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RESEARCH REPORT

Toxicity of the two insect growth regulators, pyriproxyfen and Lufox® against cotton bollworm

M Khorshidi[1], R Farshbaf Pour Abad[1,2*], A Zibaee[3*], M Saber[1]

[1]Department of Plant Protection, Faculty of Agricultural Sciences, University of Tabriz, Tabriz, Iran
[2]Department of Plant Protection, Faculty of Agricultural Sciences, Ege University,Izmir, Turkiye
[3]Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

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Abstract

This study investigated the field effects of two insect growth regulators, pyriproxyfen and lufenuron+fenoxycarb (A commercial combination), on some biological and physiological characteristics of third instar larvae of the cotton bollworm, *Helicoverpa armigera* Hubner. The insecticides significantly affected the weight and duration of larval and pupal development, the longevity of males and females, the egg laying and hatching rates. The larvae treated with sublethal concentrations showed an increase in the total number of hemocytes, plasmatocytes and granulocytes. A significant increase in aminotransferase activity was also observed in the hemolymph of treated larvae. The increased activity of acid phosphatase and alkaline phosphatase was also observed in the treated *H. armigera* larvae. The total activity of esterase and glutathione S-transferase as detoxifying enzymes also increased significantly after pyriproxyfen and lufenuron+fenoxycarb treatments compared to control. In contrast, the amount of storage macromolecules, protein, triglycerides and glycogen, which are related to the energy cost of pesticide treatment, decreased in the treated larvae compared to the control. According to the results of the present research, the pesticides pyriproxyfen and lufox can be used to control the population of *H. armigera* by interfering with the intermediate metabolism and cellular immunity of the larvae, but their efficiency and compatibility should be considered with other control methods, especially biological factors.

Key Words: *Helicoverpa armigera*; IGR; physiology; hemocyte; energy cost

Introduction

Helicoverpa armigera, the cotton bollworm, is a dangerous pest of many agricultural crops such as cotton, peas, tomatoes, etc. (Fitt, 1989). The widespread use of insecticides, multiple generations of the pest and the year-round availability of host crops have led to the development of resistance to many groups of insecticides (Haile *et al*., 2021). Therefore, it is necessary to use alternative, safe and selective insecticides to prevent the selection pressure of resistance and to produce the appropriate and healthy product. Insect growth regulators (IGRs) can be one of the alternatives to traditional chemicals because of their high safety for mammals, rapid degradation in the environment and low chance of resistance. These insecticides interfere with the normal growth and development of the insect, causing adverse effects on the endocrine system and disrupting reproduction and metamorphosis (Zibaee *et al*., 2011; Abu-Table *et al*., 2015). Although the endocrine system is the main target of such pesticides, they affect many physiological and biochemical processes in insect metabolism as well as biological and behavioral traits (Abu-Taleb *et al*., 2015). In fact, insect growth regulators alter the processing and concentration of both enzymatic and non-enzymatic substances in insect metabolism. (Nasr *et al*., 2010; Mirhaghparast *et al*., 2015; Vojoudi *et al*., 2017). Another system that may be affected following IGR treatment is immunity, which is actually induced by the invasion of any microorganism into the insect hemocoel (Strand, 2008). In general, the immune system

Corresponding authors:
Arash Zibaee
Department of Plant Protection
Faculty of Agriculture
University of Guilan
Rasht, Iran
E-mail: arash.zibaee@guilan.ac.ir;
arash.zibaee@gmx.com
Reza Farshbaf Pour Abad
Department of Plant Protection
Faculty of Agriculture
University of Tabriz
Tabriz, Iran
E-mail: reza.farshbaf.pourabad@ege.edu.tr

Table 1 Sublethal effects of lufenuron+fenoxycarb and pyriproxyfen on larval weight and duration, pupal weight and pupal longevity of *H. armigera*

Insecticides	Mean larval Duration time (days) ± SE	Larval weight (4 days after treatment) (mg)± SE	Larval weight (6 days after treatment) /mg)± SE	Pupal Duration time (days) ± SE	Pupal weight (mg) ± SE
pyriproxyfen	13.67±0.211 b	0.014±1.04 b	0.056± 0.01 b	16.00±0.11 a	0.267±11.18b
Lufenuron+fenoxycarb	17.00±0.000a	0.015±.001b	0.022±0.06c	17.00±0.000a	0.25±.010b
Control	8.74±0.372 c	0.16±2.38 a	0.44± 0.02 a	12.60±0.22 b	0.367± 10.30a

responds with two types of humoral and cellular responses upon detection of invading microorganisms (Lavine and Stand, 2002). Phagocytosis, encapsulation and nodulation are the three major processes involved in cellular immunity. Humoral defense is mediated by the activity of the prophenoloxidase system, antimicrobial proteins, oxygen and nitrogen intermediates (Manniello *et al*., 2021). The phenoloxidase enzyme is one of the oxidoreductase enzymes, which causes the oxidation of the substrate by transferring electrons from the substrate molecule to other molecules (Kasianov *et al*., 2017).

In our previous study, it was found that insect growth regulators, hexaflumuron, lufenuron and chlorfluazuron, had negative effects on biological factors and the intermediary metabolism of cotton bollworms (Khorshidi *et al*., 2019). Therefore, the present study was carried out in order to expand our knowledge on the effects of Pyriproxyfen and Lufox against the cotton bollworm and to investigate the lethal, sublethal, physiological, and cellular immunity of Pyriproxyfen and Lufox in the third instar larvae of the cotton bollworm. Pyriproxyfen is a juvenile hormone mimic that prevents regulation of molting and development of larvae from different species (Ishaaya and Horowitz, 1992; Mirhaghparast *et al*., 2014 and 2015). Lufox is a mixture of lufenuron and fenoxycarb in which Lufenuron is classified as a chitin synthesis inhibitor used to control biting and sucking insects (Wilson and Cain, 1997). Fenoxycarb is an O-ethyl carbamate ester derivative that has demonstrated efficacy against various insect pests, including Lepidoptera, aphids, and whiteflies (Goncu and Parlak, 2012). In details, we determined the effects of pyriproxyfen and Lufox on biological traits of larval-pupal duration and weight, adult longevity and fecundity, enzymatic and non-enzymatic components of intermediary metabolism and number of hemocytes.

Material and methods

Insect rearing

Eggs of *H. armigera* were provided from a colony kept in the research greenhouse of the Department of Plant Protection, University of Tabriz. The hatched larvae were fed separately with an artificial diet including 205 g cowpea powder, 14 g powdered agar, 3.5 g ascorbic acid, 1.1 g sorbic acid, 2.2 g methyl-p-hydroxybenzoate, 35 g yeast, 30 g wheat germ, 2.5 ml 37% formaldehyde solution, 5 ml vegetable oil and 650 ml distilled water (Shorey and Hale, 1965) under laboratory conditions of 28 ± 1 °C, 70 ± 5% relative humidity (RH) and a 16 h light:8 h dark (LD 16:8) photoperiod. The emerged adults were fed with a 10% honey solution and placed on 20×15×5 cm containers (equipped with ventilation cloth) for oviposition. The rearing was continued for two generations to obtain larvae for all the experiments.

Bioassay

Five concentrations based on field-recommended dose (Field dose, ½ field dose, ¼ field dose, 1/6 field dose and 1/8 field dose) of each insecticide were prepared in Tween®-80 solution (0.05%) and separately exposed to third instar cotton bollworm larvae by addition to their artificial diet. An appropriate amount of each concentration was combined with the larval diet consisting of the pesticide solution at a ratio of 9:1. Mortality was recorded at 24, 48 and 72 hours and the LC_{10}, LC_{30}, LC_{50} and LC_{90} values were determined using POLO-PC software. Active ingredient of pyriproxyfen and Lufox were 10% EC and 105 g/L EC, respectively.

Table 2 Sublethal effects of lufenuron+fenoxycarb and pyriproxyfen on adult longevity, fecundity and egg hatch rate of *H. armigera*

Insecticide	Female longevity (days)	Male Longevity (days)	Fecundity	Egg hatch rate (%)
Pyriproxyfen	4.000± 0.667 b	4.667±1.154 b	112.20±7.939 b	12.326±6.805 c
Lufenuron+Fenoxycarb	4.254± 0.321b	4.333± 0.333b	123±0.001 b	27.461±3.875b
Control	10.500±1.018 a	9.625± 0.885 a	487.00±20.580 a	70.570±2.546 a

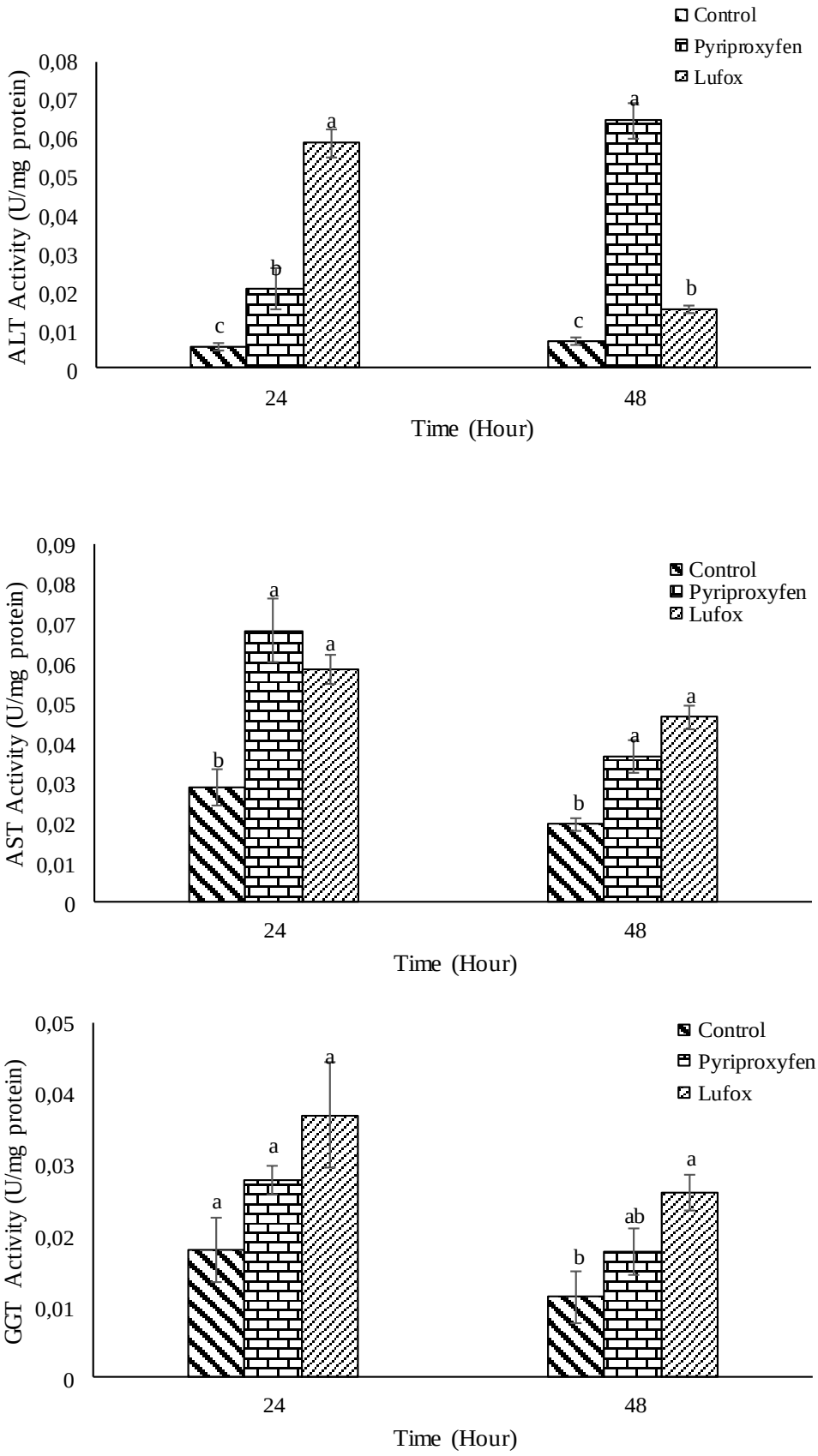

Fig. 1 Effects of insecticides on the activities of aminotransferases (U/mg protein) in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3)

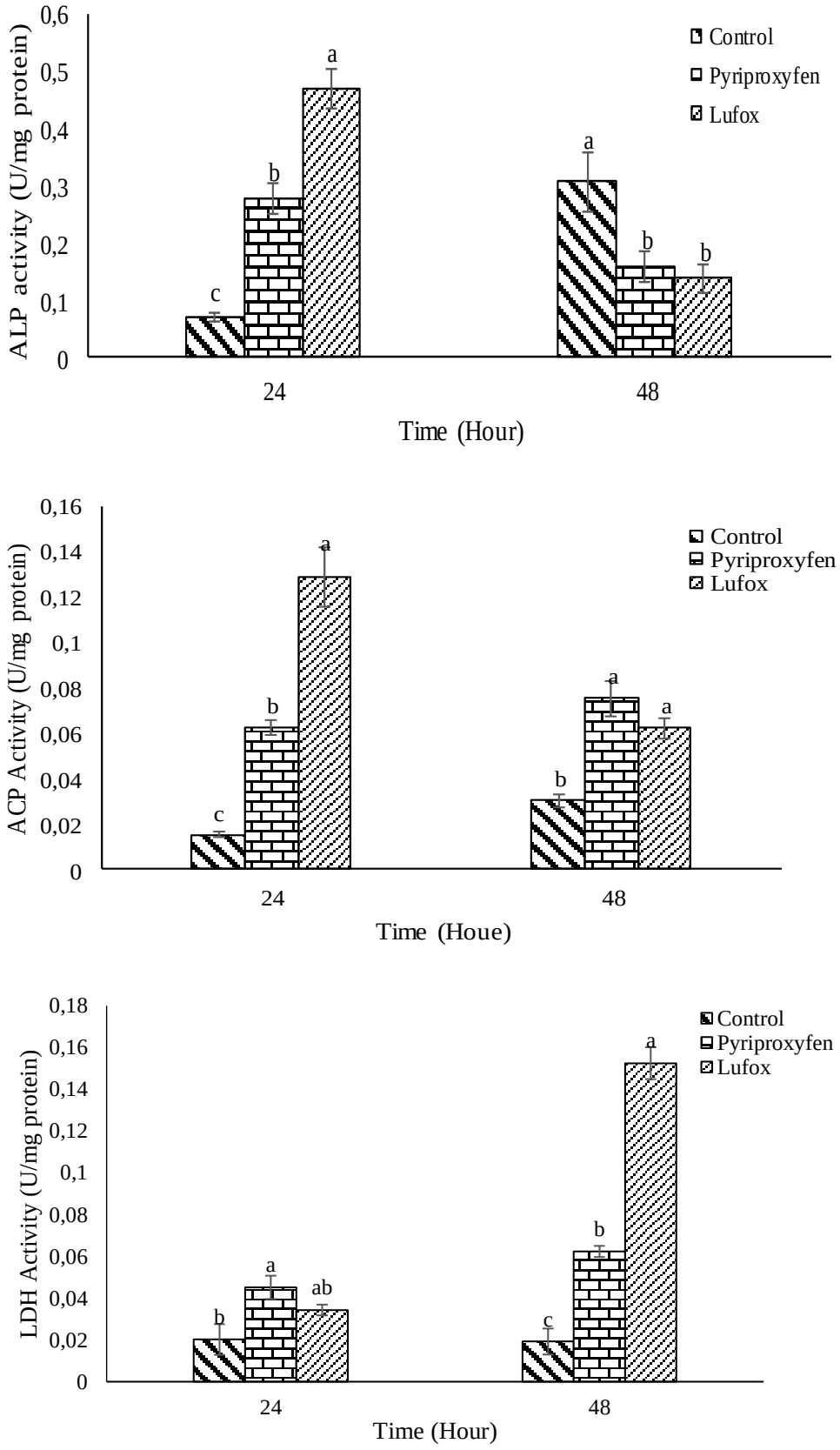

Fig. 2 Effects of insecticides on the activities of phosphatases and lactate dehydrogenase (U/mg protein) in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3)

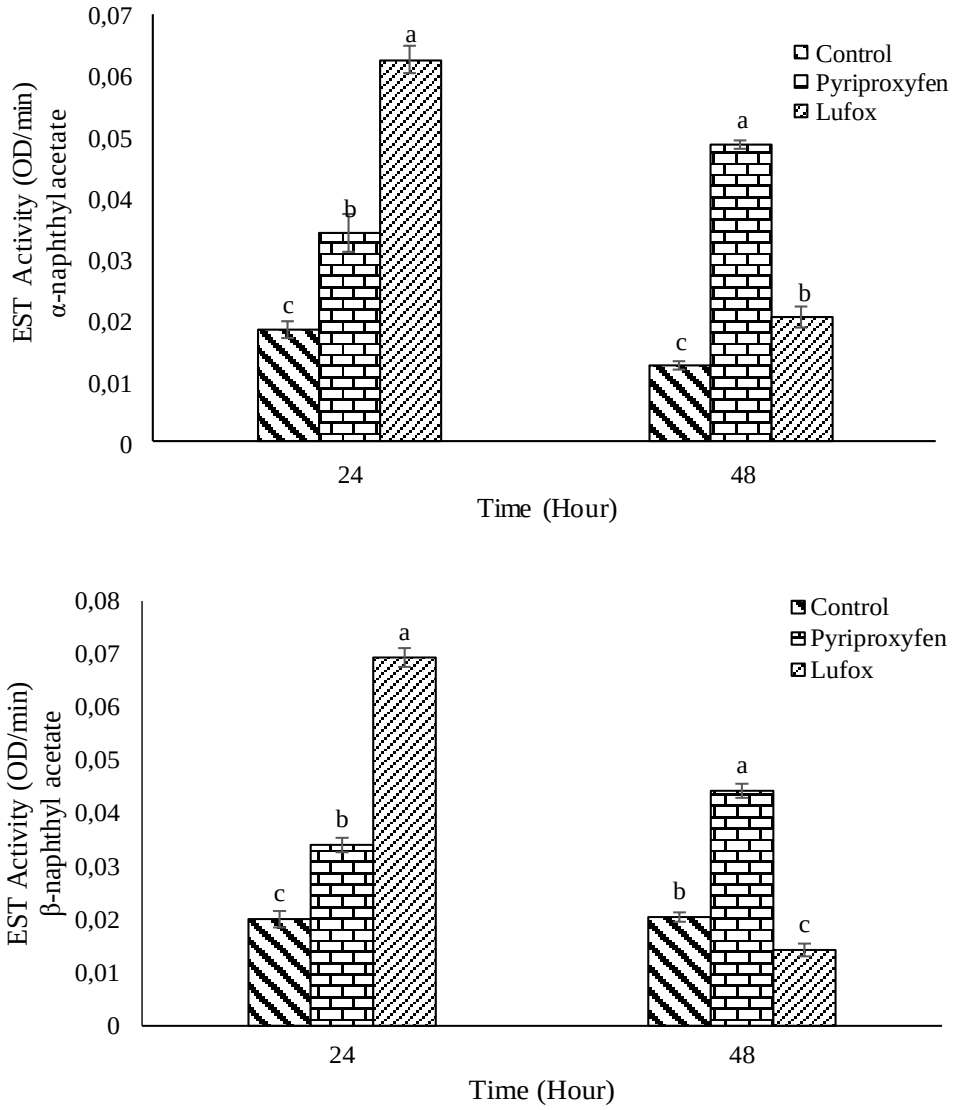

Fig. 3 Effects of insecticides on the activity of general esterases(EST) (U/mg protein) in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3)

Sublethal effects of the insecticides on H. armigera *biological parameters*

Sublethal effects associated with lufox and pyriproxyfen were assessed by oral exposure of newly molted third instar larvae on control and treated diets to the LC_{10} concentration of each insecticide separately until pupation. One hundred larvae were randomly selected and fed 1.5 g of control and insecticide treated diets separately. Larvae were checked at 24 and 72 h intervals, while surviving larvae were placed on untreated diets. Larvae were weighed and checked daily until pupation. Pupae were weighed and transferred to containers individually, separated by sex, and checked daily until adulthood to assess longevity and pupal viability. After emergence as adults, parameters related to fecundity, fertility and longevity were measured in a cylindrical container with one male and one female fed with 10% honey solution. In addition, 15 replications of each treatment were used to determine reproductive parameters. The total number of eggs laid by each female was counted daily until the female died. In order to follow hatching of the eggs, a bundle containing approximately 100 eggs, between one and 24 hours old, was placed in petri dishes (10 cm diameter and 2 cm depth). In addition, the longevity of each male and female was recorded independently.

Enzymatic assays

Preparation of samples

The 3rd instar larvae fed on the sublethal concentration of each compound separately were randomly selected and transferred individually into 1.5 g diet. The larvae treated by

lufenuron+fenoxycarb, pyripyroxyfen, and control were homogenized independently in distilled water after 24 and 48 hours, then moved to 1.5 mL centrifuge tubes. The tubes were then centrifuged at 25000×*g* for 20 minutes at 4 °C. Ultimately, supernatant was gathered and stored for biochemical analysis at -20 °C.

Assay of alanine (ALT; EC 2.6.1.1) and aspartate (AST; EC 2.6.1.1) aminotransferase

The activity of ALT and AST was assayed using the method of Thomas (1998) with a biochemical kit manufactured by Biochem (Tehran, Iran) containing pyruvate combined with 2,4-dintitrophenylpyruvate and synthetic pyruvate hydrazine. Reagent D was separately incubated with Reagent A (for AST) and Reagent B (for ALT) for five minutes. After that, an additional 60 minutes of incubation were continued after adding 10 µL of the enzyme solution. Finally, reagent C was subsequently incorporated, and the absorbance was measured at 340 nm**.**

Assay of γ-Glutamyl transferase (EC 2.3.2.2)

The γ-glutamyl transferase activity was measured by the method of Szasz (1976) using a produced kit by ZiestChem Diagnostic Company (Tehran, Iran). In summary, 20 µL of substrate reagent, 10 µL of enzyme solution, and 50 µL of buffer reagent were mixed with L-ɤ-glutamyl-3-carboxy-4-nitrianilide. After incubation for 3 minutes, the absorbance was read at 405 nm.

Assay of lactate dehydrogenase (EC 1.1.1.27)

Based on the method of King (1965), 10 µL of the sample was mixed with 20 µL of NAD+ solution and 100 µL of the buffered substrate. In control tubes, water (20 µL) was used instead of NAD+ solution. After an incubation for 15 minute at 37 °C, test tubes were filled with 100 µL of 2, 4-dinitrophenyl hydrazine as the reagent and left to continue incubating for an extra 15 minutes. After cooling in room temperature, 50 µL of NaOH was added into tubes and absorbance was read at 340 nm after 60 s.

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

The activity of ALT and AST was calculated with the technique of Bessey *et al*. (1946). For five minutes, 10 µL of the enzyme solution was incubated with the buffered substrates (Tris-HCl, 20 mM, pH 8, for ALP, and Tris-HCl, 20 mM, pH 5, for ACP). The absorbance at 405 nm was calculated following the addition of 100 µL of 1 M NaOH.

Determination of esterase activity

Esterase activity was measured using the procedure outlined by Han et al. (1998). Fifty microliters of fast blue RR salt (1 mM) and 20 µL of each substrate (α- and β-naphthyl-acetates (10 mM)) were added to the tubes containing 80 µL of universal buffer [Glycine, 2-morpholinoethan sulfuric acid, and succinate, 20 mM, pH 7]. After gently shaking, 20 µL of the enzyme solution was added, and optical density was measured at 545 nm every minute for the next five minutes.

Assay of glutathione S-transferase activity (GST)

GST activity was assayed using the procedure described by Oppenorth *et al*. (1979). Accordingly, each tube was filled with 80 µL of universal buffer (20 mM, pH 7), incorporated 20 µL of sample with 40 µL of 1-chloro-2,4,-dinitrobenzene (CDNB, 20 mM) and 1,2-Dichloro-4-nitrobenzene (DCNB, 40 mM). Then, 50 µL of reduced glutathione (20 mM) was added and shaked for 30 seconds. Finally, 50 µL of enzyme solution was added and the absorbance was read at 340nm after 3 min.

Protein determination

Protein concentration was measured based on Lowry *et al*. (1951) using a total protein assay kit manufactured by ZiestChem Diagnostic Company (Tehran, Iran). In this method, 20 µL of homogenate sample was incubated with 100 microliters of the reagent for 15 minutes, and the absorbance was recorded at 545 nm.

Triglyceride determination

Based on Fossati and Principe (1982), 70 µl of reagent, 10 µL of sample, and 10 microliters of deionized water were combined. The media was kept at 25 °C for 20 minutes before reading the absorbance at 545 nm (manufactured by PARS-AZMOON. Co., Tehran, Iran). The following equation was used to determine triglyceride content:

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

Glycogen determination

Glycogen concentration was measured based on Chun and Yin (1998) method. The tubes covered with foil containing larvae and 1 ml of 30% KOH w/Na2SO4 were boiled for 20–30 min. After shaking and cooling on ice, 95% EtOH (2 mL) was injected to the glycogen precipitation. Once more, the samples were shaken and allowed to cool on ice for half an hour. After centrifugation of the samples (13000 rpm for 30 min), the s pellet was separated and used after dissolving in one milliliter of water. Afterwards phenol 5% was used to prepare glycogen standards (0, 25, 50, 75, and 100 mg/mL) and allowed to incubate for 30 minutes in an ice bath. Finally, the absorbance was measured at 490 nm.

Assay of phenoloxidase activity (EC 1.14.18.1)

The Robb (1984)'s method was used to determine phenoloxidase activity in the hemolymph of control and treated larvae. For this purpose, 75 µL of phosphate buffer (1X, pH 7.1) was combined with 50 µL of L-dopa as substrate and 25 µL of the prepared enzyme solution. After 10 min, the absorbance was measured at 335 nm.

Effect of insecticides on hemocyte numbers

To investigate the effect of lufenuron+fenoxycarb and pyriproxyfen on the number of hemocytes, the larvae (after 4 hours of

starvation) were fed on the diet containing LC_{10} concentration of each insecticide, separately. Control larvae were just treated with distilled water. At 1, 4, 24, and 48-hour intervals, the larval hemolymph was extracted by cutting the first abdominal proleg and diluted with an anticoagulant solution (0.026 M citric acid, pH 4.6, 0.1 M glucose, 0.062 M NaCl, and 0.01 M ethylenediamine tetraacetic acid) ratio of 1:4 according to the procedure described by Azambuja *et al*. (1991). Then, the number of hemocytes, plasmocytes, and granulocytes was counted by a hemocytometer.

Statistical analysis

Using SAS software, one-way analysis of variance (ANOVA) was carried out to analyze all data from a complete randomized design in three replicates, and Tukey's test was then performed with a 5% probability using SAS software.

Results

Sublethal Effects of lufenuron+fenoxycarb and pyriproxyfen on biological traits of H. armigera

Both insecticides increased larval duration time although the treated larvae by lufenuron+fenoxycarb demonstrated the longer duration time comparison with pyriproxyfen (Table 1; Pr > F: 0.0001, Df: 2, 48). Pupal duration time was also higher than the control in the lufenuron+fenoxycarb and pyriproxyfen treatments (Pr > F: 0.0001, Df: 2, 21)**.** Also, the average larval and pupal weight showed a significant increase in both treatments compared to control (Pr > F: 0.0001, Df: 4, 83; Pr > F: 0.0001, Df: 2, 40). It was found that treatment of the larvae with both IGRs significantly reduced longevity of males and females (Table 2; Pr > F: 0.0007, Df: 2, 20; Pr > F: 0.0004, Df: 2, 20). By counting the quantity of eggs and hatched eggs, it was found that fertility

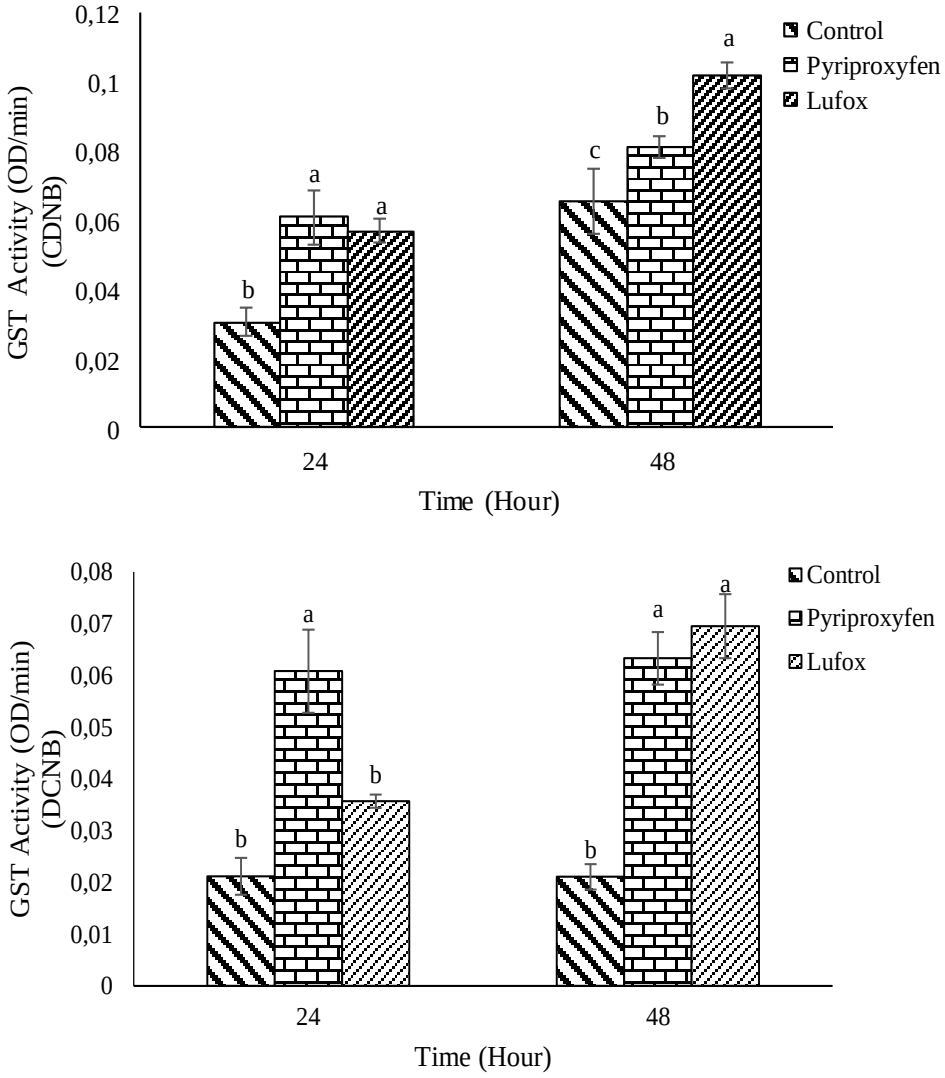

Fig. 4 Effects of insecticides on the activity of glutathione *S*-transferases (GST) (U/mg protein) in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3)

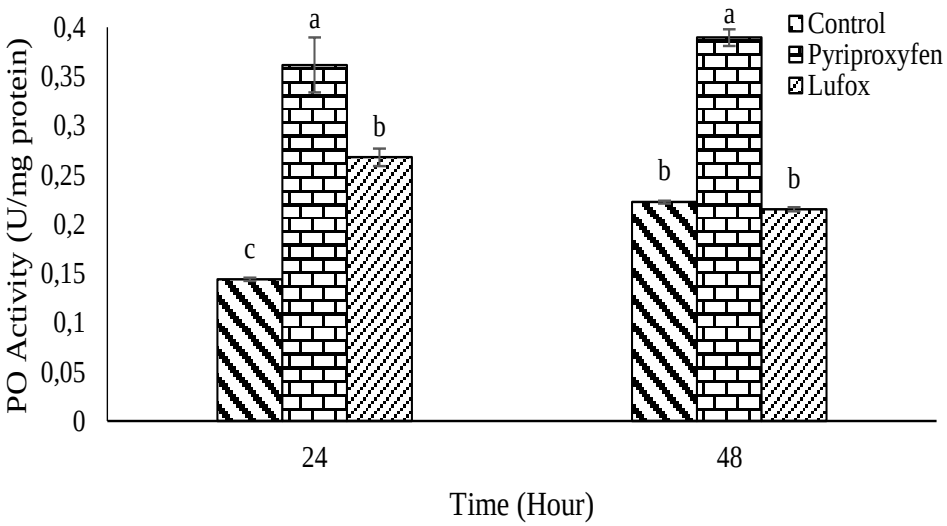

Fig. 5 Effects of insecticides on the activity of polyphenol oxidase (PO) (U/mg protein) in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; $p \leq 0.05$ (mean ± SE, n = 3)

and fecundity significantly affected by lufenuron+fenoxycarb and pyriproxyfen (Pr > F: 0.0001, Df: 2, 20).

Effects of lufox and pyriproxyfen on intermediary metabolism

The third instar larvae of *H. armigera* were treated with the mentioned concentrations of lufox and pyriproxyfen, and the results were recorded after 24 and 48 hours. Both insecticides changed ALT activity after 24 and 48 hours in comparison with the control. After 24 hours, the larvae that were treated with Lufox showed the highest level of enzyme activity (Fig.1; Pr > F: 0.0003, Df: 2, 8), but 48 hours after treatment, the highest activity was observed in larvae treated with Pyriproxyfen (Fig.1; Pr > F0.0003, Df: 2, 8). In the larvae treated with both IGRs, aspartate aminotransferase activity was higher than in the control larvae although the highest activities of AST was performed on the larvae treated with pyriproxyfen at intervals of 24 hours after treatment. (Fig.1; Pr > F: 0.0001, Df: 2, 6). No statistical difference of GGT activity was recorded in the larvae treated by the insecticides after 24 hours, (Fig.1; Pr > F: 0.0001, Df: 2, 8), but 48 hours after the treatment, activity of the enzyme was significantly higher than the control (Fig.1; Pr > F: 0.0001, Df: 2, 9).

Pyriproxyfen and Lufox had different effects on Acid (ACP) and alkaline (ALP) phosphatases in the hemolymph of *H. armigera*. Activities of ACP and ALP Significantly raised in the treated larvae by both insecticides 24 hours after treatment (Fig. 2; Pr > F: 0.0001, Df: 2, 8; Pr > F: 0.0001, Df: 2, 7). Although the activity of ACP was higher than that of the control after 48 hours (Fig.2; Pr > F: 0.0001, Df: 2, 7), ALP activity in the control was significantly higher than that of the treatment (Fig.2: Pr > F: 0.0001, Df: 2, 9).

Both IGRs significantly increased GST activity following larval treatments with pyriproxyfen and lufox, with the exception of b-naphtyl acetate after 48 hours of lufox treatment (Fig. 3; Pr > F: 0.0001, Df: 2, 11; Pr > F: 0.0001, Df: 2, 11). Similar results were recorded for glutathione S-transferase. Regarding the use of CDNB as a reagent, the highest enzyme activity was observed in Pyriproxyfen and Lufox treatments at time intervals of 24 and 48, respectively. In the case of DCNB, the highest enzymatic activity was observed 24 hours after pyriproxyfen treatment (Fig. 4; Pr > F: 0.0001, Df: 2, 11; Pr > F: 0.0001, Df: 2, 12). The activity of lactate dehydrogenase highly increased in the larvae treated by insecticides; the highest activity was recorded48 hours after lufox treatment (Fig. 2; Pr > F: 0.0001, Df: 2, 9; Pr > F: 173.22, Df: 2, 7).

Treatment of the third instar larvae with lufox and pyriproxyfen affected the amount of non-enzymatic compounds in addition to the enzymatic compounds. After 24 hours, total protein content reduced in the larvae treated with pyriproxyfen and lufox, but there were no noticeable changes in the levels of protein after 48 hours (Fig.6; Pr > F: 0.0001, Df: 2, 6; Pr > F: 0.0001, Df: 2, 3). At 24 hours of post-exposure, the amount of triglyceride in the treated larvae showed a significant decrease compared to the control. The highest reduction was observed in the larvae treated with Lufox (Fig.6; Pr > F: 0.0001, Df: 2, 8; Pr > F: 0.0001, Df: 2, 9). As a reserved macromolecule of hemolymph, glycogen level in the treated e significantly reduced in both time intervals of 24 and 48 hours (Fig.6; Pr > F: 0.0033, Df: 2, 52; Pr > F: 0.0011, Df: 2, 38).

Effect of lufox and pyriproxyfen on cellular immunity

Effects of the two IGRs, lufox and pyriproxyfen, were investigated on the total and differentiated hemocyte (granulocytes and plasmatocytes) numbers

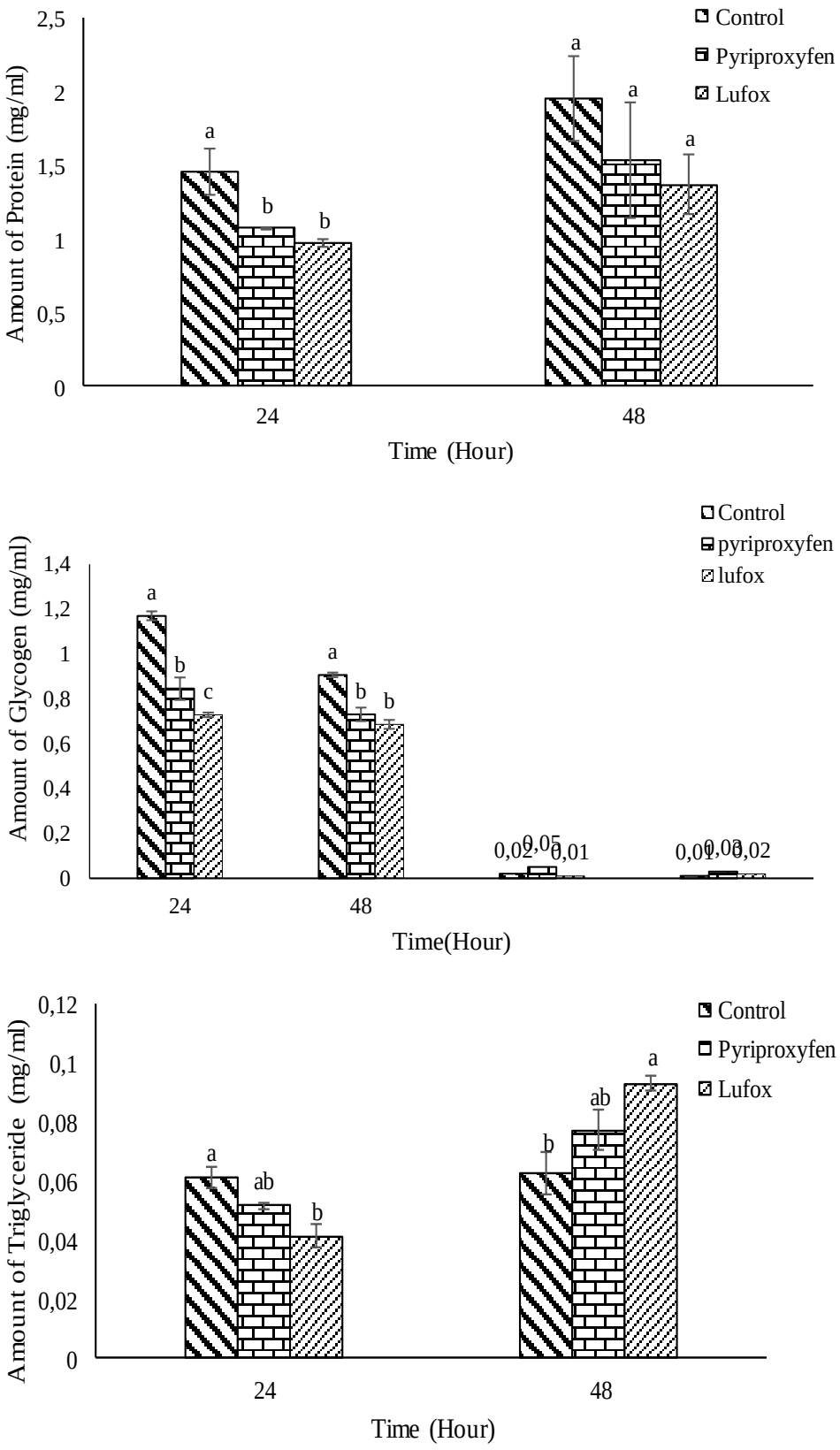

Fig. 6 Effects of insecticides on the amounts (mg/ml) of storage macromolecules in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters at Tukey test; p ≤0.05 (mean ± SE, n = 3)

of *H. armigera*. Based on these results, a significant increase was recorded in the total hemocyte number of the larvae treated with both insecticides at all-time intervals after treatment (Fig. 7; Pr > F: 0.0001, Df: 2, 7; Pr > F: 0.0001, Df: 2, 8; Pr > F: 0.0003, Df: 2, 6). One hour after pyriproxyfen treatment, the treated larvae showed the greatest number of total hemocytes (Fig. 7; Pr > F: 0.0003, Df: 2, 8). Similar results were found in the effects of pyriproxyfen and Lufox on the number of plasmatocytes by showing statistical differences at all times after treatment except for 4 hours (Fig.7; Pr > F: 0.0003, Df: 2, 8; Pr > F: 0.0003, Df: 2, 8; Pr > F: 0.0001, Df: 2, 6). Both treatments of insecticides exerted a significant effect on granulocyte count except for 4 hours, but the most relevant enhancement of granulocytes was observed in larvae treated by pyriproxyfen 1 hour after treatment (Fig.7; Pr > F: 0.0001, Df: 2, 7).

Discussion

One of the main management measures against *H. armigera* is the use of chemical insecticides both conventional and biorational ones. Although these have been shown to be effective in preventing population outbreaks, there are always serious concerns due to the adverse effects of non-selective insecticides (Khorshidi *et al*., 2017). The use of insecticides, in addition to their adverse environmental effects, has led to the development of resistance after regular use nevertheless there is still a significant demand for insecticides (Ahmad *et al*., 2002). Therefore, the use of IGRs may be a suitable measure due to their higher selectivity, environmental safety and lower potential for resistance. In our previous research, we reported the effects of three IGRs, hexaflumuron, lufenuron and chlorfluazuron, on certain biological and physiological parameters of *H. armigera* (Khorshidi *et al*. 2019). Results of the current study is another part of our project aiming to combat this devastating pest of agricultural crops. The results clearly showed that Pyripyroxfen and Lufox significantly increased the length of the larval and pupal periods, and on the contrary, they significantly decreased the weight of these two developmental stages. This means that the two insecticides, while disrupting the feeding process of the larvae, cause a kind of energy waste to reduce their destructive effects on the tissues and to detoxify them from the hemocoel. As a result, the insect has to spend more time to have optimal nutrition and to reach the appropriate size for molting. On the other hand, these two insecticides reduced the longevity of males and females, fecundity and egg hatching rate, which is related to the waste of energy and lack of proper feeding of larvae. In insects such as lepidopterans, larval physiology is such that by having maximum nutrition and improving macromolecular reserves for reproduction, it ensures the survival of adults and their maximum fertility. Such a shortage may be clearly observed on the results if intermediary metabolism in subsequent sections.

Amino acids play an important role in osmotic activity, energy supply and cuticle sclerotization of insects (Andersen, 2010). Transamination converts amino acids into pyruvate, acetyl-CoA and other citric acid cycle intermediates. During this process, the amino group of the amino acid (glutamate, aspartate and alanine) is transferred to keto acids to form ammonia. ALT is a transaminase enzyme that catalyzes two phases of the alanine cycle related to proline metabolism (Klowden, 2007; Nation, 2008). Aspartate aminotransferase is another transaminase enzyme that helps convert aspartate and alpha-glutarate to oxaloacetate and glutamate and vice versa in the insect Krebs cycle (McGill, 2016). In fact, insecticides cause damage to hemocytes and fat bodies, and more amino acids are needed to repair the tissue and energy produced during these processes. Gamma-glutamyl transferase plays an important role in the gamma-glutamyl cycle for the degradation and synthesis of glutathione, which delivers gamma-glutamyl to the receptor and produces glutamate (Balakrishna and Prabhune, 2014). Our results revealed the increased activity of all transaminases in the treated larvae compared to control. Such an increase is attributed to the role of transaminases to rehabilitate energy cost for detoxification or tissue damages following insecticide treatments. Zibaee *et al*. (2012) found an increase in ALT and AST activity at 24 and 48 hours after treatment with pyriproxyfen. Mirhaghparast *et al*. (2015) also found an increase in aminotransferase activity 12 and 24 hours after treatment with hexaflumuron.

Acid and alkaline phosphatases hydrolyse the phosphate group from molecules through dephosphorylation in acidic and alkaline environments, respectively. They also play an important role in fat digestion in the alimentary canal (Ramzi *et al*., 2014). In the present study, ACP and ALP activities increased in the haemolymph of treated larvae compared to controls. This result is consistent with our previous study on the effects of hexaflumuron, lufenuron and chlorfluazuron on *H. armigera* (Khorshidi *et al*., 2019). Mirhaghparast *et al*. (2015, 2016) also reported that the use of pyriproxyfen and hexaflumuron as two insect growth regulators increased the activity of phosphatases in the haemolymph of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). Phosphatases have also been included in the list of detoxifying enzymes (Ramakrishnan et al.,2024). The increase in phosphatase activity after insecticide treatment may be due to the phosphorylation of macromolecules stored in the body to provide energy for physiological processes. In addition to macromolecules, phosphatases also cause the phosphorylation of insecticides (Saad *et al*., 2015).

Lactate dehydrogenase is an enzyme that catalyses the conversion of pyruvate (the final product of glycolysis) to lactate, while simultaneously converting NAD and NaDH to each other under anaerobic conditions (Mazzei *et al*. 2016). In this study, we observed that treatment of *H. armigera* larvae with pyriproxyfen and lufenuron+fenoxycarb increased LDH activity in both time intervals. Similar results were found in our previous study with other IGR insecticides (Khorshidi *et al*., 2019). In similar studies, an increase in LDH activity was found after insecticide treatments (Mirhaghparast *et al*., 2016; Hanan *et al*.,

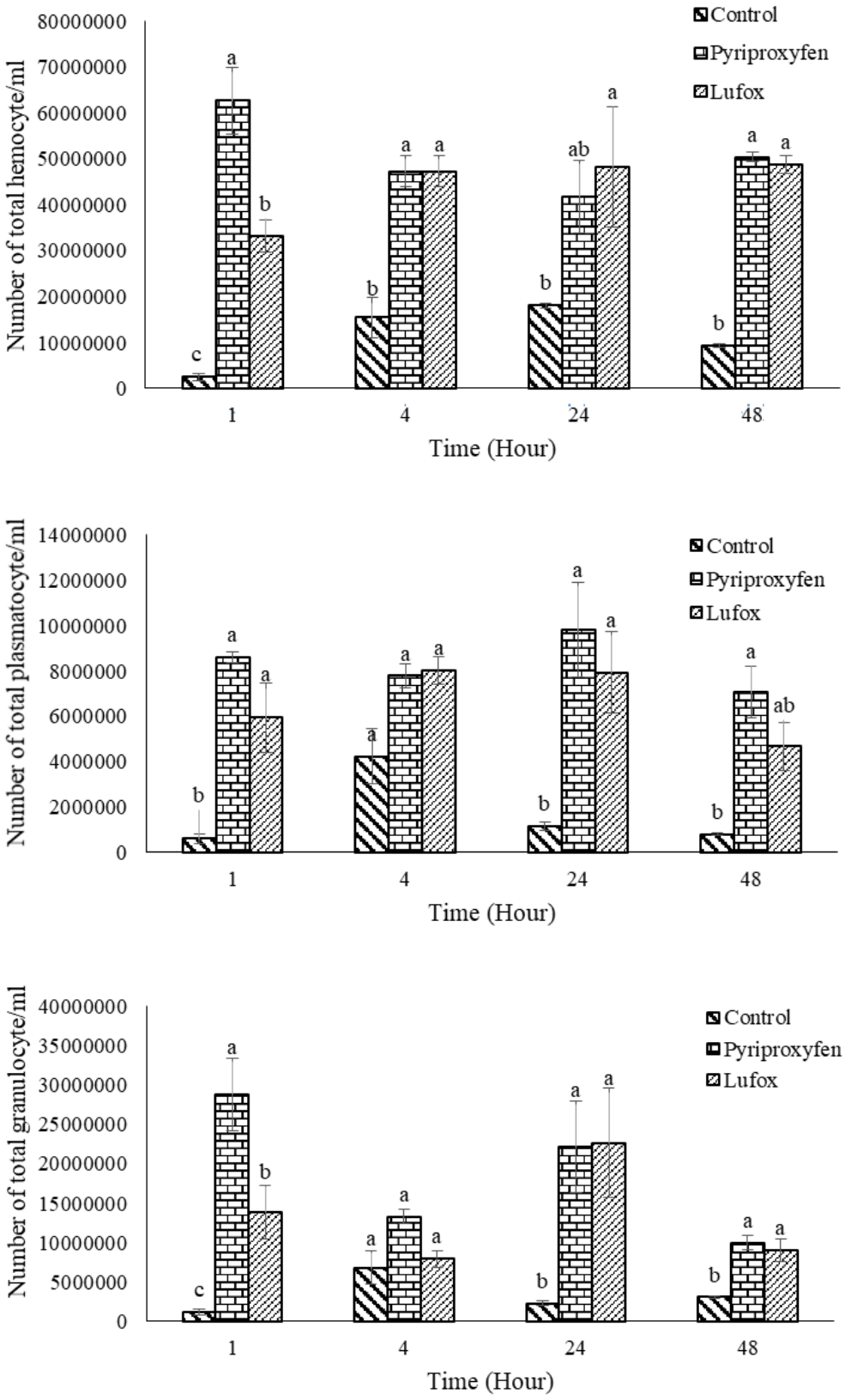

Fig. 7 Effects of lufenuron+fenoxycarb and pyriproxyfen on total and differentiated hemocyte numbers in the 3rd larvae of *H. armigera*. Statistical differences have been done within each time intervals and marked by different letters (Tukey test, p b 0.05)

2023). Such an increase may indicate the exposure of organic tissues to chemical stress, indicating the need for more energy through glycolysis. Since pyriproxyfen and Lufox imposed significant delay in larval-pupal duration as well as weight it may be more realistic tissue damages.

Two important groups of enzymes that play an important role in the removal of foreign substances such as insecticides are esterase and glutathione S-transferase (Khorshidi *et al*., 2019; Lapenna, 2023). Esterase hydrolyses choline esters, aliphatic and aromatic esters, and organic phosphorus compounds, converting them to acids and alcohols. Glutathione S-transferases detoxify xenobiotics by binding electrophilic substrates to the reduced form of glutathione (Sadek, 2003). Exposure of insects to insecticides induces an immune response and activates detoxifying enzymes. Previous studies have shown that EST and GST are important for the detoxification of IGRs in *H. armigera* larvae. (Khorshidi *et al*. 2019). In the present study, an increase in the activity of these enzymes was also observed in pyriproxyfen and lufenuron+fenoxycarb treatments at different time intervals. In the study by Vojodi *et al.* (2017), a significant increase in the amount of esterase and glutathione S-transferase enzymes in cotton bollworms was observed due to treatment with indoxacarb and hexaflumoran.

After studying the effects of insecticides on some important enzymes in the hemolymph of *H. armigera*, changes in some non-enzymatic compounds were also investigated as a nutritional index. Insects store their extra food as protein, glycogen and triglycerides. Protein, as a major organic component, plays an important role in stress compensation mechanisms where protein is broken down into amino acids and enters the TCA cycle as a keto acid for energy production (Sugumaran *et al*., 2010). Glycogen is a stored polymer of glucose that can be readily converted to trehalose to provide energy for activities such as flight, reproduction and stress compensation (Remia *et al*., 2008; Mason-Jones et al., 2022). Under conditions of stress, compensatory mechanisms are activated to provide energy and repair damaged tissues, and lactate dehydrogenase via glycolysis provides the necessary energy from glycogen catalysis. Triglyceride is a lipid stored in the fat bodies of insects that provides the necessary energy for insects by means of β-oxidation (Fossati and Prencipe, 1982). These results, according to enzymatic evidence, do indeed indicate an energy requirement in the treated larvae, resulting in the breakdown of storage macromolecules. Macromolecules are involved in energy production through intermediary metabolisms such as proline metabolism, oxidation, the Krebs cycle, glycolysis and the electron transfer cycle. Another possible hypothesis in lower amounts of macromolecules maybe nutritional deficiencies as the larvae orally were exposed to pyriproxyfen and Lufox.

The most effective hemocytes in the cellular defense of insects are granulocytes and plasmatocytes. Apparently, plasmatocytes play a more important role than granulocytes in Lepidoptera (Zibaee *et al*., 2012). In larvae treated with lufenuron+fenoxycarb and pyriproxyfen, the number of hemocytes (plasmatocytes and granulocytes) and phenoloxidase activity increased significantly compared to the control at all time intervals. In a similar study, an increase in phenoloxidase activity was observed in bollworm larvae treated with hexaflumuron, lufenuron and chlorfluazuron (Khorshidi *et al*., 2019). In addition, Tunöçsoy and özalp (2021) showed that treatment of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae with pyriproxyfen increased the number of haemocytes 48 and 72 hours after treatment. Ghasemi *et al*. (2014) also reported an increase in the number of granulocytes in *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) larvae after treatment with pyriproxyfen. Indeed, these insecticides enter the hemocoel and are known to act as foreign bodies, stimulating haematopoietic organs to proliferate hemocytes. Since phenoloxidase is secreted by hemocytes as a zymogen, it can be concluded that the possible reason for the increase in this enzyme was the increase in hemocytes.

Conclusions

In the present study, two insect growth regulators, pyriproxyfen and Lufox, were used to determine the disruption of intermediate metabolism and cellular immunity in *H. armigera*. Pyriproxyfen and Lufox affected the duration of the immature stages, longevity and fecundity of the adults, causing changes in some metabolic parameters such as aspartate and alanine aminotransferase, gamma-glutamyl transferase, lactate dehydrogenase, acid and alkaline phosphatase, general esterases and glutathione S-transferase. In addition, the total number of blood cells, plasmatocytes and granulocytes as well as the activity of phenoloxidase showed a significant change in treated larvae compared to the control. According to the results of the present research, the pesticides pyriproxyfen and lufox can be used to control the population of *H. armigera* by interfering with the intermediate metabolism and cellular immunity of the larvae, but their efficiency and compatibility should be considered with other control methods, especially biological factors. Compared to synthetic insecticides, IGRs cause no rapid mortality in insects, but they impair normal growth and physiological mechanisms so their long-term exposure suppress population increase and damages impose to agricultural pests. Also, they are recommended to control pest population in the numbers below economic thresholds to gains more protection. In contrast, the use of synthetic insecticides is more suitable for rapid pest management and severe effects on outbreaks. Modern pest management programs emphasize on the use of so-called “soft” pesticides with the aim of reducing the high mortality of natural enemies caused by insecticides. Accordingly, IGRs are more suitable than synthetic insecticides because of their rapid biodegradation in the environment and low toxicity to natural enemies.

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