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Research Article

Influencing larval development: how insect growth regulators modulate fat, protein, and enzyme levels in *Helicoverpa armigera*

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Abstact

The current study was designed to determine the toxicity of pyriproxyfen, lufenuron and tebufenozide on the 3rd instar larvae of *Helicoverpa armigera*. The insecticides were incorporated into the diet and exposed to the larvae then three concentrations of LC_{30}, LC_{50} and LC_{70} of pyriproxyfen, tebufenozide and lufenuron were tested to assay larval weight, amount of fat and protein as well as the activity of three digestive enzymes. Bioassay revealed the LC_{50} values of 3.7, 12.49 and 1.56 mg ai/L, respectively. Increased concentrations of the insecticides significantly decreased the larval weight and the amount of fat and protein compared to control as the least value was recorded on LC_{70} concentration of lufenuron. The LC_{50} concentrations of tebufenozide and lufenuron caused the least activity of α-amylase, lipase and protease in the treated *H. armigera* larvae compared to control. The results demonstrated that not only pyriproxyfen, tebufenozide and lufenuron have proper entomotoxicity against *H. armigera* larvae but also they significantly affect some physiological traits in both sublethal and lethal concentrations.

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Introduction

The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is a significant pest of several agricultural crops that has spread to almost all regions of the world. This pest has been widely distributed in Asia, Africa, Oceania (Australia, New Zealand) and Europe (Capinera 2022). *Helicoverpa armigera* is a highly polyphagous species, with its primary agricultural hosts being tomatoes, cotton, chickpeas, sorghum, pigeon peas, okra, groundnut, soybeans, tobacco, potatoes, corn, fruit trees and etc (Karim 2000). Different control measures have been reported to control outbreaks and severe damages of *H. armigera* including pheromone traps, chemical insecticides, biological control agents mainly predators and parasitoids as well as cultural methods based on field hygiene (Karim 2000).

Insect Growth Regulators (IGRs) are one of the important alternative chemicals against insect pests because of their selectivity, efficiency, low toxicity on mammals and rapid degradation in the environment (Ghasemi *et al*. 2010). These compounds primarily control insects by disrupting metamorphosis and reproduction (Riddiford & Truman 1978). Compounds designed to interfere with metamorphosis can lead to the development of adult insects that do not produce offspring. Because they impose sterility or abnormal reproductive organs, which hinders the mating process or the ability to produce fertile individuals (Merzendorfer 2013). IGRs are categorized among selective insecticides based on their mode of action as: chitin synthesis inhibitors that inhibit formation of insect cuticle, the compounds that interfere with the functioning of insect hormones, including juvenile hormones and ecdysteroids (Tunaz & Oygun 2004). Chitin synthesis inhibitors are compounds that prevent the synthesis of chitin and disrupt the molting process in immature stages of insects. These insecticides are also known as Acylurea because not only they interfere with molting of immature insects but also, they affect reproduction and lifespan of adult insects (Merzendorfer 2013). Chitin synthesis inhibitors encompass a variety of structurally diverse compounds, including pyrimidine nucleoside peptides, benzoylureas, thiophthalimides, thiadiazines, thiazolidines, tetrazines, chromophores, and fluorophores. Benzoylureas, thiadiazolines, and oxazolines disrupt a specific stage of chitin synthesis, preventing its formation. Benzoylureas also inhibit the formation of the epithelial tissue of the midgut (Merzendorfer 2013). Insecticides in this group include tebufenozide, lufenuron, diflubenzuron, hexaflumuron, and chlorfluazuron (Merzendorfer 2013). Lufenuron is an insect growth inhibitor that leads to cuticular lesions and disrupts chitin synthesis. It belongs to the benzoylurea group and is considered a chitin synthesis inhibitor, exhibiting both contact and oral effects (Merzendorfer 2013).

Any disruption in the natural balance of hormones causes disturbances in the growth and development of insects. Juvenile Hormones (JHs) control various processes in insects such as embryogenesis, molting, metamorphosis, reproduction, diapause, migration, flight, silk production, and phase change. Many analogs of juvenile hormones (JHAs) are used to control insect pests because of their ease of synthesis and selective action compared to other peptide and steroid hormones (Eto 1990). Pyriproxyfen is a well-known insect growth regulator that mimics the action of JH. It is structurally similar to fenoxycarb and belongs to the 4-phenoxyphenoxy group but differs chemically from methoprene and JH III (Palma *et al*. 1993). In the larval stage of target insects, pyriproxyfen prevents the transformation of larvae into pupae or creates intermediate states like larva-pupa, which disables reproduction (Palma *et al*. 1993). Tebufenozide is a selective insecticide for target pests and has low toxicity to mammals that acts as a molting hormone, causing premature molting in larvae. Tebufenozide is now widely used to control leaf-feeding pests in the order Lepidoptera worldwide (Carlson 2000).

The use of selective and low-risk pesticides for beneficial insects and mammals is one of the main principles of chemical control of pests. Insect growth regulators are among these insecticides that should be screened to determine their efficacy on target insects. In the present study, the toxicity of three compounds pyriproxyfen, tebufenozide and

lufenuron was investigated on the third instar larvae of *H. armigera* and then their physiological effects were evaluated on larval weight, fat and protein contents and three digestive enzymes.

Materials and Methods

Insect

The initial population of *H. armigera* was prepared from the greenhouses of plant protection department at the University of Tabriz. The cultivation encompassed all developmental stages, with conditions rigorously controlled at a temperature 25 ± 2 ˚C, a photoperiod of 16 hours light to 8 hours dark, and a relative humidity of 70%. The larvae were fed on a diet containing 204 gr cow pea powder, 30 gr wheat germ powder, 30 gr yeast, 3.5 gr ascorbic acid, 1.3 gr sorbic acid, 2.7 mL formalin, 4 mL cooking oil of sun flower, 14 gr agar and 600 mL water (Shorey & Hale 1965).

Treatments and bioassays

The insecticides used in the current study were pyriproxyfen (Admiral®, 10 EC), lufenuron (Match ® 5 EC), tebufenozide (Mimic® 20SC). The bioassay was conducted within 6-centimeter diameter plastic containers with a one-centimeter hole covered with high-mesh fabric. After initial experiments and obtaining the main concentrations for each insecticide, 1 milliliter of the desired concentration, combined with 9 gr of artificial diet. To ensure insecticide integration with the nutritional substance, a green food dye was used in 10-microliter amounts for each concentration. It is worth noting that a positive control (solely with food dye) was employed for the experiments and the whole experiment was repeated three times. After 24 h, mortality was recorded and the data was analyzed by POLO-plus software. After determination of lethal concentrations, the three values of LC_{30}, LC_{50} and LC_{70} for lufenuron (0.720, 1.56, and 3.38, mg ai/L), tebufenozide (5.77, 12.49, and 27.6, mg ai/L) and pyriproxyfen (1.94, 3.70, and 7.07, mg ai/L) were selected to be combined with artificial food and administered for larval feeding to determine changes in larval weight, fat and protein contents as well as the activity of three digestive enzymes. Data collection occurred 48 hours later.

The weight of treated larvae

Initially, 3rd instar larvae of the *H. armigera* were fed on the artificial food containing LC_{30}, LC_{50} and LC_{70} (mg ai/L) of each insecticide, respectively and kept at 25±2 °C, under a light-dark cycle of 16:8 hours, with a relative humidity of 50%. After 48 hours, 10 larvae from each treatment were selected and their weights were measured with a sensitive scale (accuracy 0.1 gram). Control larvae were fed on the artificial diet without any treatment.

Total lipid extraction

Chloroform-methanol mixture (2:1 by volume) was utilized for lipid extraction, following the method of van Handel (1985). Initially, 10 larvae from the control and treatment were placed separately in the freezer for several minutes, then powdered in a mortar using liquid nitrogen and the powder was dried at 70 °C for 24 hours. In the next step, 1 ml of chloroform-methanol solution (2:1 ratio) was poured onto the sample and vortexed for 30 seconds. Subsequently, the sample was centrifuged at 13,000 rpm for 5 minutes at 4 °C. After removing the solvent, another 1 mL of chloroform-methanol solution (2:1 ratio) was poured, vortexed, and centrifuged under the afore-mentioned conditions. Following solvent removal, the sample was dried at 80 °C for 48 hours and then weighed. The obtained weight represents the lipid content of the target sample. The experiments were conducted in triplicate.

Protein Extraction

Similarly, 10 control and treated larvae were separately selected and put into ceramic container. One ml of Tris buffer (27 ml, pH 7) was added, homogenized and centrifuged at 12,000 rpm for 20 minutes at 4 °C. The liquid phase was selected and transferred to a 1.5 ml microtube. The new microtubes were moved to a temperature of -20°C for further experiments. Protein concentration was determined using Bradford (1976) with bovine serum albumin standard at concentrations of 0.1, 0.25, 0.42, 0.6 and 1 mg/ml. Briefly, 10 µl of

samples were mixed with 80 µl of Bradford reagent, incubated for 10 min and read the absorbance at 595 nm.

Enzyme extraction

Sample extraction for the enzyme study from the midgut of *H. armigera* larvae were done separately in control and treated ones. The larvae were dissected in saline solution and their midguts were transferred to 2 ml microtubes containing extraction buffer (phosphate buffer, 1X, pH 6.8). The microtubes were placed on ice until homogenization. After 26 seconds of homogenization using a homogenizer, the microtubes were kept in a refrigerator at 4 °C for an hour to dissolve digestive enzymes in the buffer. Following this, the microtubes were centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was transferred to other microtubes and stored at -20 °C for enzyme assay.

α-amylase activity

α-Amylase activity was assessed using 3,5-dinitrosalicylic acid (DNS) and 1% starch as a substrate (Bernfeld 1955). Briefly, 10 µl of each enzyme sample, along with 20 µl of starch solution were incubated at 35 °C for 30 minutes within 100 µl of phosphate buffer (1X, pH 7). The reaction was halted by adding 100 µl of DNS, followed by a 15-minute heat treatment in boiling water and a subsequent 5-minute immersion in ice water. After centrifugation at 10,000 rpm for five minutes, the absorbance of the supernatant was measured at 540 nm. The experiments were conducted in triplicate

Protease assay

Azocasein was used as a substrate to assay total protease activity. Briefly, 1 ml of enzyme sample was mixed with 200 µl of glycine-NaOH buffer (0.2 mM, pH 10) containing 5 mM calcium chloride. The tubes were maintained at 37 °C for 10 minutes. Subsequently, 200 µl of 1% azocasein (w/v) was added, and the reaction was allowed to proceed for an additional 60 minutes under the same conditions. The reaction was stopped by adding 300 µl of 10% trichloroacetic acid solution. After centrifugation, the absorbance was measured at 450 nm. The control solution was prepared by replacing the substrate with an equal volume of buffer (Heydari-Zad *et al*. 2019).

Lipase assay

A ZiestChem Diagnostic Lipase Kit was used to assay lipase activity in the control and the treated larvae of *H. armigera* based on the method of Kwon and Rhee (1986). Briefly, 1 ml of reagent buffer R1 was mixed with 200 µl buffer reagent R2 before to add 50 µl of enzyme solution. After 10 min, the absorbance was read at 578 nm.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine statistical differences which were marked with different letters at the probability less than 5%.

Results

Bioassay of the all three insecticides; pyriproxyfen, tebufenozide, and lufenuron, showed mortality on the 3rd instar larvae of *H. armigera* in a dose-dependent manner (Figure 1). Based on the obtained LC_{50} values, lufenuron had the highest toxicity of 1.56 mg ai/L, while tebufenozide showed the lowest toxicity of 12.49 mg ai/L against 3rd instar larvae of *H. armigera* (Table 1). Considering the non-significant X^2 factor, *H. armigera* population has a consistent response to the pesticides, indicating homogeneity. Moreover, the regression relationships and the slope of the line in Figure 1 may confirm that an increase in concentration of all three insecticides elevate mortality percent in the 3rd instar larvae. The slope of the tebufenozide (1.563 ± 0.617) and lufenuron (1.562 ± 0.617) lines showed the same value which indicates a similar mode of action so it can be stated that 3rd instar larvae of *H. armigera* exhibited a similar response to both insecticides. Finally, the slope of pyriproxyfen dose-response line (1.868 ± 0.65) showed the highest value among the insecticides. Therefore, a slight increase in concentration leads to a significant increase in mortality of the treated larvae.

Table1. Toxicity of the insecticide treated on the 3rd instar larvae of *Helicoverpa armigera*.

Insecticide	LC30(mg ai/L) (95% CL)	LC50(mg ai/L) (95% CL)	LC70(mg ai/L) (95% CL)	Slope ± SE	Factor X2		
					Df	P	X^2
pyriproxyfen	1.94 (0.54 - 3.09)	3.7 (2.2 - 8.56)	7.07 (4.2 - 50.42)	1.868 ± 0.65	4	0.95	0.697
tebufenozide	5.77 (0.27 – 11.7)	12.49 (4.04 – 38.59)	27.06 (13.23 – 558.1)	1.563 ± 0.617	3	1	0.015
lufenuron	0.72 (0.03 - 0.1)	1.56 (0.5 – 4.8)	3.38 (1.65 - 69.81)	1.562 ± 0.617	3	1	0.015

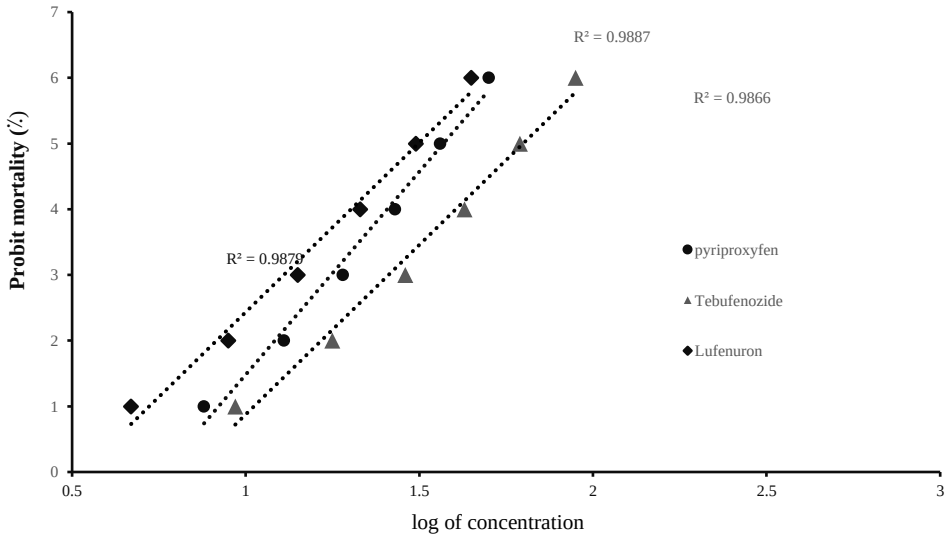


Figure1. Dose-response line of pyriproxyfen, tebufenozide and lufenuron 3rd instar larvae of *Helicoverpa armigera*.

Insecticide effects on larval weight

A statistically significant difference was recorded in larval weight between control and treated larvae by pyriproxyfen, tebufenozide, and lufenuron at a 5% significance level (Df9, 20 = 138.907; Pr > f: 0.0001) (Figure 2). The highest larval weight reduction was observed in LC_{70} concentration of lufenuron, amounting to 65.33 mg ($p<0.005$) (Figure 2). The least weight reduction was observed in the larvae treated by LC_{30} and LC_{50} concentrations of pyriproxyfen (Figure 2). These findings indicated that lufenuron, regardless of concentration, is highly effective at inhibiting larval growth, which could reflect its mechanism of action that disrupts chitin synthesis and, consequently, larval development. The minimal impact of pyriproxyfen at lower concentrations might suggest a more moderate effect on larval growth or a greater capacity for the larvae to metabolize or detoxify it in the given levels.

Effect of used insecticides on the amount of larval fat and protein

The insecticidal treatments caused a statistically significant difference in the fat amount of larvae compared to control at a 5% significance level (Figure 3). The highest fat reduction was recorded in the larvae treated by LC_{70} concentration of lufenuron and tebufenozide, respectively (Figure 3). In contrast, the larvae treated by LC_{30} and LC_{50} concentrations of pyriproxyfen showed the least fat reduction compared to control (Figure 3).

Pyriproxyfen, Tebufenozide and Lufenuron significantly affected protein amount in the treated

larvae compared to control (Df 9,20 = 77.82; Pr > f: 0.0001 at the 5% probability level) (Figure 3). Specifically, the larvae treated by the LC_{70} concentration of lufenuron exhibited the lowest protein concentration among the treatment groups. Similar results were recorded by the LC_{70} and LC_{50} concentrations of tebufenozide, which also showed reduced protein levels, albeit to a lesser extent than lufenuron (Figure 3). The pattern of results across the concentrations indicates a dose-dependent effect of the insecticides on protein concentration.

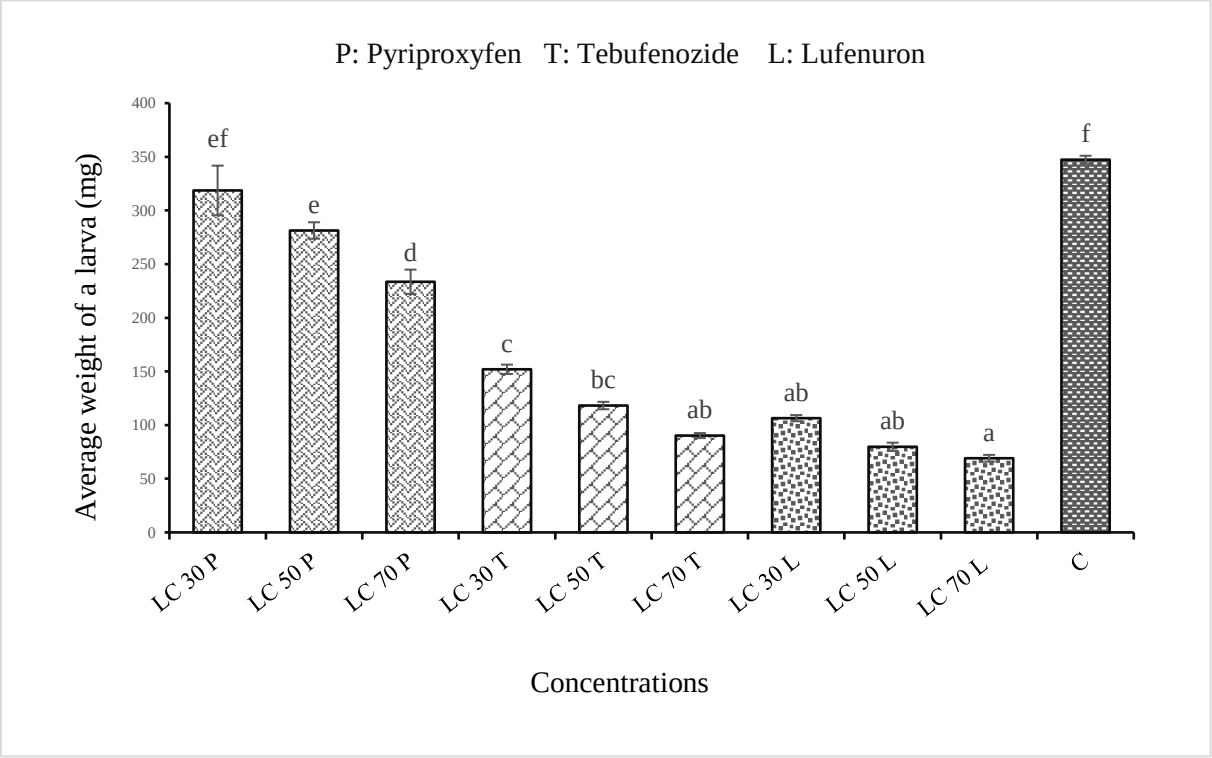
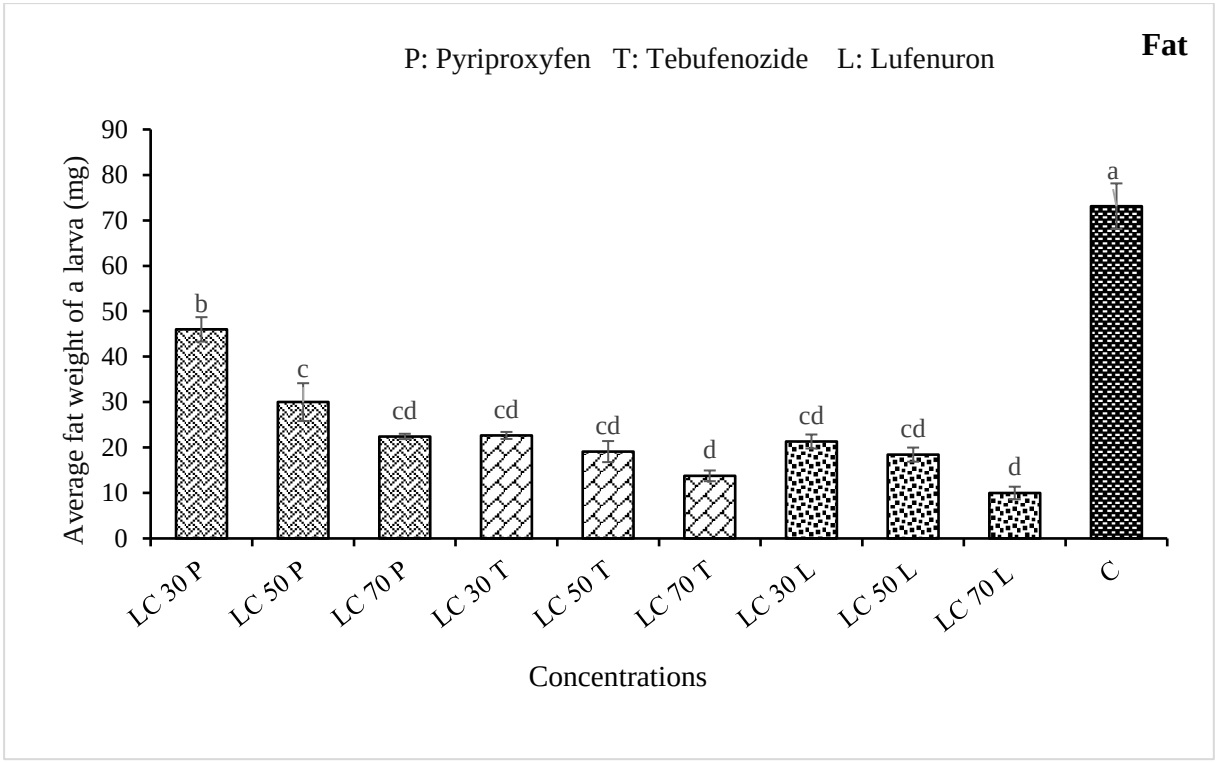


Figure 2. Average weight in the control and the treated larvae with concentrations of LC_{30}, LC_{50}, and LC_{70} of pyriproxyfen, tebufenozide, and lufenuron. Different letters indicate significant difference probability less than 5% (Tukey test).

Digestive enzyme activity

The activity of α-amylase was compared between control and the treated larvae by LC_{30} and LC_{50} concentrations of pyriproxyfen, tebufenozide, and lufenuron shows, at a 5% probability level (Df 6,21 = 42.32 Pr > f: 0.0001) (Figure 4). The results showed the least activity in the larvae treated by LC_{50} concentration of lufenuron and tebufenozide. Also, there was no significant difference between LC_{30} concentration of pyriproxyfen and control (Figure 4). Similarly, lipase activity significantly decreased in the all treated larvae by insecticides compared to control although the least activity was found in the larvae treated by LC_{50} concentration of lufenuron and tebufenozide (Df 6, 21 = 60.42 Pr > f: 0.0001) (Figure 4). Although no significant difference was recorded in protease activity of control and LC30-treated larvae by pyriproxyfen, other treatments showed a significant decrease of protease activity with the least value in LC_{50} concentration of lufenuron and tebufenozide (Df 6,21 = 69.32, Pr > f: 0.0001) (Figure 4).



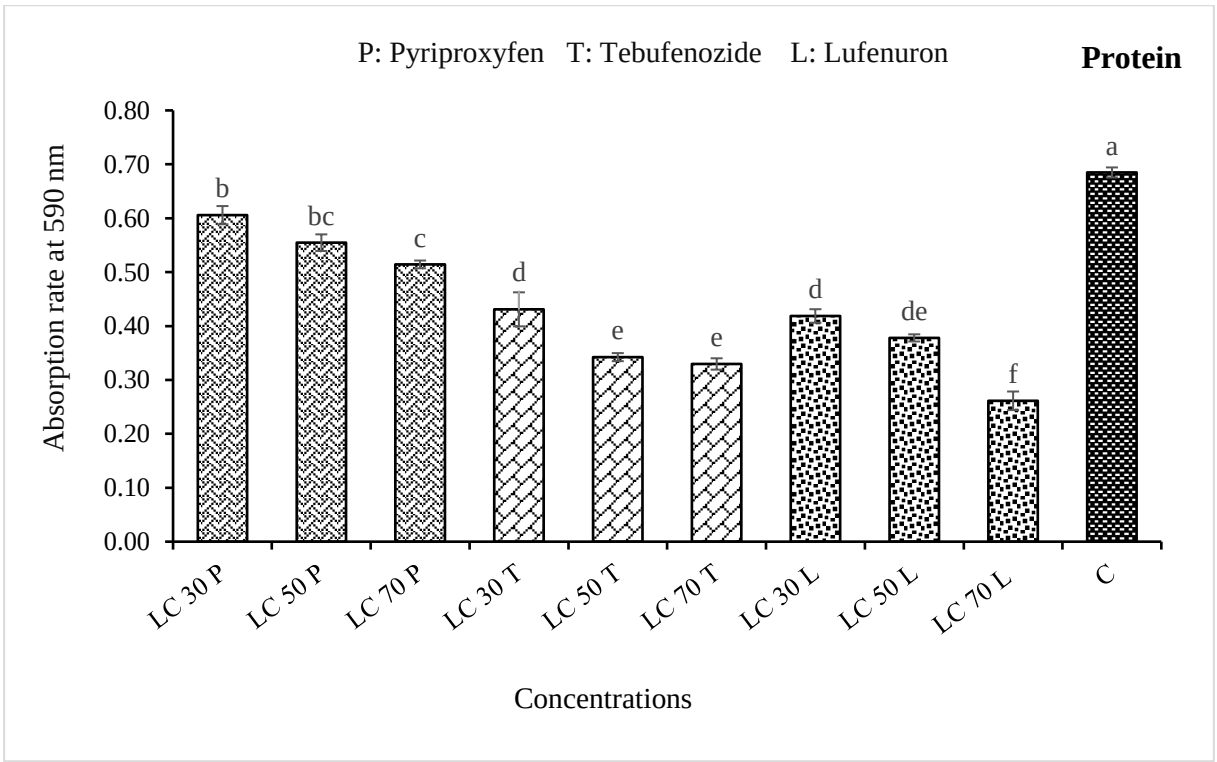
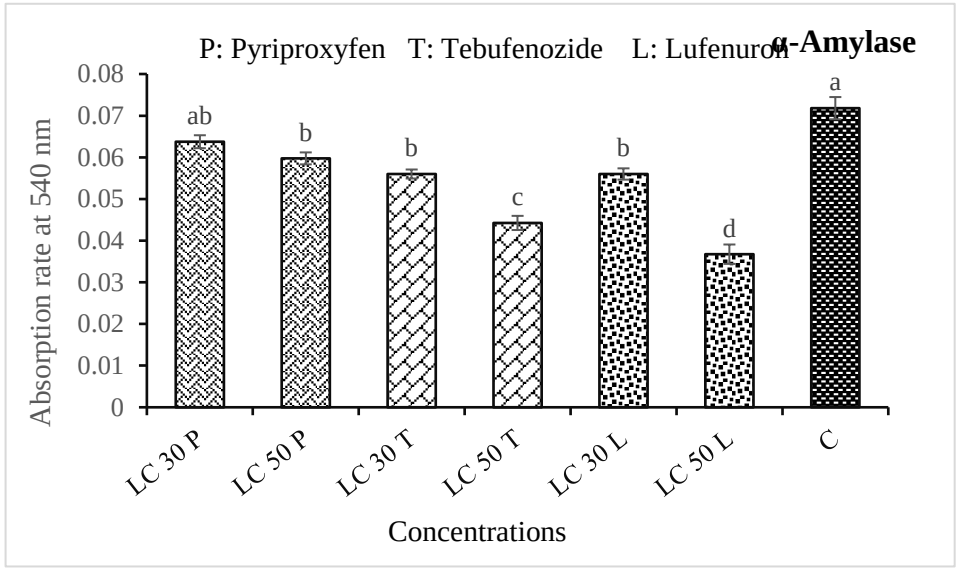
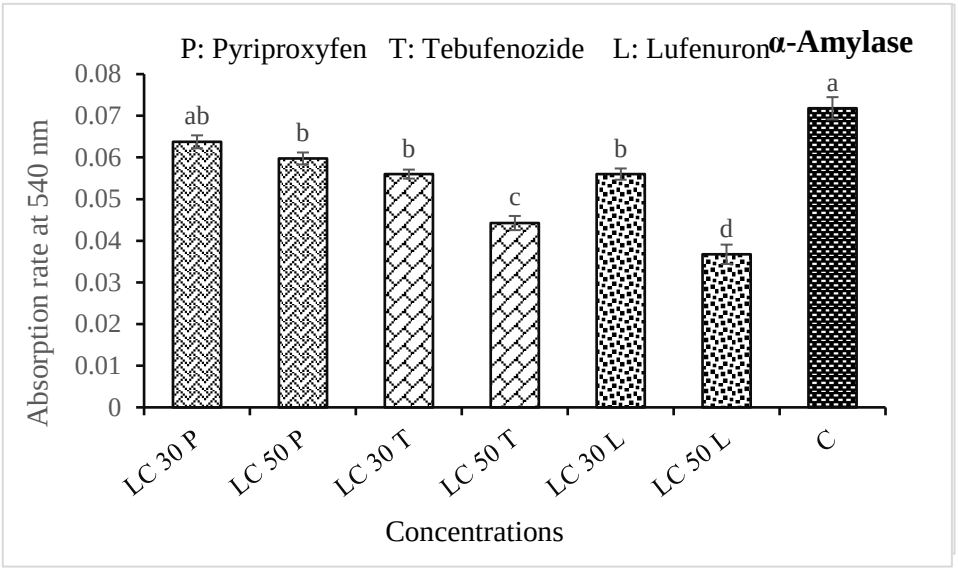


Figure 3. The average fat weight and the amount of protein in the control and the treated larvae with concentrations of LC_{30}, LC_{50}, and LC_{70} of pyriproxyfen, tebufenozide, and lufenuron. Different letters indicate significant difference probability less than 5% (Tukey test).





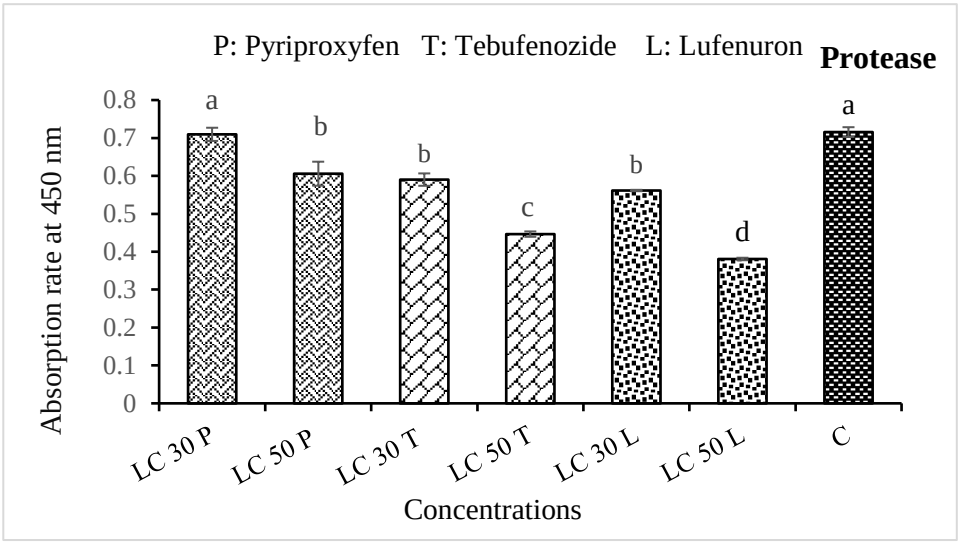


Figure 4. Optical Absorbance of α-amylase, lipase and protease in the control and the treated larvae with concentrations of LC_{30} and LC_{50} of pyriproxyfen, tebufenozide, and lufenuron. Different letters indicate significant difference probability less than 5% (Tukey test).

Discussion

The current study demonstrated the insecticidal influence of the three IGRs including pyriproxyfen, lufenuron and tebufenozide on *H. armigera* larvae. Our results revealed significant effect of lufenuron against the larvae compared to tebufenozide and pyriproxyfen. Lufenuron is an insect growth regulator that inhibits the synthesis of chitin and molting of larvae. This insecticide affects integrity of the external skeleton of insects during molting and leads to improper attachment of new cuticle during molting process. So the treated larvae become deformed or succumb to starvation because of improper splitting of the new cuticle and exhibit a swollen head. Khatri *et al*. (2014) reported that lufenuron, flufenoxuron, chlorfluazuron, and diflubenzuron showed mortality on 3rd instar larvae of *H. armigera* after 48, 72, 96, and 120 hours. They reported that larvae treated with lufenuron had the highest mortality after 120 hours among the treatments. Khorshidi *et al*. (2019) also reported LC_{50} of 6.16 mg ai/L of lufenuron against *H. armigera* after 72 hours. El-Sheikh & Aamir (2011) investigated the effects of lufenuron, flufenoxuron, and triflumuron against second- and fourth-instar larvae of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). Lufenuron demonstrated the highest toxicity against both larval stages at the earliest time by evaluating LT_{50}. Ghasemi *et al*. (2010) demonstrated significant increase of the larval duration in *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) by elevating pyriproxyfen concentration although a significant reduction was recorded in adult longevity and average egg laying. Pyriproxyfen, on the other hand, displayed a more moderate effect at lower concentrations, which might be reflective of a different mode of action or an adaptive metabolic or detoxification response by the larvae (Zibaee *et al*. 2011). Silva et al. (2023) reported significant mortality on *Euschistus heros* (F.) (Heteroptera: Pentatomidae) after treatment by different concentrations of tebufenozide and lufenuron.

In greenhouse, the insecticides significantly decreased fecundity and egg viability as well as adult deformation. Lv et al. (2023) showed that lufenuron exhibits high insecticidal activity against *S. frugiperda* Smith (Lepidoptera: Noctuidae) that significantly prolong the larval developmental duration and reduce the rates of pupation and emergence. Also, the authors reported lufenuron treatments can significantly reduce the expression of the genes involved in larval molting. The minimal effect of pyriproxyfen might suggest that it affects other aspects of insect physiology, such as juvenile hormone analog activity, rather than directly inhibiting chitin synthesis (Zibaee *et al*. 2011). This observation may be crucial for integrated pest management (IPM) strategies as using pyriproxyfen at lower concentrations could reduce the risk of developing resistance while still contributing to larval control (Subramanyam & Hagstrum, 1995).

Sublethal effects of the given IGRs significantly decreased the larval weight, amount of fat and protein as well as activity of digestive enzymes. It was found a significant decrease of the afore-mentioned parameters in the larvae treated by lufenuron and tebufenozide with the least value caused lufenuron. Decrease in the activity of digestive enzymes including α-amylase, lipase and protease led to malnutrition of the larvae which negatively affected larval weight and amount of the two macromolecules. Al-shannaf *et al*. (2012) reported that chlorfluazuron and pyriproxyfen caused a significant decrease in amylase activity by 61.9% and 59.9% respectively compared to the control group, suggesting an inhibitory effect on the larvae's ability to metabolize carbohydrates effectively. Cruz *et al*. (2021) demonstrated that lufenuron treatment caused weight loss and less accumulation of protein and lipids in boll weevil because of digestion disorders. Moreover, the treated females produce less viable eggs compared to control. In details, the authors observed the oocytes from lufenuron-treated females contained less protein so they concluded that the treatment caused probably vitellogenin (AgraVg) downregulation.

The current study contributes to the growing body of evidence supporting the strategic use of pyriproxyfen, lufenuron, and tebufenozide as part of

integrated pest management programs targeting the cotton bollworm. By providing a detailed understanding of these IGRs' modes of action and their ecological and evolutionary implications, this research may show a way to develop more effective, sustainable, and environmentally responsible pest management strategies.

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موثر بر نمو لاروی؛ چگونه تنظیم‌کننده‌های رشد حشرات سطوح چربی، پروتئین و آنزیم را در *Helicoverpa armigera* تنظیم می‌کنند

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چکیده

پژوهش حاضر برای تعیین سمیت پیروپیروکسی‌فن، لوفنورون و تبوزید علیه لاروهای سن سوم کرم غوزه پنبه طراحی شد. حشره‌کش‌ها با رژیم غذایی مخلوط و در معرض لاروها قرار گرفتند. سپس، سه غلظت LC_{30}، LC_{50} و LC_{70} پیروپیروکسی‌فن، لوفنورون و تبوزید برای ارزیابی وزن لاروی، مقدار چربی و پروتئین و همینطور فعالیت آنزیم‌های گوارشی سنجش شدند. زیست‌سنجی مقدار LC_{50} ۳/۷، ۱۲/۴۹ و ۱/۵۶ میلی‌گرم ماده موثره بر لیتر را نشان داد. افزایش غلظت حشره‌کش‌ها به طور معنی‌داری وزن لارو و مقدار چربی و پروتئین را در مقایسه با شاهد در کمترین مقدار LC_{70} لوفنورون کاهش داد. غلظت LC_{50} تبوفنوزید و لوفنورون سبب حداقل فعالیت آلفا-آمیلاز، لیپاز و پروتئیاز در لاروهای تیمار در مقایسه با شاهد شد. نتایج نشان دادند که نه فقط پیروپیروکسی‌فن، تبوفنوزید و لوفنورون تاثیر حشره‌کشی مناسبی علیه لاروهای کرم غوزه پنبه داشتند، بلکه آنها به طور معنی‌داری بر برخی ویژگی های فیزیولوژیک در دو غلظت کشنده و زیرکشنده موثر بودند.

کلمات کلیدی: کرم غوزه پنبه، نمو، تنظیم‌کننده‌های رشد حشرات، فیزیولوژی