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Effects of hexaflumuron, lufenuron and chlorfluazuron on certain biological and physiological parameters of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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ABSTRACT

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the most economically devastating pests of agricultural crops around the world. In this study, the effects of hexaflumuron, lufenuron and chlorfluazuron were evaluated on some biological and physiological parameters of *H. armigera* larvae. Effects of the insecticides were assessed by adding different concentrations into the larval artificial diet. Dose-response bioassay showed LC_{50} values of 6.16, 61.31 and 31.75 mg ai/l for hexaflumuron, lufenuron and chlorfluazuron, respectively. Subsequently, sublethal effects of hexaflumuron, lufenuron and chlorfluazuron were assessed using LC_{10} concentration to find their possible effects on the biological and physiological parameters of *H. armigera* larvae. The results showed significant changes in biological parameters, where the mean larval and pupal weights significantly decreased following treatment, while their developmental time significantly increased compared to the control. Meanwhile, the longevity of adult males and females, number of laid eggs per female, and rate of egg hatch were significantly reduced due to the insecticide treatments. The activities of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase, acid- and alkaline phosphatases, lactate dehydrogenase, glutathione *S*-transferase were increased following the treatments. Sublethal exposure of the insecticides on larvae led to lower amounts of glycogen, triglyceride and protein compared to the control.

1. Introduction

Insect growth regulators (IGRs) are promising chemical compounds by intervening within endocrine system which affects the development, reproduction and metamorphosis of insects in several orders. IGRs are categorized into three main groups, namely juvenile hormone mimics, chitin synthesis inhibitors (CSIs) and ecdysone agonists (Yu, 2008; Tabozada, 2014). CSIs disrupt the mechanisms involved in chitin synthesis, cause cuticle malformations and reduce the proper amount of required chitin during metamorphosis (Merzendorfer, 2013). These compounds include pyrimidinenucleoside peptides, benzoylurea, oxazolines, thiazolidines, tetrazines, thiadiazines, thiophthalimides, and certain chromo- and fluorophores. Benzoylurea, oxazolines, and thiazolidinones may have a common mode of action and block a post-catalytic step of chitin synthesis (Mezendorfer, 2013). Although benzoylureas prevent chitin synthesis in many insects, they have no or only mild effects on fungi, parasitoids, and other natural enemies of insects. Therefore, these insecticides may be considered as potential candidates with important roles in a successful IPM program (Mezendorfer, 2013).

American bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the most important pests of agricultural crops around the world entailing high economic damages due to the intensive feeding on all parts of the host plant, the long period of larval development and the high reproductive ability (Fitt, 1989). The primary and usual approach to controlling *H. armigera* is to use insecticides, which, on the other hand, causes myriad serious problems such as environmental pollution and resistance development due to frequent spraying (Rui et al., 1999; Ahmad et al., 2002). Although there exist other control techniques such as traps baited with sexual pheromones, resistant cultivars, destruction of crop remnants, and releasing natural enemies, such as *Trichogramma* sp., the trends toward using chemical insecticides for the immediate alleviation of population outbreaks, have led to the discovery of suitable, mainly ecofriendly, insecticides. CSIs could be one of the most appropriate candidates owing to their selective efficacy against insect pests on one hand, and minimum effects on non-target organisms on the other. Chitin synthesis inhibitors have shown

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considerable effects by imposing biological parameters like weight, growth, fecundity, fertility and physiological turbulences(Pineda et al., 2007; Saber et al., 2013; Rehan and Freed, 2015; Mirhaghparast et al., 2015; Vojoudi et al., 2017). Hexaflumuron, lufenuron and chlorfluazuron are among the most commonly used CSI against lepidopteran larvae in Iran. These compounds belong to benzoylurea which disrupt chitin formation through abnormal endocuticular deposition and abortive molting (Ghanim and Ishaaya, 2011). Specifically, they reduce the firmness and elasticity of cuticle by intervening hormonal imbalances, resulting in the inhibition of DNA synthesis, and discrepancies in the activities of carbohydrases, phenoloxidases and microsomal oxidases (Ghanim and Ishaaya, 2011). It is imperative that the ecological and physiological effects of CSIs on target insects be determined so as to elucidate the most optimal compound responsible for suppressing population and the subsequent economic damages. In detail, alterations in activities and amounts of biomolecules involved in intermediary metabolism of insects could be crucial to finding the suitability of CSIs (Mirhaghparast et al., 2015). Several studies have reported significant changes in biochemical compounds (enzymatic and non-enzymatic) of insects followed by CSIs (Nasr et al., 2010; Zibaee et al., 2011; Abou-Taleb, 2015; Mirhaghparast et al., 2015). Therefore, the present study aimed to determine the possible toxicity, and the biological and physiological discrepancies of the three chitin synthesis inhibitors, namely hexaflumuron, lufenuron and chlorfluazuron against the larvae of *H. armigera*.

2. Materials and methods

2.1. Insect rearing

The individuals of *H. armigera* was obtained from a colony maintained at the research greenhouse in the Department of Plant Protection, University of Tabriz where were reared on artificial diet including cowpea powder (205 g), powdered agar (14 g), ascorbic acid (3.5 g), sorbic acid (1.1 g), methyl-p-hydroxybenzoate (2.2 g), yeast (35 g), wheat germ (30 g), 37% formaldehyde solution (2.5 ml), vegetable oil (5 ml) and distilled water (650 ml), in a growth chamber at 26 ± 2 °C, 60 ± 10% RH and a photoperiod of 16:8 h (L:D). The adults were fed on 10% honey solution at the same laboratory condition. A daily check of both larvae and adults were done to refresh food staple and clean rearing containers.

2.2. Insecticides

The insecticides used in the current study were the commercial formulations of Hexaflumuron (10% EC, Golsam company, Gorgan, Iran), Chlorfluazuron (Atabron® 5% EC, Zhejiang, China) and Lufenuron (Mach® 50% EC, Syngenta Agr AG, Switzerland).

2.3. Bioassays

Effects of Hexaflumuron, Chlorfluazuron and lufenuron were assessed on the newly molted 3rd instar of *H. armigera* by incorporating different concentrations of the given compounds prepared with Tween®–80 solution (0.05%) into the artificial diet. Based on the preliminary experiments, five concentrations were chosen for each compound. The range of concentrations were 5.5–8, 40–75 and 1.75–4.5 mg a.i./L for hexaflumuron, lufenuron and chlorfluazuron, respectively. Only Tween®–80 solution (0.05%) was added into the control diet. The sufficient amount of each concentration was added into the larval artificial diet at a proportion of 9:1 (artificial diet: insecticide solution). Then, twenty 3rd instar larvae were individually placed on the containers including 1.5 g of treated diets. The containers were checked daily and the mortality was recorded at 24, 48 and 72 h after initial exposure.

2.4. Sublethal effects tests on biological parameters of H. armigera

Sublethal effects of hexaflumuron, lufenuron and chlorfluazuron were assessed using LC_{10} concentration of each insecticide. About 100 newly molted 3rd instar larvae were selected randomly and transferred individually on 1.5 g insecticide - incorporated diet. After 72 h, weight of the larvae was recorded and the survived larvae were transferred on untreated diet. Larval mortality and larval developmental time, pupal weight and period were also recorded in treated and control groups. Moreover, since adult emergence, a pair of male and female were placed in the plastic pots (with 10% honey solution) to determine fecundity and fertility of adults.

2.5. Effects of the insecticides on physiological parameters of H. armigera

Sublethal effects of hexaflumuron, lufenuron and chlorfluazuron on physiological parameters were investigated on the 3rd larval instar of *H. armigera* using LC_{10} concentration of each compound. In details, 40 newly molted 3rd instar larvae were randomly selected per each time intervals and transferred individually on the containers including 1.5 g of insecticide-incorporated diet. After 24 and 48 h, the survived larvae were weighted and handled for sample preparation. Briefly, the larvae from each treatment (insecticide) and control were separately homogenized in distilled water and centrifuged at $25000 \times g$ for 20 min at 4 °C. The supernatant was collected and kept at −20 °C for biochemical assays. In this experiment, 40 larvae were considered for each time interval as 10 larvae for control, 10 for hexaflumuron, 10 for lufenuron and 10 for chlorfluazuron and three replicates containing three larvae were prepared as biochemical samples.

2.5.1. Assay of alanine (ALT; EC 2.6.1.1) and aspartate (AST; EC 2.6.1.1) aminotransferases

A biochemical kit manufactured by Biochem company (Tehran, Iran) was used to assay ALT and AST activities in which pyruvate is combined with 2,4-dintitrophenyl pyruvate and synthesized pyruvate hydrazine (Thomas, 1998). Briefly, reagent A (for AST) and reagent B (for ALT) were incubated separately with reagent D. After 5 min, 10 µL of the enzyme solution was added and incubation was done for 60 min. At the end, reagent C was added into the solution prior to read absorbance at 340 nm.

2.5.2. Assay of ϒ-glutamyl transferase (γ-GT) (EC 2.3.2.2)

Based on a kit manufactured by ZiestChem Diagnostic Company (Tehran, Iran) and the method of Szasz (1976), 20 µL of substrate reagent and L-ϒ-glutamyl-3-carboxy-4-nitrianilide were added into 50 µL of buffer reagent. Then, 10 µL of enzyme solution was added, mixed thoroughly prior to read the absorbance at 405 nm after 3 min.

2.5.3. Assay of lactate dehydrogenase (EC 1.1.1.27)

Briefly, 20 µL of NAD^+ solution and 20 µL of water were added into test and control tubes, separately. Then, 100 µL of the buffered substrate and 10 µL of the sample was added into the test tubes and incubated for 15 min at 37 °C. After that, 100 µL of the reagent (2,4-dinitrophenyl hydrazine) was also added and the reaction mature was reincubated for 15 min. Tubes were cooled at room temperature prior to add 50 µL of NaOH (0.4 M). Finally, the absorbance was read at 340 nm after 60 s (King, 1965).

2.5.4. Assay of acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatase (ACP, ALP)

Based on the method of Bessey et al. (1946), 10 µL of enzyme solution was added into the buffered substrate (Tris-HCl, 20 mM, pH 8) for ALP and (Tris-HCl, 20 mM, pH 5) and incubated for 5 min. Afterward, 100 µL of NaOH (1 M) was added and the absorbance was read at 405 nm.

2.5.5. Determination of esterase activity

Esterase activity was determined using a method described by Han et al. (1998). 20 µL of α- and β-naphthyl-acetates (10 mM) and 50 µL of fast blue RR salt (1 mM) were added into 80 µL volume of universal buffer [Glycine, 2-morpholinoethan sulfuric acid and succinate, 20 mM, pH 7]. After gentle shacking, 20 µL of larval sample was added and optical density was recorded every 1 min up to 5 min at 545 nm.

2.5.6. Glutathione S-transferase (GST) assay

GST activity was measured using the method of Oppenoorth et al., 1979 in which 20 µL of 1-chloro-2,4,-dinitrobenzene (CDNB, 20 mM) and 1,2-Dichloro-4-nitrobenzene (DCNB, 40 mM) were separately pipetted into 80 µL of universal buffer (20 mM, pH 7). Afterward, 50 µL of reduced glutathione (20 mM) was added and gently shacked for 30 s. Finally, 20 µL of enzymatic solution was added and optical density was recorded every 1 min up to 5 min at 340 nm.

2.5.7. Measurement of total protein

Protein concentrations in the treatments and control larvae were determined using bovine serum albumin based the method described by Lowry et al. (1951) (Manufactured by Ziest Chem. Co., Tehran-Iran).

2.5.8. Measurement of triglyceride

The amount of triglyceride was determined by a diagnostic kit provided by PARS-AZMOON® Company (Tehran, Iran). Reagent solution contained phosphate buffer (50 mM, pH 7.2), 4-chlorophenol (4 mM), adenosine triphosphate (2 mM), Mg^{2+} (15 mM), glycerokinase (0.4 kU/L), peroxidase (2 kU/L), lipoprotein lipase (2 kU/L), 4-aminoantipyrine (0.5 mM) and glycerol-3phosphate-oxidase (0.5 kU/L). Then, 10 µL of sample with 10 µL of distilled water were added to 70 µL reagent and incubation was made for 20 min at 25 °C (Fossati and Prencipe, 1982). Absorbance of sample and reagent were measured at 545 nm and amount of triglyceride was calculated with the following equation:

$$mg/dl = \frac{OD\ of\ sample}{OD\ of\ standard} \times 0.01126$$

2.5.9. Measurement of glycogen

The control and treated larvae were immersed in 1 ml of 30% KOH w/Na2SO4. Tubes which contained the samples were covered with foil (to avoid evaporation) and were boiled for 20–30 min. Tubes were shaked and cooled in ice. Then, 2 ml of 95% EtOH was added to precipitate glycogen from digested solution. The samples were again shaked and incubated in ice for 30 min. Tubes were centrifuged 13000 rpm for 30 min. Supernatant was removed and pellets (glycogen) were re-dissolved in 1 ml of distilled water before being shaked. Glycogen standard (0, 25, 50, 75 and 100 mg/ml) was prepared before adding phenol 5%. Incubation was performed on ice bath for 30 min. Standards and samples were read at 490 nm and distilled water was used as blank (Chun and Yin, 1998).

2.6. Data analysis

All experiments were done within a completely randomized design including at least three replicates. The bioassay data were subjected to probit analysis by SAS (SAS Institute, 2004) software. The data were analyzed by one-way analysis of variance (ANOVA) and mean comparisons were compared by Tukey's test at the probability of 5% using SAS software.

3. Results

3.1. Acute toxicity

Table 1 shows the bioassay results of hexaflumuron, lufenuron and chlorfluazuron on the 3rd instar larvae of cotton bollworm. The LC_{50} values for hexaflumuron, lufenuron and chlorfluazuron were 31.75, 6.16 and 61.31 mg a. i./L following 72 h of exposure, respectively. The results revealed that lufenuron had the highest toxicity against *H. armigera* compared to other insecticides (Table 1).

3.2. Sublethal effects

Table 2 shows the larval/pupal weight and also their duration time in the control and the individuals treated by the chemicals. All tested insecticides reduced the mean larval and pupal weight following treatment by LC_{10} concentration, while chemical treatments significantly increased the developmental duration of both larval and pupal stages (Pr > F: 0.0001, Df: 4, 83; Pr > F: 0.0001, Df: 4, 40). The time of larval developmental was recorded as 8.74, 13.64, 15.6 and 14.67 days for control, chlorfluazuron, hexaflumuron and lufenuron, respectively (Table 2; Pr > F: 0.0001, Df: 4, 48). Additionally, chlorfluazuron and lufenuron significantly reduced pupal duration, while hexaflumuron prolonged the duration compared to the control (Table 2; Pr > F: 0.0001, Df: 4, 21). The LC_{10} concentration of chlorfluazuron, hexaflumuron and lufenuron significantly decreased the longevity of males and females, number of laid eggs and their hatching rate compared to the control (Table 3; Pr > F: 0.0007, Df: 4, 20; Pr > F: 0.0004, Df: 4, 20; Pr > F: 0.0001, Df: 4, 20). Finally, our results demonstrated that the chemicals significantly lowered the rate of oviposition in the treated females exposed to the insecticides residue at larval stage (Table 3; Pr > F: 0.0001, Df: 4, 20).

3.3. Biochemical effects

Oral exposure of *H. armigera* larvae to chlorfluazuron, hexaflumuron and lufenuron caused alterations in the activities of the assayed transaminases concerning both time intervals (Fig. 1). The insecticides significantly increased ALT activity at 24 h post-exposure because the highest activity was obtained following chlorfluazuron. Similar results were obtained following 48 h, except for lufenuron which was not significantly different from the control (Fig. 1; Pr > F: 0.0003, Df: 3, 16; Pr > F: 0.0006, Df: 3, 11). After 24 h, the activity of AST increased in the larvae exposed to chlorfluazuron and lufenuron compared to the control, but hexaflumuron treatment showed no statistical differences (Fig. 1; Pr > F: 0.0001, Df: 3, 18). Although AST activity in the larvae treated with chlorfluazuron was higher than control after 48 h, the activity was significantly reduced following treatment with hexaflumuron and lufenuron (Fig. 1; Pr > F: 0.0022, Df: 3, 22). The larvae treated by hexaflumuron showed the highest GGT activity following 24 h, although the enzymatic activity in other treatments was higher than the control (Fig. 1; Pr > F: 0.0004, Df: 3, 32). At 48 h post-exposure, only the larvae treated by lufenuron had the highest GGT activity compared to the control (Fig. 1; Pr > F: 0.0048, Df: 3, 16).

The activity of ACP and ALP significantly increased in the larvae treated with insecticides after 24 h, as the highest activities were recorded after treatment with lufenuron and chlorfluazuron, respectively (Fig. 2; Pr > F: 0.0003, Df: 3, 11; Pr > F: 0.0016, Df: 3, 24). At 48 h post-exposure, the highest activities of ACP and ALP belonged to the larvae treated with chlorfluazuron and hexaflumuron, respectively; however, no significant differences were observed in the larvae treated with lufenuron for ACP and chlorfluazuron and lufenuron for ALP, compared to control (Fig. 2; Pr > F: 0.0014, Df: 3, 28; Pr > F: 0.0061, Df: 3, 32). The larvae treated with hexaflumuron and lufenuron showed the highest LDH activity after 24 h, but maximum enzymatic activity was recorded in the larvae treated with hexaflumuron following 48 h (Fig. 2; Pr > F: 0.024, Df: 3, 22; Pr > F: 0.037, Df: 3, 34).

Using α-naphthyl acetate as substrate, the highest esterase activity was found in the larvae treated with chlorfluazuron after 24 h, while no larvae treated with the insecticides showed any significantly different esterase activity compared to control after 48 h (Fig. 3; Pr > F: 0.0021,

Table 1
Acute Toxicity results of the insecticides on third instar larvae of *Helicoverpa armigera*.

χ^2	LC_{90} (mg ai/l) (95% CI)	Slope ± SE	LC_{50} (mg ai/l) (95% CI)	Insecticides
17.16	76.22 (59.22–121.95)	3.37 ± 0.54	31.75 (29.17–35.14)	Chlorfluazuron
14.69	8.82 (8.04–10.67)	8.24 ± 1.561	6.16 (5.87–6.40)	Hexaflumuron
7.49	101.46 (87.51–131.98)	5.86 ± 0.89	61.31 (58.38–66.65)	Lufenuron

Table 2
Sublethal effects of chlorfluazuron, lufenuron and hexaflumuron on larval weight and duration, pupal weight and pupal longevity of *Helicoverpa armigera*.

Pupal weight (mg) ± SE	Pupal Duration time (days) ± SE	Larval weight (6 days after treatment) ± SE	Larval weight (4 days after treatment) (mg) ± SE	Mean larval Duration time (days) ± SE	Insecticides
259 ± 21.68b	16.11 ± 0.35 a	0.104 ± 0.01 b	0.027 ± 3.96 b	13.64 ± 0.491 b	Chlorfluazuron
215 ± 16.61b	13.20 ± 0.80 b	0.024 ± 0.01 b	0.016 ± 1.13 c	15.60 ± 0.979 a	Hexaflumuron
257 ± 11.18b	18.00 ± 0.11 a	0.068 ± 0.01 b	0.015 ± 1.04 c	14.67 ± 0.211 a	Lufenuron
367 ± 10.30a	12.60 ± 0.22 b	0.440 ± 0.02 a	0.16 ± 2.38 a	8.74 ± 0.372 c	Control

Df: 3, 44; Pr > F: 0.48, Df: 3, 56). Once β-naphthyl acetate was used as substrate, the highest esterase activity was recorded in the larvae treated with chlorfluazuron and lufenuron at 24 and 48 h, respectively; other treatments at 48 h showed no statistical differences with the control (Fig. 3; Pr > F: 0.0002, Df: 3, 18; Pr > F: 0.036, Df: 3, 42). The activity of GST using CDNB as reagent was the highest in the larvae treated with hexaflumuron after 24 h, while both larvae exposed to chlorfluazuron and hexaflumuron resulted in the highest GST activity after 48 h (Fig. 4; Pr > F: 0.0003, Df: 3, 28; Pr > F: 0.00022, Df: 3, 34). The larvae treated with chlorfluazuron led to the highest GST activity using DCNB as reagent at both time intervals (Fig. 4).

At 24 h post-exposure, the amount of protein decreased in the larvae treated with all insecticides except for hexaflumuron, but no significant changes were recorded regarding the amount of protein after 48 h (Fig. 5; Pr > F: 0.0001, Df: 3, 16; Pr > F: 0.6472, Df: 3, 42). The amount of glycogen was significantly reduced in the larvae treated with the insecticides after both time intervals compared to the control (Fig. 5; Pr > F: 0.0033, Df: 3, 52; Pr > F: 0.0011, Df: 3, 38). Similar results were obtained concerning the amount of triglyceride except for chlorfluazuron after 48 h, where no significant differences were recorded compared to the control (Fig. 5; Pr > F: 0.0014, Df: 3, 28; Pr > F: 0.0008, Df: 3, 34).

4. Discussion

The current study determined the potential effects of three chitin synthesis inhibitors, namely chlorfluazuron, hexaflumuron and lufenuron against the 3rd instar larvae of *H. armigera*. Our study showed that these insecticides were acutely toxic against 3rd instar larvae of *H. armigera*, where hexaflumuron requires the least concentration to cause 50% mortality (LC_{50}). There are many studies on the effects of IGRs against lepidopteran insects (Buter et al., 2003; Eziah et al., 2008; Nehare et al., 2010). A previous study on *H. armigera* reported an LC_{50} value of 0.31 mg ai/l against the first instar larvae fed on the diet containing hexaflumuron (Rafiee et al., 2008). Zhu et al. (2012) reported that the LC_{50} value of hexaflumuron was 0.5 mg a.i./ml on the larvae of *H. armigera*. Butter et al. (2003) examined the toxicity of lufenuron on different larval instars of *H. armigera* with leaf dipping method, in which the LC_{50} value of lufenuron was reported to be 0.38–0.61 mg/l for 1–5 instars larvae. Aboutaleb et al. (2015) studied the effects of lufenuron and chlorfluazuron on the 4th instar larvae of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). They reported LC_{50} values of 1.7 and 2.2 ppm for lufenuron and chlorfluazuron, respectively. In the present research hexaflumuron was able to cause 50% mortality against *H. armigera* larvae with minimum concentration, whereas lufenuron caused the same number of mortality with the highest concentration. Primarily, such a difference may indicate the structural-insecticidal relationships of benzoylurea CSI which highlight the role of benzoyl moiety and diverse functional groups within CSI structure (Sun et al., 2015). Apart from CSI structure itself, the exposure procedure, larval instar, source of stock insect referring to potential genetical/geographical variations, and the time of larval exposure to CSI are among other factors affecting toxicity against larvae.

The larval diet containing hexaflumuron, lufenuron and chlorfluazuron (LC_{10}) lowered the larval and pupal weight at both time intervals (4 and 6 days after treatment), which may be attributed to the reduced food intake during the larval period or high metabolic costs required for the detoxification of ingested insecticides. Similar results have been observed in other studies (Saber et al., 2013; Carneiro et al., 2016; Vojudi et al., 2017). For example, Vojudi et al. (2017) reported that LC_{30} concentration of hexaflumuron reduced the larval and pupal weight of *H. armogera* following 72 h of exposure. The authors believed that the lower weight of the treated larvae might be due to the disruption of chitin synthesis and loss of body fluid during the molting stage. Moreover, we observed that LC_{10} concentration of the insecticides increased the developmental time of both larvae and pupae, subsequently reducing the population growth and increasing the time of exposure to parasitoid and predators (Mahmoudvand et al., 2010, 2011; Zhu et al., 2012; Vojoudi et al., 2017). Saber et al. (2013) also reported that the higher larval period would increase population doubling time, and reduce population growth. Regarding adult longevity, it was observed that a lower number of males and females were exposed to the chlorfluazuron, hexaflumuron and lufenuron compared to control as regards longevity. Similarity, Mahmoudvand et al. (2010, 2011)

Table 3
Sublethal effects of chlorfluazuron, lufenuron and hexaflumuron on adult longevity, fecundity and egg hatch rate of *Helicoverpa armigera*.

Egg hatch rate (%)	Fecundity	Male Longevity (days)	Female Fecundity (days)	,Insecticide
27.621 ± 2.938 b	239.29 ± 6.753 b	5.429 ± 0.202 b	5.286 ± 0.421 b	Chlorfluazuron
56.250 ± 13.750 a	167.25 ± 7.909 bc	6.470 ± 0.750 b	6.250 ± 1.190 b	Hexaflumuron
13.326 ± 6.805 b	123.20 ± 7.939 c	5.667 ± 1.154 b	5.000 ± 0.667 b	Lufenuron
70.570 ± 2.546 a	487.00 ± 20.580 a	9.625 ± 0.885 a	10.500 ± 1.018 a	Control

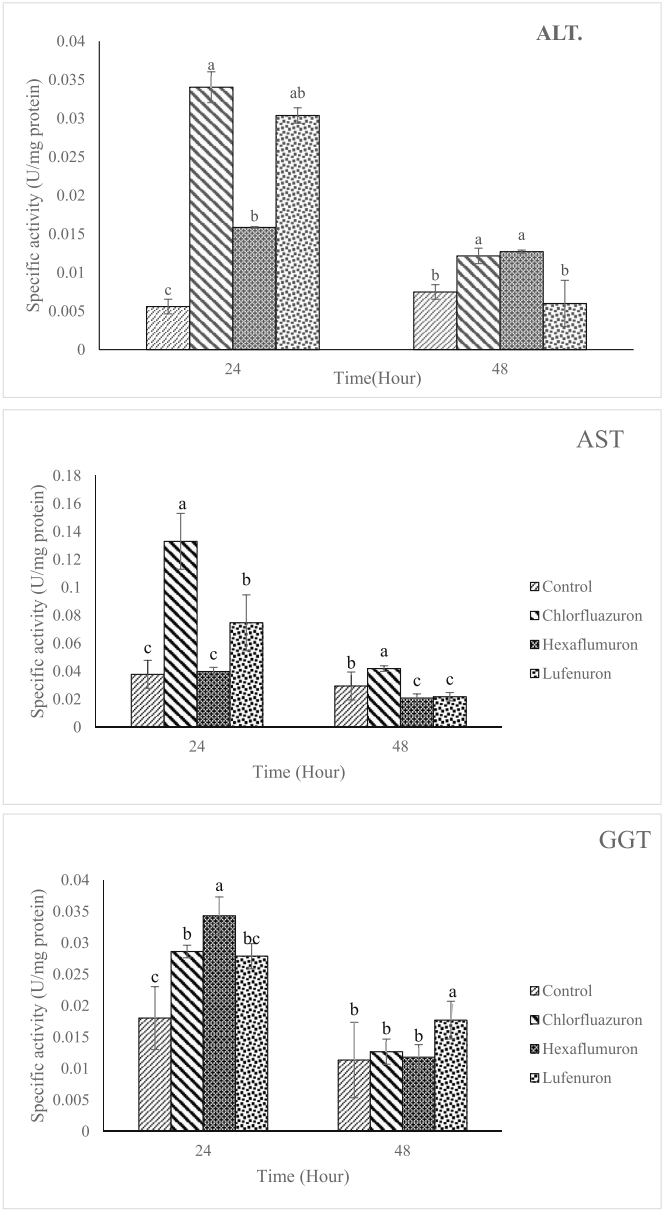


Fig. 1. Effects of insecticides on the activities of aminotransferases (U/mg protein) in the 3rd larvae of *Helicoverpa armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GGT: ϒ-Glutamyl transferase.

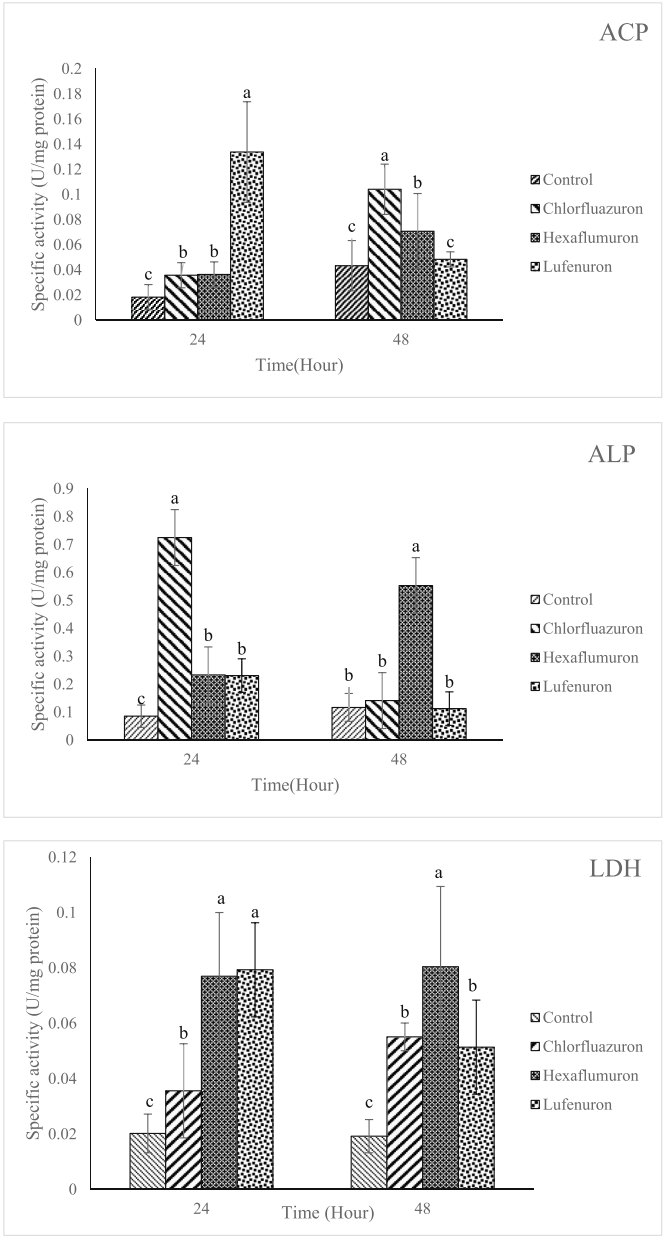
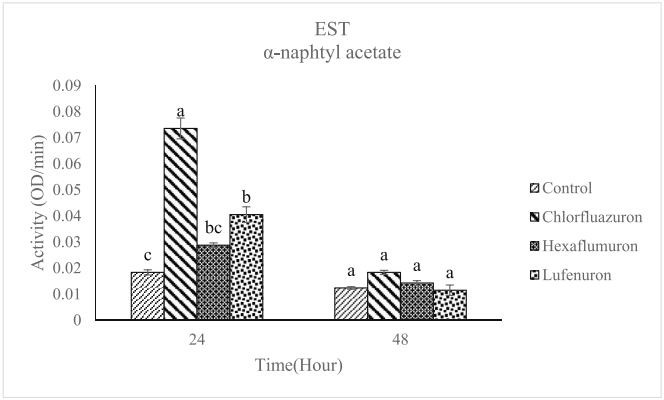
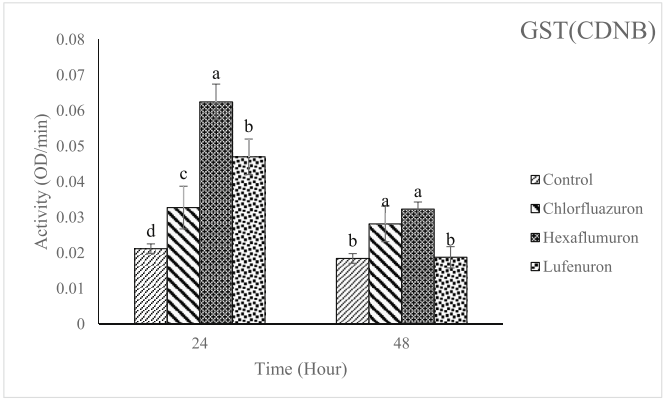


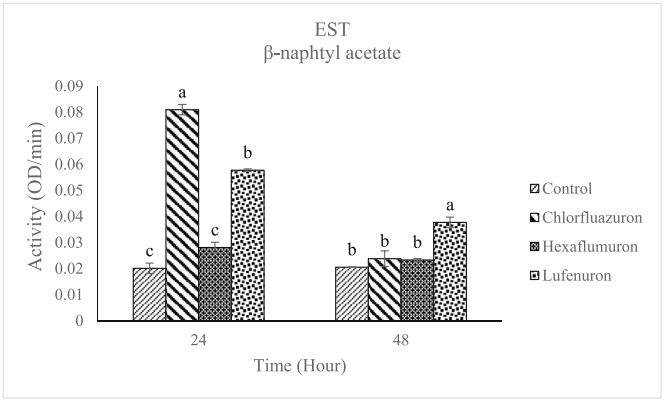
Fig. 2. Effects of insecticides on the activities of phosphatases and lactate dehydrogenase (U/mg protein) in the 3rd larvae of *Helicoverpa armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3). ACP: Acid phosphatase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase.

reported that LC_{25} concentration of hexaflumuron reduced adult longevity of *Plutella xylostella* (L.); Vojudi et al. (2017) further observed that sublethal concentration of hexaflumuron decreased the adult longevity of *H. armigera* compared to the control. Finally, there was a significant decrease in the number of eggs laid by females of *H. armigera* treated by chlorfluazuron, hexaflumuron and lufenuron compared to the control ones. Other studies have indicated the negative effects of chitin synthesis inhibitors on the reproductive parameters of insects such as fecundity and fertility (Galvan et al., 2005; Kellouche and Soltani, 2006; Mahmoudvand et al., 2010, 2011; Vojudi et al., 2017). The negative effects of CSI on the weights and duration of developmental stages as well as the reproductive performance of females may be confirmed by biochemical and molecular approaches. However, we believe in imposing energetic demands on the treated larvae, where such larvae should divert the energy stream from development and reproduction to detoxification or rehabilitation of tissues following possible damages. Moreover, hormonal imbalances after CSI treatments may cause diverse physiological discrepancies to be taken care of by the survived larvae, which requires energy.

Our results revealed the interference of chitin synthesis inhibitors used in certain biochemical enzymes in the intermediary metabolism of *H. armigera* larvae. Such a disruption may chronically affect the overall physiological status and potential disability in growth and development. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the main enzymes which conduce to transamination within the intermediary metabolism of insects to make amino acids available for biochemical demands. ALT catalyzes the interconversion of alanine and α-ketoglutarate to pyruvate and glutamate, while AST converts aspartate and α-ketoglutarate to oxaloacetate and glutamate (Klowden, 2007; Nation, 2008). γ-GT is yet another transaminase engaging in γ-glutamyl chain through the decomposition and production of







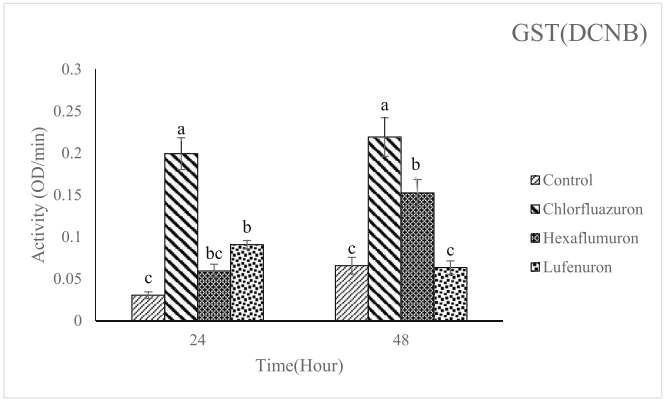


Fig. 3. Effects of insecticides on the activity of general esterases(EST) (U/mg protein) in the 3rd larvae of *Helicoverpa armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3).

Fig. 4. Effects of insecticides on the activity of glutathione *S*-transferases (GST) (U/mg protein) in the 3rd larvae of *Helicoverpa armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3).

glutathione (Mirhaghparast et al., 2016). Aboutaleb et al. (2015) demonstrated the higher activities of ALT and AST in *S. littoralis* treated by lufenuron and chlorfluazuron following 24 and 48 h. Mirhaghparast et al. (2015) reported that the highest activity of these transaminases occurred 12 and 24 h following the treatment of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) with hexaflumuron. Overall, the present results on transaminases demonstrate the higher activities in the larvae treated by insecticides compared to the control. In case of AST and ALT, the higher enzymatic activity may refer to higher demands for amino acids due to possible tissue damage or energy requirements in the treated larvae. Furthermore, the higher activity of GGT may refer to its detoxifying properties to make glutathione available for the activity of GST in the final stage. Such an interpretation may be obtained regarding the higher activity of GST, which will be discussed later.

ACP and ALP are two important phosphatases regarding the intermediary metabolism of insects, which not only hydrolyze phosphate groups from the ingested toxic molecules and naturally occurring ones, but also their activities may refer to the efficiency of digestion and transportation within insect body (Senthil-Nathan et al., 2006; Nation, 2008; Zibaee et al., 2011). Zibaee et al. (2011) reported the higher ACP activity in *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) following treatment with pyriproxifen. Aboutaleb et al. (2015) showed a significant increase phosphatase activity in *S. littoralis* following 48 and 72 h of lufenuron and chlorfluazuron treatments. Mirhaghparast et al. (2015) showed that *C. suppressalis* larvae had higher ACP and ALP activities following treatment with hexaflumuron at all time intervals. The higher activity of these enzymes in the current study may refer to their role in the transportation of lipids, such as monoacylglyceroles, through low density lipoproteins to supply the energy demands of the treated larvae. Such a conclusion may be in accordance with the lower amount of storage triglycerides in the larvae treated with all the employed insecticides. Moreover, lactate dehydrogenase contributes to the conversion of lactate to pyruvic acid and vice versa. It is further used to indicate tissue damage in response to chemical stresses (Zibaee et al., 2011; Mirhaghparast et al., 2016). The higher activity of LDH in *H. armigera* treated with chlorfluazuron, hexaflumuron and lufenuron may show the damages imposed by these insecticides or the higher energy demands through glycolysis, which may later be inferred through the lower amount of glycogen in the larvae compared to control.

Insects highly depend on detoxifying mechanisms to remove xenobiotics such as insecticides. Monoxygenases, esterases and GSTs are the three main detoxifying enzymes which crucially contribute to insect survival. Esterases hydrolytically break esters into an acid and an alcohol, while GSTs increase the solubility of insecticides within the physiological media of insects through binding electrophile substrates to glutathione (Oppenoorth et al., 1979; Han et al., 1998; Yu, 2008). The larvae of *H. armigera* treated by chlorfluazuron, hexaflumuron and lufenuron showed the elevated activities of both esterases and GSTs mainly after 24 h. Our results clearly indicate the role of these enzymes in removing or reducing the concentration of these insecticides to suppress their negative effects. Further demonstrated is the potential involvement of one isozyme of esterase assayed using α-naphthyl acetate as detoxifying esterase toward chlorfluazuron, hexaflumuron and lufenuron because the other isozyme assayed with β-naphthyl acetate was not statistically different from the control and treated larvae. Vojoudi et al. (2017) reported an increase in the activity of GST *H. armigera* toward the sublethal doses of the indoxacarb and hexaflumuron. Mirhaghparast et al. (2015) also observed an increase in the activity of esterases and GSTs in *C. suppressalis* larvae 1–12 h after

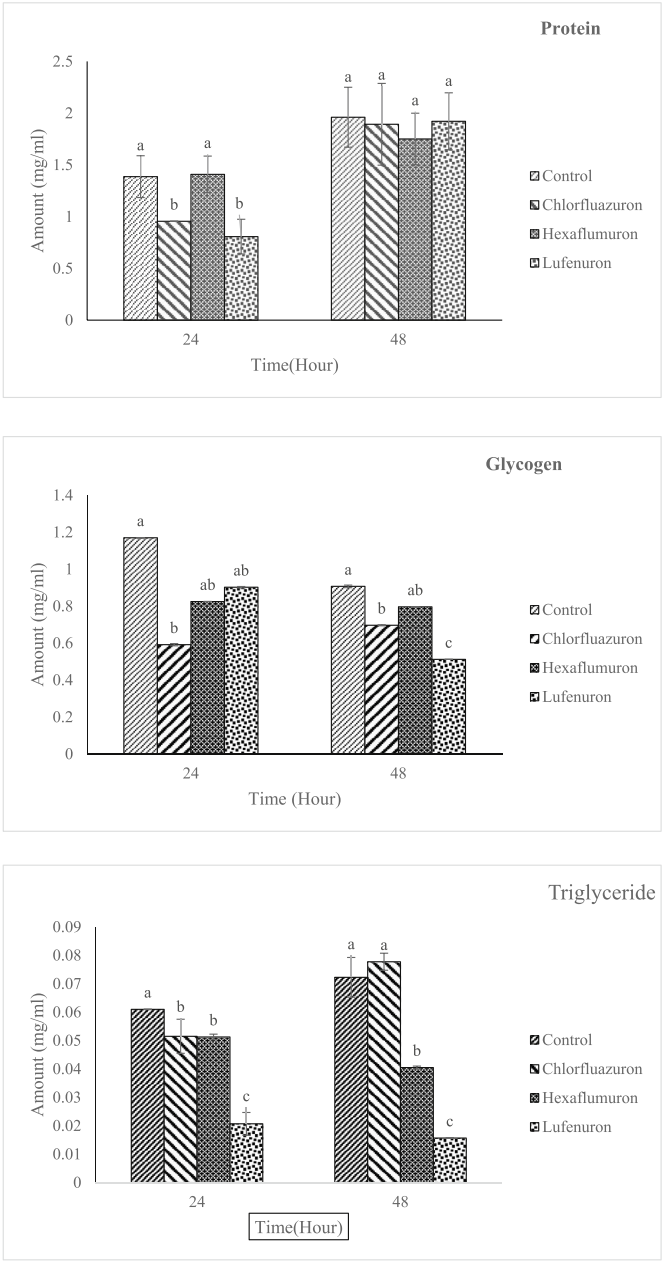


Fig. 5. Effects of insecticides on the amounts (mg/ml) of storage macromolecules in the 3rd larvae of *Helicoverpa armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3).

treatment by hexaflumuron, which is also reported by Delkash-Roudsari et al. (2014) on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae).

Storage macromolecules, glycogen and triglyceride were also affected in the larvae of *H. armigera* treated with chlorfluazuron, hexaflumuron and lufenuron at both time intervals after 48 h. The lower number of these molecules in the treated larvae clearly indicate the imposed energetic/physiological costs on the treated larvae due to insecticides. In fact, it may be inferred that insecticidal treatment led to the activation of compensatory mechanisms via storage macromolecules to provide energy and substrates for tissue repair, equilibrating physiological status and detoxification of the entered insecticides. In case of protein, we believe in i) compensatory mechanism within larval body which may be more feeding, ii) providing the required energy from macromolecules such as triglyceride and glycogen, iii) detoxification of the entered CSI 48 h post-treatment, iv) low concentration of CSI after 48 h to intervene the physiological process.

5. Conclusions

All the three insect chitin inhibitors, namely chlorfluazuron, hexaflumuron and lufenuron, showed toxicity against the larvae of *H. armigera*, although hexaflumuron, with the lowest LC_{50} concentration (6 mg ai/l), caused proper mortality on the larval population. Moreover, these insecticides increased larval/pupal duration compared to the control, but they significantly reduced the weight of immature stages. Activities of transaminases, phosphatases, lactate dehydrogenase and detoxifying enzymes were also affected in the larvae treated with the significantly lower numbers of storage macromolecules. Although field based trials are recommended to gain more reliable and constant results, we believe that these insecticides may suppress annual population outbreaks of *H. armigera* in Iranian agroecosystems because of their direct toxicity and intervening properties concerning larval physiology. Because of their selectivity and biodegradation in nature, these insecticides may impose minimum effects on non-targets and the potential pollution of environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101270.

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