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Insect Growth Disruptors Cause Mouthpart Malformations, Inhibition of Feeding, and Mortality in the Neotropical Brown Stink Bug *Euschistus heros* (Hemiptera: Pentatomidae)

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Abstract

The Neotropical brown stink bug, *Euschistus heros,* is the most important sucking pest of soybean crops, quantitatively and qualitatively affecting grain and seed. This study aimed to determine mouthpart deformities, feeding inhibition, and *E. heros* nymphs' mortality when in contact with insect growth disruptors (IGDs). The effects of two insecticides of the chitin biosynthesis inhibitor group (CBIs), lufenuron and diflubenzuron, and the juvenile hormone analog pyriproxyfen were tested in the laboratory. CBI application promoted mouthpart deformities, feeding inhibition, alteration of metamorphosis, and mortality of *E. heros* nymphs. Although pyriproxyfen did not cause mouthpart deformation, it caused certain mortality for nymphs, possibly due to physiological disorders. Lufenuron caused feed inhibition and mortality in less time when compared with diflubenzuron and pyriproxyfen. It was also more toxic to 4th instar nymphs. The insecticides, when used in higher concentrations, increased nymph's mortality in less time. Nymphs with deformed mouthparts were unable to feed and died. All IGDs in the tested concentrations caused 100% mortality and prevented adult formation.

Keywords: Hemiptera, physiological disorder, metamorphosis, deformity

1. Introduction

The stink bug *Euschistus heros* (Fabr.) (Hemiptera: Pentatomidae) is the most important pest of soybean crops in Brazil (Maciel & Bueno, 2022), and is difficult to manage. This stink bug stands out for its tolerance to insecticides of high toxicity, abundance, and distribution in various regions and crops of economic importance (Sosa-Gómez et al., 2010). Maintenance of stink bug population below pest threshold in soybean is often difficult because of its increased ability to reproduce and colonize. Successful control of *E. heros* involves implementing integrated pest management (IPM). However, the chemical control practices commonly used by soybean growers are carried out without criteria and often without necessity (Maciel & Bueno, 2022). As a result, conventional control methods are sometimes inefficient, leading to the need to review the management strategies of the populations of these insects.

Second instar nymphs and adults feed on the plant using their mandibular stylets to pierce tissues by inserting them in developing seeds. In the process, salivary secretions are injected into the seeds with a mix of digestive enzymes prior to uptake, thus reducing the productivity and quality of the soybean seeds (Depieri & Panizzi, 2010; Panizzi, Lucini, & Mitchell, 2021). In addition to physical damage caused by stink bugs, the holes resulting from the feeding process can serve as entrance for the fungus *Nematospora coryli* Peglion accelerating the decomposition of soybean (Sosa-Gómez et al., 2010). The main target of control is usually adults. However, second instar nymphs have a high damage capacity and make up most of the stink bug population (around 70%) during flowering and maturation (Corrêa-Ferreira et al., 2009). Thus, a strategy to reduce the damage caused to soybeans by stink bugs and increase the pest mortality is to affect their oral apparatus (mouthparts), hindering or inhibiting feeding by nymphs. Penetration depth of the stylet increases significantly as the insect develops through its nymphal phases, as well as after the length of the rostrum (Esquivel, 2011). After inserting the stylets into the plant tissue, they can be oriented in different directions, causing lysis of the tissues through disruption of

the cell walls and membranes (Schoonhoven et al., 2005). The primary damage to soybean seeds is due to the depth of insertion of the stylets and the biochemical components present in their saliva that destroy cellular structures (Depieri, Siqueira, & Panizzi, 2010).

Stink bugs have a needle-shaped face composed of the labrum and labium (pl. labra and labia, respectively), articulated in three segments, and a set of stylets that includes the insect punching–sucking mechanism. The stylet set is formed by two juxtaposed maxillary stylets in the center that interconnect the food and salivary canals, and two mandibular stylets (mandibles) involving the maxillary stylets. The stylets come out of the anterior dorsal part of the head. When at rest, they are held against the ventral surface of the head between the buccula. The remaining length of the stylets lies on the groove along the labium (Esquivel, 2011; Snodgrass, 1993).

Insect growth disruptors (IGD) act on the target pest development based on modes of action that elicit hormone activity. Chitin biosynthesis inhibitors (CBIs) disrupt the normal development of nymphs of pentatomids (Bagheri et al., 2011; Cremonez et al., 2019; Roggia et al., 2011). Another insect growth disruptor, pyriproxyfen, acts as a juvenile hormone (JH) analog, affecting the normal development of late nymphal stages (Cremonez et al., 2017; Matsumoto et al., 2021). Studies carried out by these researches showed that both group of insecticides caused deformations in the external and internal anatomy of stink bugs, but they do not mention changes in the mouthparts.

Thus, the work aimed to verify the occurrence of malformations of the oral apparatus, inhibition of food activity, and mortality of *E. heros* nymphs resulting from the application of lufenuron, diflubenzuron, and pyriproxyfen.

2. Method

2.1 Insects Rearing

The insects were obtained from an established colony in the Laboratory of Entomology of the Institute for Rural Development (IDR), Londrina, Brazil (courtesy of Dr. Ana M. Meneghin). The insects were kept in an air-conditioned chamber and reared under controlled conditions (25±2°C, 65±5% RH, 14:10 L:D photophase), and food based on common bean pods, peanut and soybean seeds was provided *ad lib*, as described elsewhere (Cremonez, Marcomini, Pinheiro, & Neves, 2022).

2.2 Application and Evaluation

Fourth instar nymphs (N4), with approximately two days after molting, were treated with different concentrations of IGDs, as follows: lufenuron (Match® EC, Syngenta, Sao Paulo, Brazil) at 10, 30, and 50 mL 100 L^{-1}; diflubenzuron (Dimilin® WP, UPL, Ituverava, SP) at 5, 10, and 15 g 100 L^{-1}; and pyriproxyfen (Tiger® 100 EC, Sumitomo Chemical, Sao Paulo, Brazil) at 200, 300 and 500 mL 100 L^{-1}. The choice of concentrations was made based on preliminary studies, using sublethal concentrations to cause morphological deformation of nymphs during molting and that may prevent ecdysis. For each concentration of the three insecticides (lufenuron, diflubenzuron, and pyriproxyfen) and check (distilled water), an aliquot of 2 mL of each treatment was sprayed on a group of 10 N4s inside a Petri dish (9 cm diam.) using a Potter's tower (Burkard Scientific, Uxbridge, UK) attached to an air compressor calibrated at 68.9 kPa (Cremonez et al., 2022).

The treated nymphs with insecticides and distilled water were individually separated into plastic containers (2 cm diam.) containing cotton moistened with pure distilled water and a single seed of white bean *Phaseolus vulgaris* L. (Fabaceae) cv. 'IPR Garça,' previously soaked in water for 30 min. The seeds were dyed with 1% acid fuchsin (Bowling, 1980) and observed for fifteen days under a stereoscopic microscope (25x magnification) for the observation and quantification of salivary sheaths. The seeds were changed daily. Nymphs were observed daily under a stereoscopic microscope to detect malformations in the mouthparts. Mortality was also recorded after all remaining insects reach adulthood.

2.3 Scanning Electron Microscopy (SEM)

To keep a record of the observed malformations, resulting fifth instar nymphs (N5s) presenting deformed mouthparts were selected and cold anesthetized (-4 °C) until death. The defrosted head was carefully removed with the aid of iridectomy scissors and moved to a fixative solution (glutaraldehyde 2% + paraformaldehyde 2%) for 12 h in a refrigerator (3±1 °C). They were then washed three times (15 min each) with phosphate-buffered saline (PBS) solution (0.1 M, pH = 7.2). Subsequently, they were dehydrated in serial concentrations of 70, 80, 90, and 100% ethanol for 10 min each. Drying at the critical point, *i.e.*, to reduce surface tension of the sample close to nullity and allow metal coating, was performed by compression and CO_2 injection into the sample. The heads were then glued to the stubs with double-sided carbon adhesive tape and sputter coated with gold (Bal-Tec® SCD050, Balzers, Liechtenstein). Photomicrographs were generated using a scanning electron

microscope (FEI® Quanta 200, Eindhoven, Netherlands). The characterization of mouthpart malformations was performed by comparing the samples with normal species from the check, following the classification used by Singha, Thareja, & Singh (2007).

2.4 Statistical Analysis

A completely randomized design was used, with three replicates of 10 nymphs per treatment (products at different concentrations and control). Mortality, the total time required to cause mortality, and feeding inhibition data were submitted to ANOVA, and means were compared using the Scott–Knott test at a 5% probability. The feeding activity was analyzed by comparing the average salivary sheaths per N4 treated with the products before and after N5 molting. Malformations of the mouthparts, *i.e.*, anatomical separation or deformation of the component pieces that forms the oral apparatus and impair its function, were recorded in frequency (%) based on the number of nymphs with normal mouthparts and malformations, following the classification used by Singha et al. (2007). The corrected mortality (efficacy) of insecticides was calculated using Schneider-Orelli's formula (Püntener, 1981), considering the number of insects killed in the treatment and the control.

3. Results

Lufenuron and diflubenzuron caused malformations in the mouthparts of *E. heros* in the late nymphal stages (N4-N5). All concentrations of the insecticide treatments caused mortality, with no adults forming. Mortality occurred mainly in the molting process from N4 to N5, as the insect was trapped in its old exuviae. Nymphs that reached the fifth instar and had malformed mouthparts were unable to feed and eventually died (Figure 1 and Table 1). Several types of morphological alterations in the mouthparts were caused by lufenuron and diflubenzuron. In this study, pyriproxyfen did not cause molting disruption in stink bugs. The highest proportion of dead N5s occurred with pyriproxyfen and diflubenzuron (Figure 1).

The highest frequency of malformations in the mouthparts occurred with lufenuron at a concentration of 10 mL^{-1}. Of the nymphs, 30% had partial separation of their stylets, 13.33% had total separation, *i.e.*, separated in all its length on the labial grove, 6.66% had malformations in the labrum, and 3.33% had malformations in the labium. The latter occurred independently or sometimes along with other deformities. With the increase in concentration to 30 and 50 mL^{-1}, the frequency of N5s with the normal mouthparts decreased to 13.00 and 3.33%, respectively (Table 1).

The most relevant lethal and sublethal effects for N4s were caused by lufenuron at 50 and 30 mL^{-1}, causing malformations of the mouthparts in 23 and 33% of the nymphs that reached N5, respectively. Diflubenzuron at 5, 10, and 15 mL^{-1} caused substantiate effects on the mouthparts but with lower N4 mortality (6, 10, and 13%, respectively). Pyriproxyfen did not affect the mouthparts; however, at concentrations of 200, 300, and 500 mL^{-1}, N4 mortalities were 16, 20, and 26%, respectively, and N5 mortalities were 80, 83, and 73%, respectively (Figure 1). Mortality of N4s increased with higher concentrations of insecticides, preventing molting to N5 (Figure 1). All insecticides used were effective compared to the water check, and the most relevant results were caused by lufenuron, followed by diflubenzuron and pyriproxyfen.

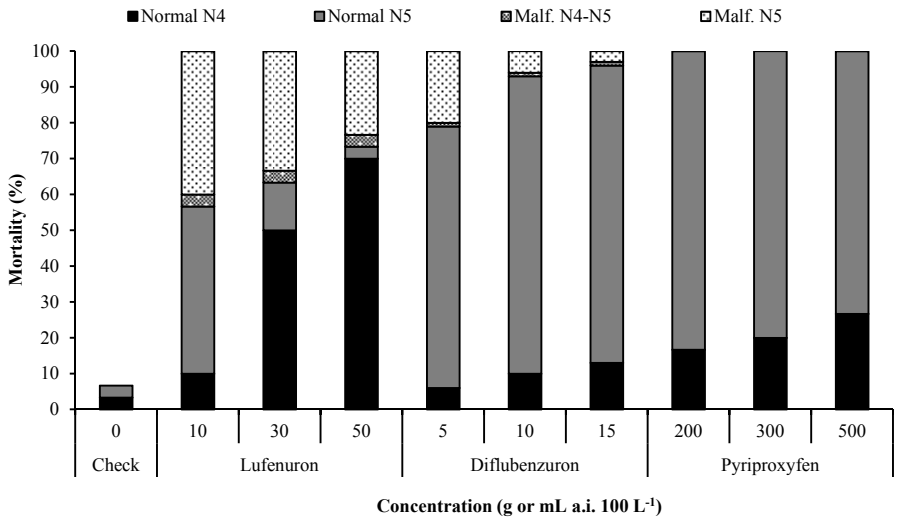

Figure 1. Mortality (%) and frequency (column proportion) of *Euschistus heros* nymphs in late nymphal stage (N4 and N5) with normal or malformed (Malf.) mouthparts treated with insect growth disruptors. All treatments are significantly different from the untreated control

Table 1. Feeding activity of *Euschistus heros* nymphs treated in the 4th instar with insect growth disruptors and surviving 5th instar nymphs

Treatment	Concentration (mL or g 100 L^{-1})	Salivary sheaths (avg.) and mouthpart malformation frequency (%)											
		4th instar								5th instar			
		Normal	%	PS²	%	TS²	%	TS²	%	DLa²	%	DLb²	%
Lufenuron	10	0.90	100.00	0.27	47.00	0.00	30.00	0.00	13.33	0.00	6.66	0.00	3.33
	30	1.53	100.00	0.42	13.00	0.00	13.33	0.00	13.33	0.00	3.33	-	-
	50	1.00	100.00	0.66	3.33	0.00	20.00	0.00	6.66	0.00	3.33	-	-
Diflubenzuron	5	0.56	100.00	0.54	73.33	0.00	10.00	0.00	10.00	-	-	-	-
	10	0.46	100.00	0.56	83.33	0.00	3.33	-	-	-	-	-	-
	15	0.30	100.00	0.60	83.33	0.00	3.33	-	-	-	-	-	-
Pyriproxyfen	200	0.23	100.00	0.50	86.66	-	-	-	-	-	-	-	-
	300	0.36	100.00	0.34	80.00	-	-	-	-	-	-	-	-
	500	0.30	100.00	0.50	73.33	-	-	-	-	-	-	-	-
Check	Distilled water	1.30	100.00	2.76	100.00	-	-	-	-	-	-	-	-

Note. Mouthpart deformities categories: PS = partially separated stylets; TS = totally separated stylets or labial grove; DLa = malformed labra; DLb = malformed labia (Singha et al., 2007).

Feeding activity was reduced in all treatments tested, evidenced by a significant reduction of the inhibition time (Figure 2). Lufenuron and diflubenzuron were more effective on reducing the feeding time, especially in higher concentrations. Moreover, mortality results were correlated with the reduction of feeding activity, confirming that the products affect the insects' capability to cause damage.

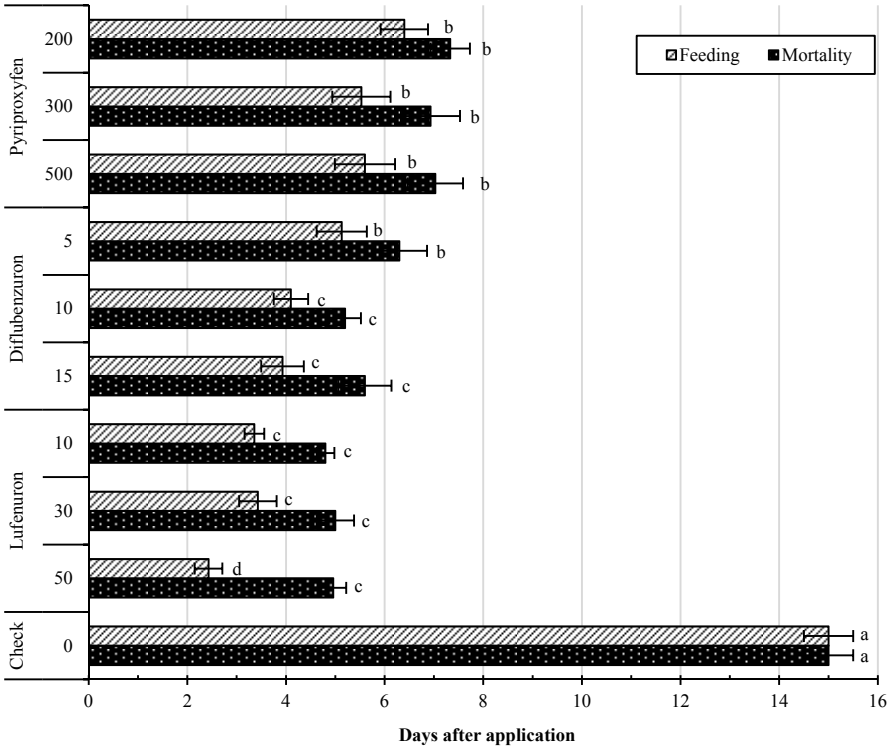

Figure 2. Total lethal and food inhibition time (avg.±SE) of *Euschistus heros* nymphs treated with insect growth disruptors. Means separation by Scott-Knott test ($p \leq 0.05$)

Mouthparts were analyzed by scanning electron microscopy (SEM) photomicrographs and are presented below (Figure 3). Overall, it is possible to observe the labrum (la) and labium (lb) in the untreated insect rostrum (Figure 3A). In treated insects, malformed insects were commonly found. A malformation of the mouthparts shows an irreversible dislodge of the mandibular stylets (st) from the labial grove (Figure 3B). In a more detailed perspective, some deformities were visible from the stylet insertion in the labrum section. In contrast, normal mouthparts were well constructed (Figure 3C), and malformed insects present a total separation of the stylets from this proximal portion (Figure 3D). From the terminal portion of the labia, a partial separation of the mandibular stylet also prevents the insect from piercing the plant material and feeding. A detailed comparison between a normal labia-mandibular stylets bundle (Figure 3E) and a malformed set (Figure 3F) is also presented.

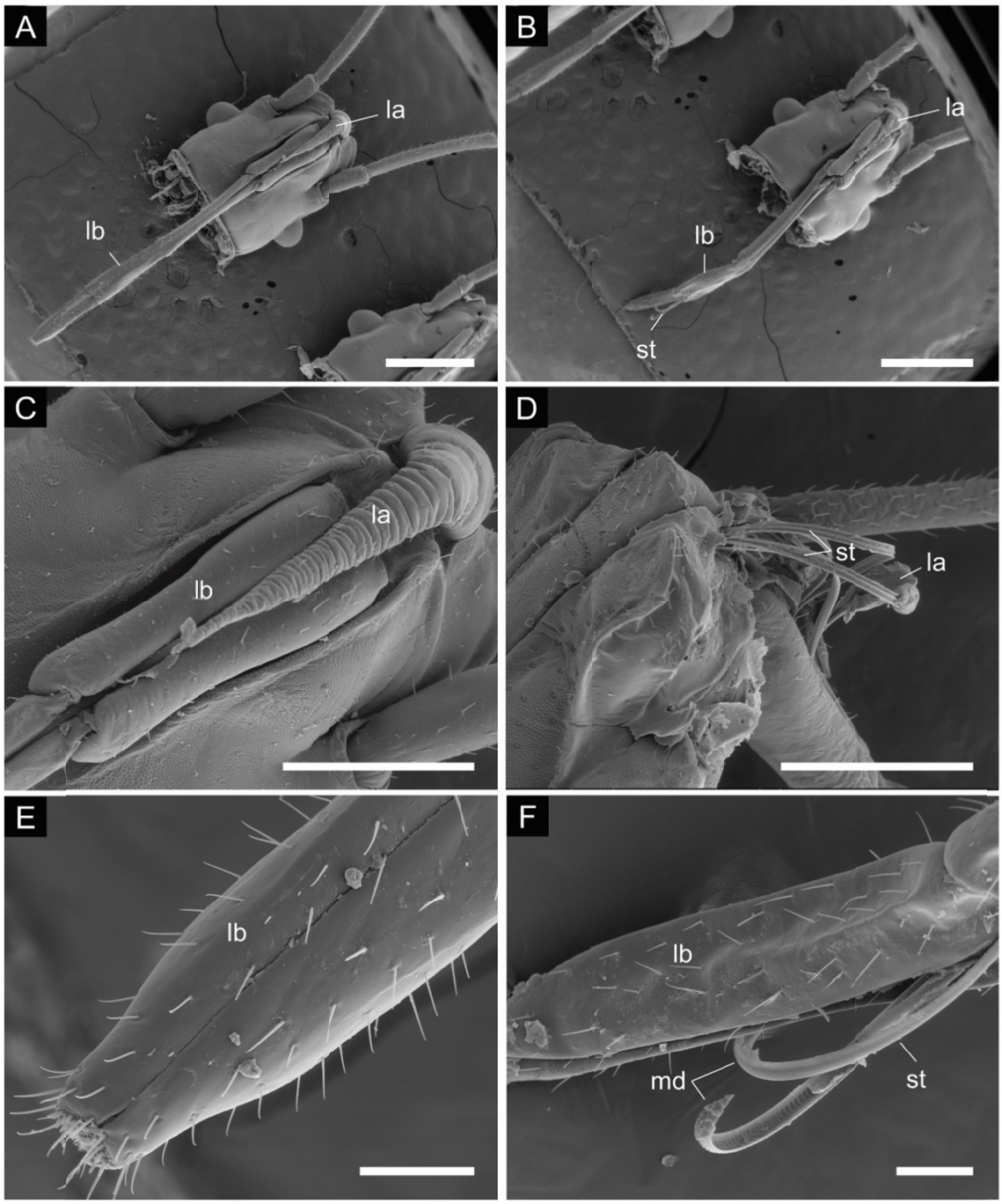

Figure 3. Photomicrographs of mouthparts of the Neotropical brown stink bug *Euschistus heros*. A: normal (bar = 1000 µm); B: malformed by lufenuron (bar = 1000 µm); C: normal labrum (bar = 500 µm); D: malformed labrum (bar = 500 µm); E: normal labium without separation of the stylets (bar = 100 µm); F: malformed labium with partial separation of the mandibular stylets (bar = 100 µm). la: labrum; lb: labium; st: mandibular stylets; md: mandible. Scanning electron microscopy

4. Discussion

Chitin biosynthesis inhibitors, such as the insecticides lufenuron and diflubenzuron, act at the time of molting, increasing the catalytic activities of kinases in the cuticle that degrade chitin, inhibiting the activity of chitin synthase, and reducing chitin production. They may also promote the abnormal translocation of chitin synthase or reorder the assembly of chitin microfibrils. By structural similarity with sulfonylureas, they interact with their homolog, competing for the sulfonylurea receptor in the insect, a key event in chitin formation. Thus, the newly

formed damaged cuticle does not support hemolymph pressure or muscle tension. Although this knowledge is known, the mechanism of action of these compounds has not yet been elucidated (Abo-Elghar, Fujiyoshi, & Matsumura, 2004; Cohen, 1987, 2001; Merzendorfer, 2006; Zhang & Zhu, 2006). Chitin is a necessary component of the cuticle in insect mouthparts, especially for sap-feeding species that require a hard and steady mouthpart. As lufenuron and diflubenzuron affect the synthesis of the new cuticle, thus interrupting its development and altering the normal deposition of chitin during cuticular formation, morphological changes in the treated nymphs are expected. Mouthparts are mainly affected during head pulling from old exuviae, a terminal moment in the ecdysis process. The malformations are then irreparable, as the cuticle hardening process is already advanced. Ultimately, with an unusable rostrum, feeding activity is inhibited, and the insect starves. Most sublethal effects caused by malformations on the mouthparts were found in the lowest concentrations of lufenuron and diflubenzuron since the insect carried out the compounds through N4-N5 molting.

Pyriproxyfen is an IGD similar in chemical structure and function to the insect juvenile hormone (JH), competing with the endogenous hormone for its receptor binding site (Sullivan & Goh, 2008). In *E. heros*, pyriproxyfen may cause a hormonal imbalance in nymphs that alters their normal development, preventing adult formation. In other studies, pyriproxyfen did affect the development of the gonads of male adults when applied over late instar nymphs (Matsumoto et al., 2021). Studies conducted by Rill, Grafton-Cardwell, and Morse (2007) showed that with the increase in pyriproxyfen concentration, there was an increase in the mortality of the early nymphal stages of the citrus plague *Aonidiella aurantia* Maskell (Hemiptera: Diaspididae). Mahdian, Van Leeuwen, Tirry, and De Clercq (2007) also showed that pyriproxyfen was highly toxic to fourth instar nymphs of the predator *Pricomerus bidens* L. (Hemiptera: Pentatomidae), and the highest mortality occurred during the N5-adult molting.

Malformations in the mouthparts were irreversible, and although mortality was not immediate, the number of salivary sheaths or damage to the bean seeds was reduced in a descending manner until death. These results coincide with those of Singha et al. (2007); however, these authors verified that the degree of malformation in mouthparts increased due to the concentration of neem, as opposed to our findings. In the highest concentrations of insecticides, the new cuticle was not developed, and the insect died in the N4 stage. Of the group of nymphs that reached fifth instar (N5), most suffered malformations in the mouthparts and were not able to feed, as evidenced by the absence of salivary sheaths. In our study, the synthetic insecticides used presented an efficient potential for stink bug control, causing mortality in part of the population and an additional sublethal effect capable of reducing damage by causing malformations.

The salivary sheath presence in the substrate or food is an indicator of the stink bug's feeding activity (Bowling, 1980). However, malformed nymphs did not produce salivary sheaths on the offered seeds. This indicates that stink bugs were not able to start feeding because of mouthpart malformation. Their disposition was affected, and mandibular stylets could not perform the puncture and start the feeding process (Singha et al., 2007). In similar studies, Roggia, Corrêa-Ferreira, Bueno, and Alves (2011) observed that the application of lufenuron and diflubenzuron on 5th instar nymphs of *E. heros* reduced the number of salivary sheaths and the feeding of stink bugs when reaching adulthood, attributing poor health to leg deformations that hindered their locomotion to seek food. In the pentatomid predator *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) treated with neem oil, the locomotion was also impaired, due to malformations in the legs and wings, with negative effects on the natural enemy (Zanuncio et al., 2016). The studies concluded that these malformations can be deleterious by ultimately limiting dispersal and feeding activity.

The reduction in the number of stink bug feeding sheaths was reported by Singha et al. (2007). And showed that topical application of neem stratum *Azadirachta indica* A. Juss resulted in deformations of the oral tract, inhibiting feeding and subsequently causing mortality of *Nezara viridula* L. (Hemiptera: Pentatomidae). Similarly, Pinheiro and Quintela (2010) observed deterrence, reduction of salivary sheaths, and damage in rice panicles by *Oebalus poecilus* Dallas (Hemiptera: Pentatomidae) with the application of neem oil. The inhibiting effect of feeding *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caterpillars due to the ingestion of leaves treated with lufenuron was reported by Adal (2012). This author also observed histopathological effects, blockade of endocuticle production, and very delicate cuticle formation only with an epicuticle not properly attached to the epidermis that does not resist muscle connection during the seedling process. Under the epidermis, the cells were irregularly distributed under the cuticle, with several mitotic divisions and fissures. The products also present negative effects close-related stink bug predators, such as observed in *P. nigrispinus* fed with lufenuron-treated (Lira et al., 2020). The digestive tract is coated with a cuticle formed from chitin, proteins, lipids, and hydrocarbons. The midgut is the section of the digestive tract responsible for food intake; thus, it is the first part affected by the ingestion of insecticide-treated food. The inner membrane that protects the midgut

epithelium, known as the peritrophic membrane in holometabolous insects, and perimicrovillar membranes in the hemimetabolous, seem to be affected by the activity of chitinases involved in its formation, permeability, and degradation (Benedito Filho et al., 2002; Berini et al., 2016; Shao, Devenport, & Jacobs-Lorena, 2001).

How quickly a product acts to reduce, damage, or kill the pest is an important factor that should be considered in pest management. Although IGDs do not cause an instant lethal effect, physiological disturbances that prevent the growth and normal insect development manifest after two or three days. Lufenuron and diflubenzuron were quicker than pyriproxyfen to cause deleterious effects. Inhibition of feeding occurs mainly due to malformation of the mouthparts. It was also observed that nymphs with normal mouthparts in a moribund phase manifested difficulty in locomotion or were kept steady in a supine position, thus also presenting with limited feeding activity and posterior death. Considering the mechanism of action of CBIs, the results indicate that, in addition to the effects on the mouthparts, there are more morphological and physiological changes that might affect the behavior of the insect, preventing it from feeding. Pyriproxyfen may cause internal physiological disorders, molting delays, and behavioral changes, such as reduced or no locomotion. A visually perceptible morphological alteration was the darkening of the cuticle, normally linked to sclerotization and hardening caused by the new exoskeleton.

The total lethal time and feeding decreased due to the mechanism of action of the insecticide and its concentration. Lufenuron and diflubenzuron were able to kill and inhibit the feeding of nymphs in a shorter time compared to pyriproxyfen. The inhibition of feeding occurred days before death, a fact of importance because nymphs stop causing damage to the grains quickly and later die. The speed of containing the damage to the grains and killing the pest is fundamental to the success of the product in pest management, due to which, in the field, it is necessary to reduce the damage and the population of the pest in the shortest possible time after insecticide exposure.

The deleterious effects of insect growth disruptors on the mouthparts and on the anatomy of *E. heros* nymphs is important for their application in the field due to the low toxicity of these insecticides to humans, beneficial organisms, and the environment, as well as the low probability of the pest acquiring resistance to the insecticides tested. Further field-level studies should be carried out to validate these results since, in the laboratory, there is maximum exposure of insects to products, and confinement can cause stress and other factors that can potentiate the effects of insecticides.

5. Conclusion

Lufenuron and diflubenzuron caused malformations in the mouthparts, inhibiting feeding, affected metamorphosis, and consequently caused mortality of nymphs of *Euschistus heros*. Pyriproxyfen, at the concentrations used, did not cause malformations in the oral apparatus and did not inhibit feeding but caused physiological disturbances responsible for the mortality of Nymphs of *E. heros*. The higher the concentrations of lufenuron, diflubenzuron and pyriproxyfen, the faster was the mortality response of *E. heros* nymphs.

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