, 16:8-hour light:dark photoperiod, and 65 ± 5% relative humidity. Forty newly emerged adult female and male (1:1) were released in a cage (20 × 20 × 30 cm) to mate. Cotton soaked in a 10% (vol/vol) honey solution was provided for the adult moths as food.

Fenoxycarb treatment

Fenoxycarb, a juvenile hormone analogue insecticide, was supplied by Sigma-Aldrich (St. Louis, Missouri, United States of America; catalogue number 34343) and dissolved in a water and acetone (1:1) solution. Third-instar larvae were fed an artificial diet (1 mg) that contained 100 µL of fenoxycarb at doses of LC10 (3.42 mg/L), LC25 (5.53 mg/L), and LC50 (9.2 mg/L; lethal dose). Control larvae were allowed to feed on artificial diet containing water and acetone (1:1) in the same ratio as that provided in the fenoxycarb treatments. The experiment was repeated three times, and each replication contained 10 third-instar larvae, each of which was kept in a separate plastic box (7 cm³). Mortality was recorded after 10 days of exposure to the insecticide-laced food, as noted in the treatments above.

Determining fenoxycarb effects on fecundity, fertility, and longevity parameters

One hundred young third-instar larvae were selected randomly and fed individually on diet containing fenoxycarb (doses: LC10 and LC25) for 10 days. Surviving larvae were then collected and reared on artificial diet and monitored daily, and larval mortality and pupal weight were determined. After adult emergence, one male and one female were transferred to a cylindrical container, and their fecundity, fertility, and longevity parameters were determined. The adults were fed on a 10% (v:v) honey solution in water. The experiments to determine the reproductive parameters were carried out with 15 replications for each treatment. The number of eggs laid by

each female was counted daily until the female died. The longevity of each male and female was also recorded.

Measuring oocyte length

To determine the effect of fenoxycarb on the size of the insect's oocytes, terminal oocytes (the largest oocyte is located at the base of the ovarioles) were measured according to the method of Van Wielendaele *et al.* (2013) in 10 females. A piece of millimetre-squared paper was used to calculate the average oocyte size.

Ecdysteroid extraction and quantification

To determine the concentration of ecdysteroid in ovaries, the enzyme immune assay method (Cayman Chemicals, Inc., Ann Arbor, Michigan, United States of America) was used (Ahmadi *et al.* 2021; Hackney *et al.* 2012).

Ecdysteroid extraction. The assay is based on competition between a 20-hydroxyecdysone-acetylcholinesterase (AChE) conjugate (20E Tracer) and 20-hydroxyecdysone for a limited number of 20E-specific rabbit antiserum-binding sites.

The ovaries extracted from each female *H. armigera* were homogenised with 100 μ L methanol, followed by centrifugation at $15\,000 \times g$, 4 °C for 15 minutes. The supernatant was removed and poured into a separate tube. This step was repeated once. The supernatant was then placed in a Speed Vac machine to evaporate the solvent, then 1000 μ L enzyme immunoassay buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 0.4 M NaCl, and 0.1% bovine serum albumin in 0.1 M phosphate buffer) was added to the samples and kept at -20 °C for further experiment.

Ecdysteroid quantification. To determine the ecdysteroid concentration in each well, 50 μ L of 20E-acetylcholinesterase (AChE) tracer, 100 μ L of enzyme immune assay buffer, 50 μ L of 20E enzyme immune assay antiserum (Cayman Chemicals, Inc.), and 50 μ L of sample solution extracted from ovaries, per the above, were poured and placed on a shaker at 4 °C for 18 hours. The plate was then emptied and rinsed six times with a washing buffer to remove any unbound reagents. Then, 200 μ L of Ellman's reagent (which contains the substrate to AChE) was added to each well for development. Then, 5 μ L of tracer (acetylcholinesterase linked to 20E) was added, and finally, the plate was covered and placed on the shaker for 90–120 minutes.

The product of this enzymatic reaction has a distinct yellow colour, determined spectrophotometrically, that is proportional to the amount of 20E tracer bound to the well, which is inversely proportional to the amount of free 20E present in the well during incubation. Using a microplate reader spectrometer (BioTek, Winooski, Vermont, United States of America), the absorbance was read at 412 nm.

Real-time quantitative reverse transcription-polymerase chain reaction

Ribonucleic acid samples were extracted from the fat bodies of female *H. armigera* using TRIzol solution, according to the manufacturer's instructions (Molecular Research Center, Cincinnati, Ohio, United States of America), then 0.2 mL of chloroform per 1 mL of TRIzol Reagent (Molecular Research Center Inc., Cincinnati, Ohio, United States of America) was added. The tubes were shaken vigorously by hand for 15 seconds and then incubated at 15–30 °C for 2 to 3 minutes. The samples were centrifuged at $12\,000 \times g$ for 15 minutes at 2–8 °C, which separated the mixture into a lower red, phenol-chloroform phase, an interphase, and a colourless upper-aqueous phase, with the RNA remaining exclusively in the aqueous phase. The aqueous phase was

Table 1. Primers used in the present study.

| Primer sequence (5'–3') | Primer name |
|----------------------------|-------------|
| GTGTGATGGTCGGTATGGGTCAGAAG | Actin-F |
| CAGATCTTCTCCATGTCTCCAGT | Actin-R |
| CGGAGACAAGAAACAGAACAC | Vg-F |
| AAGCAATAATGCGGACGAGAAT | Vg-R |
| GACAATGGAGCAACAGCAGC | EcR-F |
| AGACCCTTAGCGAATTCGAC | EcR-R |
| CATCCTGGAACGAACTGCTA | USP-F |
| GAGTTGTGGTGGCGATGTGGAC | USP-R |
| CGAGTTGGCCATTGTGAGAC | HR3-F |
| TCGCCGCTCAAATATACC | HR3-R |

transferred to a fresh tube. The RNA from the aqueous phase was precipitated by mixing with 0.5 mL of isopropyl alcohol per 1 mL of TRIzol Reagent for the initial homogenisation. Samples were incubated at 15–30 °C for 10 minutes, followed by centrifugation at 12 000 × g for 10 minutes at 2–8 °C. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol, adding at least 1 mL of 75% ethanol per 1 mL of the TRIzol Reagent used for the initial homogenisation. The sample was mixed by vortexing and centrifuged at 7500 × g for 5 minutes at 2–8 °C. At the end of the procedure, the RNA pellet was dried.

The concentration of the purified RNA was measured using a nanodrop spectrophotometer. One microlitre total RNA (containing 1 ng–5 µg) and 1 µL oligo (dT) were added to sterile nuclease-free water for 5 minutes to create a total volume of 13.41 µL on ice. This mixture was incubated for 5 minutes at 65 °C and chilled on ice. Then 4 µL M-MLV, 1 µL M-MLV, and 1 µL deoxyribonucleoside 5'-triphosphates (dNTPs) were added to the solution and incubated for 60 minutes at 55 °C, followed by incubation for 5 minutes at 85 °C. The primers used for real-time quantitative reverse transcription–polymerase chain reaction were designed using the Primer3 software. The real-time quantitative reverse transcription–polymerase chain reaction was performed to determine transcription levels of the vitellogenin gene (*Vg*; GenBank accession number: JQ723600), the nuclear hormone receptor, ultraspiracle (*USP*; GenBank accession number: EU526832.1), the ecdysone receptor (*EcR*; GenBank accession number: EU526831.1), and hormone receptor 3 (*HR3*; GenBank accession number: AF337637.3), using specific primers and Actin as reference genes (Table 1). Twenty nanograms of total genomic DNA were subjected to quantitative polymerase chain reaction as a template, using SYBR Green (Ampliqon, Odese, Denmark) with a Mic-qPCR cycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia). Polymerase chain reaction conditions were as follows: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. All the experiments repeated three times, with three biological replicates.

Statistical analysis

One-way analysis of variance, followed by Tukey's test, was used to compare the data (Tables 2, 3, 4, and 5; Figs. 1, 2, 3, and 4). Quantitative polymerase chain reaction data were analysed using the 2– $\Delta\Delta$ CT method (Livak and Schmittgen 2001). Student's *t*-test was applied. Graphs were created with PRISM, version 7 (GraphPad, San Diego, California, United States of America).

Table 2. Effects of sublethal doses of fenoxycarb (doses: LC10 and LC25) on the larval survival rate, pupation, and adult emergence of *Helicoverpa armigera*. Means followed by the same letters within a column are not significantly different (Tukey's test; $P < 0.05$). The values with different superscript letters in a column are significantly different ($P < 0.05$).

| Entries | Adult emergence (%) | Pupation (%) | Larval survival rate |
|-----------|--------------------------|--------------------------|--------------------------|
| Control | 87.76 ± 3.2 ^a | 93.61 ± 2.4 ^a | 95.00 ± 2.1 ^a |
| LC10 | 70.31 ± 4.5 ^b | 74.41 ± 4.3 ^b | 80.00 ± 3.4 ^b |
| LC25 | 56.21 ± 4.7 ^b | 63.10 ± 4.8 ^b | 73.00 ± 4.4 ^b |
| <i>F</i> | 13.33 | 14.80 | 9.95 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 |
| <i>df</i> | 2, 297 | 2, 297 | 2, 297 |

Table 3. Effects of fenoxycarb (doses: LC10 and LC25) on the pupal weight, fecundity (eggs/female), and fertility (fertile eggs/female) of *Helicoverpa armigera*. Means followed by the same letters within a column are not significantly different (Tukey's test; $P < 0.05$). The values with different superscript letters in a column are significantly different ($P < 0.05$).

| Treatment | Fertility (fertile eggs/female) | Fecundity (eggs/female) | Pupal weight (mg) |
|-----------|---------------------------------|-----------------------------|--------------------------|
| Control | 559.46 ± 38.52 ^a | 702.26 ± 47.13 ^a | 224.4 ± 5.6 ^a |
| LC10 | 388.46 ± 26.49 ^b | 589.20 ± 40.76 ^b | 207.1 ± 5.8 ^b |
| LC25 | 300.13 ± 27.76 ^b | 539.13 ± 37.44 ^b | 204.8 ± 6.8 ^b |
| <i>F</i> | 24.51 | 4.87 | 10.44 |
| <i>P</i> | 0.0001 | 0.012 | 0.0001 |
| <i>df</i> | 2, 42 | 2, 42 | 2, 87 |

Table 4. Effects of sublethal fenoxycarb (doses: LC10 and LC25) on the preoviposition period, oviposition period (day), postoviposition period (day), and peak oviposition period (day) of *Helicoverpa armigera*. Means followed by the same letters within a column are not significantly different (Tukey's test; $P < 0.05$). The values with different superscript letters in a column are significantly different ($P < 0.05$).

| Entries | Peak oviposition period (day) | Postoviposition period (day) | Oviposition period (day) | Preoviposition period (day) |
|-----------|-------------------------------|------------------------------|--------------------------|-----------------------------|
| Control | 3.46 ± 0.29 ^a | 1.8 ± 0.17 ^a | 8.53 ± 0.23 ^a | 2.2 ± 0.1 ^a |
| LC10 | 2.93 ± 0.30 ^a | 1.66 ± 0.18 ^a | 6.6 ± 0.27 ^b | 2.86 ± 0.2 ^b |
| LC25 | 2.60 ± 0.23 ^a | 1.6 ± 0.19 ^a | 5.86 ± 0.23 ^b | 3.06 ± 0.2 ^b |
| <i>F</i> | 2.49 | 0.31 | 30.60 | 6.29 |
| <i>P</i> | 0.095 | 0.738 | 0.0001 | 0.004 |
| <i>df</i> | 2, 42 | 2, 42 | 2, 42 | 2, 42 |

Mortality data from the larval toxicity bioassays were analysed to calculate LC10, LC25, and LC50 values, with 95% confidence limits, slope, and intercept, using the Probit procedure of the SPSS programme, version 23, developed by IBM (Armonk, New York, United States of America).

Table 5. Effects of sublethal fenoxycarb (doses: LC10 and LC25) on pupa longevity and female longevity of *Helicoverpa armigera*. Means followed by the same letters within a column are not significantly different (Tukey's test; $P < 0.05$). The values with different superscript letters in a column are significantly different ($P < 0.05$).

| Entries | Female | Longevity (day) |
|-----------|---------------------------|--------------------------|
| | | Pupa |
| Control | 13.07 ± 0.3 ^a | 11.57 ± 0.3 ^b |
| LC10 | 10.66 ± 0.33 ^b | 13.07 ± 0.3 ^a |
| LC25 | 10.06 ± 0.26 ^b | 13.40 ± 0.3 ^a |
| <i>F</i> | 15.33 | 20.004 |
| <i>P</i> | 0.0001 | 0.0001 |
| <i>df</i> | 2, 87 | 2, 87 |

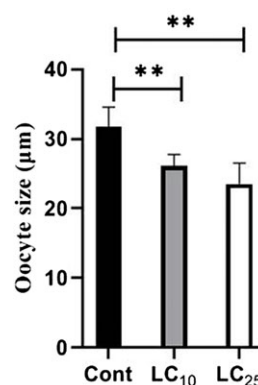


Figure 1. Effects of sublethal doses, LC10 (3.42 mg/L) and LC25 (5.53 mg/L), of fenoxycarb on oocyte size in adult female *Helicoverpa armigera*. One-way analysis of variance and Tukey's test were used to compare data (** $P < 0.01$).

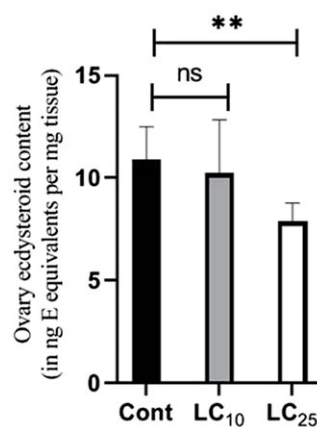


Figure 2. Effects of sublethal doses, LC10 (3.42 mg/L) and LC25 (5.53 mg/L), of fenoxycarb on ecdysteroid level in the ovaries of adult female *Helicoverpa armigera*. Ecdysteroids concentration was measured using enzyme immune assays. One-way analysis of variance and Tukey's test were used to compare data. Asterisks indicate significant differences (** $P < 0.01$), and "n.s." indicates no significant difference.

Results

Toxicity of fenoxycarb

The data presented in Table 6 indicate that the fenoxycarb clearly had high toxicity against *H. armigera*. The estimated LC90 and LC50 doses were 25.99 and 9.12 mg/L after 10 days, respectively. Also, the LC10 and LC25 values were 3.42 and 5.53 mg/L after 10 days, respectively (Table 6).

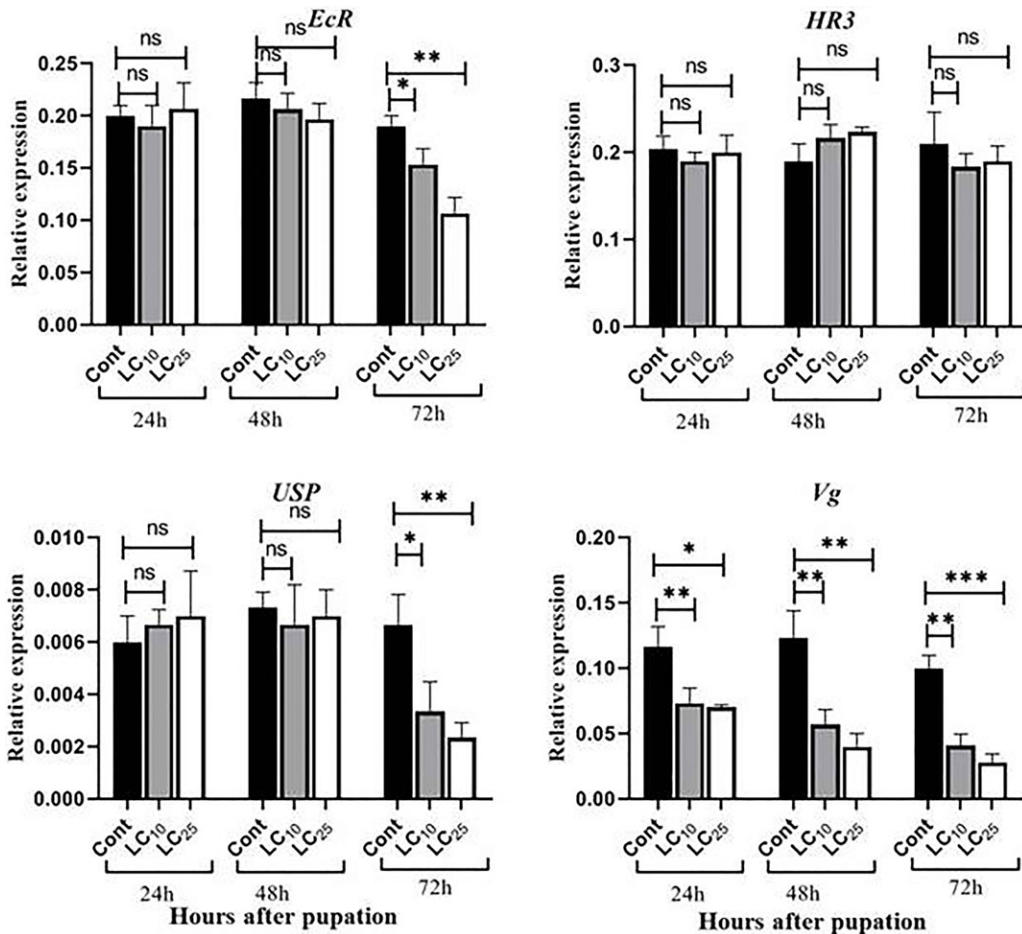


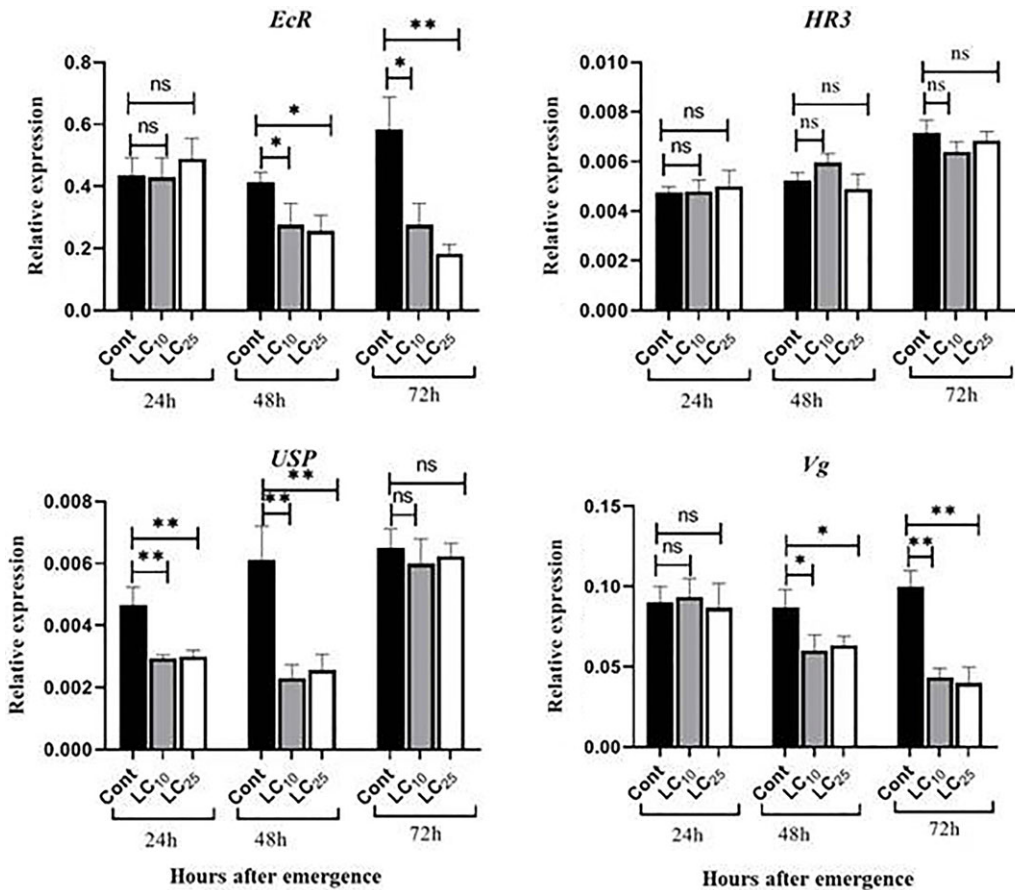
Figure 3. Effects of sublethal doses, LC10 (3.42 mg/L) and LC25 (5.53 mg/L), of fenoxycarb on *EcR*, *HR3*, *USP*, and *Vg* gene expression levels in pupal *Helicoverpa armigera*. Actin was used as reference gene. Each experiment was replicated three times with three biological replicates. The levels of *EcR*, *HR3*, *USP*, and *Vg* gene expression were analysed. One-way analysis of variance and Tukey's test were used to compare data. Asterisks indicate significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$), and "n.s." indicates no significant difference.

Effects of sublethal dose of fenoxycarb on larval survival rate, pupation, and adult emergence

Feeding on artificial diet that contained fenoxycarb (doses: LC10 and LC25) significantly decreased the percentage of larval survival rate ($F = 9.95$; $df = 2, 297$; $P < 0.0001$). The sublethal doses (at LC10 and LC25) of fenoxycarb caused losses of almost 15.8 and 23.1% of the larval survival rate, respectively. In addition, the pupation rate ($F = 14.80$; $df = 2, 297$; $P < 0.0001$) significantly decreased, as did the adult emergence rate ($F = 13.33$; $df = 2, 297$; $P < 0.0001$). Fenoxycarb at the LC10 and LC25 doses elicited a decrease of about 20.5 and 32.6% in pupation rate, respectively. Moreover, losses of 18.7 and 35.9% were observed in adult emergence when the insecticide was applied at LC10 and LC25 concentrations, respectively (Table 2).

Table 6. Toxicity of fenoxycarb on *Helicoverpa armigera*, 10 days after feeding on artificial diet containing the insecticide. LCL, lower confidence limit, at 95%; UCL, upper confidence limit, at 95%.

| Treatment | N ^a | df | Lethal and sublethal concentration (Mg/L ⁻¹) ^b | | | | Slope ± standard error | χ ² |
|------------|----------------|----|---|---------------------|----------------------|------------------------|------------------------|----------------|
| | | | LC10 (LCL–UCL) | LC25 (LCL–UCL) | LC50 (LCL–UCL) | LC90 (LCL–UCL) | | |
| Fenoxycarb | 180 | 4 | 3.42 (1.56–4.95) | 5.53 (3.36–7.28) | 9.12 (6.85–11.69) | 25.99 (18.20–34.89) | 2.91 ± 0.60 | 1.1 |

^aNumber of larvae.^b95% confidence limits in parenthesis.**Figure 4.** Effects of sublethal doses, LC10 (3.42 mg/L) and LC25 (5.53 mg/L), of fenoxycarb on *EcR*, *HR3*, *USP*, and *Vg* gene expression levels in adult female *Helicoverpa armigera*. Actin was used as reference gene. Each experiment was replicated three times with three biological replicates. The levels of *EcR*, *HR3*, *USP*, and *Vg* gene expression were analysed. One-way analysis of variance and Tukey's test were used to compare data. Asterisks indicate significant differences (** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$), and "n.s." indicates no significant difference.

Effects of sublethal dose of fenoxycarb on pupal weight, fecundity (eggs/female), and fertility (fertile eggs/female)

Pupal weight decreased by 7.7 and 8.7% with the fenoxycarb LC10 and LC25 treatments, respectively, compared to the control ($F = 10.44$; $df = 2, 87$; $P < 0.0001$). Fecundity ($F = 4.87$; $df = 2, 42$; $P < 0.012$) and fertility ($F = 24.51$; $df = 2, 42$; $P < 0.0001$) were also affected by the insecticide. Fenoxycarb at the LC25-dose treatment clearly reduced fecundity and fertility by about 23.2 and 46.3%, respectively (Table 3).

Effects of sublethal dose of fenoxycarb on preoviposition period (day), oviposition period (day), postoviposition period (day), and peak oviposition period (day)

The preoviposition period and oviposition period of *H. armigera* were significantly affected by fenoxycarb. The LC25 treatment significantly increased the preoviposition period by about 39% ($F = 6.29$; $df = 2, 42$; $P < 0.004$), but it clearly decreased oviposition period by about 31.3% ($F = 30.60$; $df = 2, 42$; $P < 0.0001$). The postoviposition period (day) and the peak oviposition period (day) were unaffected by the insecticide (Table 4).

Effects of sublethal dose of fenoxycarb on pupa longevity (day) and female longevity (day)

Feeding on artificial diet that contained fenoxycarb (doses: LC10 and LC25) significantly increased pupal longevity in *H. armigera* ($F = 20.004$; $df = 2, 87$; $P < 0.0001$) but shortened female longevity ($F = 15.33$; $df = 2, 87$; $P < 0.0001$). The LC25 treatment increased pupa longevity by about 15.81% but decreased female longevity by about 23.02% (Table 2).

Effects of sublethal dose of fenoxycarb on oocyte size

To investigate the effect of the sublethal doses of fenoxycarb on oocyte size, third-instar larvae were fed individually on artificial diet containing fenoxycarb (doses: LC10 and LC25) for 10 days. After adult emergence, the oocyte size was measured. The results showed that feeding on diet containing fenoxycarb clearly decreased oocyte size compared to the control. The LC25 fenoxycarb treatment decreased oocyte size by about 28.1% (Fig. 1).

Effects of sublethal dose of fenoxycarb on ecdysteroid concentration in the ovaries

Third-instar larvae of *H. armigera* were fed on diet containing sublethal doses of fenoxycarb for 10 days. The ecdysteroid concentration in the ovaries of the resulting adult females was measured using enzyme immune assay. The results show that larvae fed diet containing 5.53 mg/L (the LC25 treatment) of fenoxycarb lead to adult female ovaries having a significantly lower ecdysteroid concentration than did ovaries of adult female *H. armigera* raised as the control. The ecdysteroid concentration in ovaries of the control was approximately 1.5 times that found in the ovaries of the insects fed the fenoxycarb-containing (LC25) diet (Fig. 2).

Sublethal effects of fenoxycarb on *Vg*, *USP*, *EcR*, and *HR3* gene expression levels in pupae and adults of *H. armigera*

To understand the contribution of fenoxycarb to vitellogenesis in *H. armigera*, the insect was fed on diet containing the insecticide. The expression of *EcR*, *USP*, *HR3*, and *Vg* genes in the fat body dissected from both fenoxycarb-treated and control insects were investigated at 24, 48, and 72 hours after treatment. As shown in Figures 3 and 4, the fenoxycarb treatment suppressed the expression of the *USP*, *EcR*, and *Vg* genes in both pupal and adult *H. armigera*. A similar expression profile was observed for *EcR* and *USP* genes in pupa, but a different expression profile was observed in the adult insects. However, relative expressions of the *EcR* gene were 3.22- and twofold lower in fenoxycarb-treated (LC25) adult and pupa after 72 hours, respectively, compared

to those in control insects (Figs. 3 and 4). Although relative expression of the *USP* gene was not downregulated after 48 hours in fenoxycarb-treated (LC25) pupa, it became threefold lower after 72 hours, compared to the control. In the case of adult insects, it was 2.72-fold lower compared to that in the control after 48 hours. That is, although the level of *USP* expression was similar between the fenoxycarb-treated and control adult in the last 24 hours of the experiment, it significantly decreased in the fenoxycarb-treated insects during the first 48 hours of the observation period compared to the controls (Fig. 4).

Discussion

In this study, the effects of the insecticide fenoxycarb on the female reproductive system of *H. armigera* were determined. Many studies confirm the toxicity of fenoxycarb on some insects. Mahmoudvand and Moharramipour (2015) tested its effects on some biological parameters of *Plutella xylostella* and found the calculated LC10 and LC25, and LC50 values of the insecticide were 21.58 and 43.25 mg/L on third-instar larvae of that insect, respectively. Martínez *et al.* (2021) evaluated potential of fenoxycarb to control *E. elaeasa*, assessing the insecticide against *E. elaeasa* larvae for toxicity, feeding inhibition, and mortality. Those authors found that the LC50 of fenoxycarb was 0.199 g/L⁻¹.

In the present study, we report the sublethal effects of fenoxycarb on *H. armigera*. In previous studies, fenoxycarb's effects on fecundity varied. For example, Horton and Lewis (1996) found the chemical enhanced the fecundity in *Cacopsylla pyricola* (Foerster) (Hemiptera: Psyllidae), whereas the present study shows that fenoxycarb decreased fecundity and fertility in *H. armigera*. Zhang *et al.* (2019) showed that silencing the *Vg* gene decreased fecundity and ovary development in *Agasicles hygrophila* (Selman) (Coleoptera: Chrysomelidae), but in the present study, we found that fenoxycarb decreases *Vg* expression, thereby inhibiting fertility and egg hatching rates.

Treatment of the larvae with fenoxycarb in the present study caused *H. armigera* pupae to be underweight compared to the controls. This result is consistent with those of studies of Mahmoudvand and Moharramipour (2015) and Biddinger and Hull (1999). The present study's findings are also consistent with those of Mahmoudvand and Moharramipour (2015), who reported that LC10 and LC50 fenoxycarb affects longevity, fecundity, fertility, and pupa weight in *P. xylostella*. Moreover, those authors reported that fenoxycarb did not affect the postoviposition period in *P. xylostella*, and Xu *et al.* (2015) showed no significant difference in the ovipositing peak of *S. littoralis* when different concentrations of pyriproxyfen were applied. The present study found similar results.

In the present study, fenoxycarb significantly increased the preoviposition period but shortened the oviposition period, compared to the control. Yusoff *et al.* (2021) observed similar effects were observed with farnesyl acetate decreasing oviposition in *P. xylostella*. According to Yusoff *et al.* (2021), decreasing oviposition time may lead to decreases in fecundity.

We demonstrated that fenoxycarb decreased the size of *H. armigera* oocytes. It also decreased the level of ecdysteroid within the ovaries. Previously, Bai *et al.* (2010) reported that methoprene, a juvenile hormone analogue, inhibited ecdysteroid biosynthesis in *Anopheles gambiae* (Giles) (Diptera: Culicidae), and Rountree and Bollenbacher (1986) reported that juvenile hormone or its analogues inhibited ecdysteroid secretion in some lepidopterous insects. In the present study, relative expression levels of the *EcR* gene decreased after *H. armigera* larvae fed on diet containing fenoxycarb. It may be possible that the low level of *EcR* expression led to a decrease in the length of the oocytes. Previously, Gujar and Palli (2016) reported that knockdown of the *EcR* gene decreased the oocyte size in *Cimex lectularius* (Linnaeus) (Hemiptera: Cimicidae).

In the present study, real-time quantitative reverse transcription–polymerase chain reaction was used to understand the effect of fenoxycarb on the relative expression levels of the *EcR*, *USP*, *HR3*, and *Vg* genes. The results showed that relative expression levels of *EcR* and *USP* were lower in fenoxycarb-treated insects than in their controls. Hossain Ahmed *et al.* (2020) showed that

pyriproxyfen considerably decreased the expression of the *EcR*, *HR3*, and *Vg* genes in *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), and Bai *et al.* (2010) reported that the transcription of *EcR* and *HR3* is downregulated in methoprene-treated *A. gambiae*, possibly due to the inhibition of 20E action by insecticide. The *EcR* and *USP* genes combine to generate a heterodimer, which 20E can combine with to regulate the expression levels of downstream genes and thus affect the ovary development (Qian *et al.* 2020). However, in the present study, fenoxycarb was found to have no effect on *HR3*, which is an important downstream hormone. This result means that *HR3* is not involved in vitellogenesis in *H. armigera*.

Our results show that fenoxycarb decreased *Vg* expression. Previously, Bai *et al.* (2010) reported that methoprene suppressed expression of *Vg* in *A. gambiae* by 30%. This may be a result of the insecticide effects on ecdysteroid levels. 20-Hydroxyecdysone-acetylcholinesterase can play an important role in regulating vitellogenesis (Sun *et al.* 2003). Fenoxycarb decreased the 20E levels, which led to decreased *Vg* gene expression.

We showed that gene expression levels were affected, according to different concentrations of fenoxycarb. This finding is consistent with the results of Xu *et al.* (2015) regarding the influence of pyriproxyfen on reproductive system in *S. litura*, which showed that the effects of pyriproxyfen on *Vg* and *USP* gene expression levels were dose dependent.

In conclusion, feeding *H. armigera* larvae a diet containing fenoxycarb decreased ecdysteroid titre in ovaries and the expression levels of *EcR*, *USP*, and *Vg*, which led to decreases in fecundity, fertility, and oocyte size. Further study is needed to clarify fenoxycarb's effects on the reproductive system in the adult female *H. armigera*.

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