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# Morphological and Morphometric Parameters of the Reproductive Organs of *Euschistus heros* (Hemiptera: Pentatomidae) Treated With a Sublethal Juvenile Hormone Analog

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**Abstract**

The stinkbug *Euschistus heros* is an important pest of Brazilian agricultural production with increasing importance in the Neotropical region. High reproductive potential and increased risk of resistance to insecticides are determining factors influencing its pest status. This work evaluated the effects of pyriproxyfen on the reproduction and morphological development of the reproductive system of *E. heros*. Pyriproxyfen was utilized at sublethal concentrations ($LC_{30}$) in fourth-instar nymphs (N4). Sublethal pyriproxyfen resulted in a reduction in fertility and fecundity of 2 and 12% when only the males were treated, 47 and 53% when only the females were treated, and 32 and 46% when females and males were treated. Basal oocytes were larger and more previtellogenic in newly emerged pyriproxyfen-treated females. The nuclear area of testicular accessory cells shrunk after 10 and 15 days of age in treated insects. Similarly, testicular shape was altered, changing from an ellipsoid to an obpyriform pattern, characterized by hypertrophy of the basal region over time. The apparent area of the organ grew until ten days of age, then shrunk at 15 days of age. In summary, pyriproxyfen treatments disrupted the normal development of N4 of *E. heros* and diminished the reproductive potential of female and male adults.

**Keywords:** neotropical brown stink bug, insect reproduction, insect growth disruptor

## 1. Introduction

The neotropical brown stinkbug *Euschistus heros* Fabricius (Hemiptera: Pentatomidae) is an agricultural pest of great importance in Brazil (Soares et al., 2018), with increasing reports of occurrence in neighboring regions like Argentina (Saluso et al., 2011). The reproductive and disseminative potential of *E. heros*, in addition to the selection of insecticide-resistant populations, have led to the reproductive success of the species in soybean in these regions. The primary crop damage caused by feeding by *E. heros* has significantly reduced the productivity and quality of soybean seeds in Brazilian production systems (Moraes et al., 2022; Silva et al., 2012). Consequently, insecticide control failures (Tuelher et al., 2018) have sparked the use of insecticide resistance management (IRM) techniques, such as rotations of different insecticide modes of action, to preserve the efficacy of active ingredients (Sparks et al., 2021). As a part of this IRM effort, growth-disrupting insecticides have emerged as excellent alternative control products that serve as a good rotation partners with broad-spectrum and highly toxic insecticides (Horowitz et al., 2009).

Studies on the development and functionality of the reproductive system of insects are essential to determine their biological status, both individually and at the population level (Esquivel, 2009). The development and maturation of the ovary and testis ensures that a set of germline cells develop for fertilization and zygote formation. This process depends on maternal development and regular hormonal control, mainly in the final juvenile stages. Moreover, these processes involve the direct and indirect interactions of juvenile hormone (JH), which act on sexual maturation, lipid metabolism, and vitellogenin biosynthesis in insects (Gijbels et al., 2019; Guo et al., 2014; Lenaerts et al., 2016; Smykal et al., 2014). Female pentatomids are highly prolific, having ovaries made up of telotrophic meroistic ovarioles presenting a cluster of nurse cells positioned in the apex of the ovariole. These

ovarioles function by transferring proteins and nutrients to the oocytes in formation via cytoplasmic extensions (Fortes et al., 2011; Huebner & Diehl-Jones, 1993). Juvenile hormone analogs (JHAs), such as pyriproxyfen, fenoxycarb, and methoprene, have similar regulatory mechanisms over reproductive parameters (Edwards et al., 1993; Jindra et al., 2015). However, the implications of using JHAs on the reproductive biology of stink bugs require further study.

Here we present evaluations on the biological characteristics and reproductive performance of females and males of the *E. heros* species following exposure to pyriproxyfen in the juvenile stage (fourth-instar nymph – N4). Similar evaluations were made on adult reproductive performance (fecundity and fertility) and the effects of pyriproxyfen on the development of reproductive organs. We hypothesized that a sublethal dose ($LC_{30}$) of pyriproxyfen applied to either the males or females of a mating pair would significantly reduce the fertility and fecundity rates of *E. heros* females.

## 2. Method

### *2.1 Insect Rearing*

Adults of *E. heros* were collected from an established colony maintained at the Entomology Laboratory of the Brazilian Agricultural Research Corporation (EMBRAPA), Soybean Research Facility, Londrina, Brazil (provided by Dr. Samuel Roggia) and reared at a lab facility at the State University of Londrina. Stinkbugs were kept in plastic containers covered with a mesh lid (v = 12 L) and fed routinely with green bean pods (*Phaseolus vulgaris* L.), peanuts (*Arachis hypogaea* L.), and soybean seeds (*Glycine max* Merril) offered *ad lib*. Cotton rolls moistened with distilled water were provided for hydration, according to the methodology of Depieri et al. (2010).

Fourth instar nymphs (N4, F1 generation, approx. 20 days after hatching) collected from the colony were used in the study. The emergence of humeral angles of the pronotum were used to identify the correct life stage (Correa-Ferreira & Panizzi, 1999). For the colony, as in all stages of the bioassays, the insects were kept under controlled conditions at 26±1ºC, 65±5% RH, and 14 h L:10 h D photoperiod.

### *2.2 Insecticide Application*

Predetermined sublethal concentrations ($LC_{30}$ = 0.688 mL a. i. $L^{-1}$) of pyriproxyfen (Tiger® 100 EC, Sumitomo Chemical, Sao Paulo, SP, Brazil) were diluted in distilled water and used as insecticide treatments for the bioassays (Cremonez et al., 2019). Additionally, a pure distilled water treatment was used as a check (control). An aliquot of 1 mL of the insecticide solution was sprayed on a group of ten N4 individuals inside a crystal polystyrene (PS) box (11.0 × 11.0 cm). A Potter tower (Burkard Scientific, Uxbridge, UK) calibrated with a working pressure of 68.9 kPa, was used for application for uniform deposition of the insecticide over the insects. Insects were separated from the main colony and offered food *ad lib*, as previously described, until the emergence of adults.

### *2.3 Couple Fertility and F1 Fecundity*

Newly emerged adults were sexed and unmated couples were arranged as follows: control female + control male (control pair, FC × MC); control female + treated male (FC × MP); treated female + control male (FP × MC), and treated female + treated male (FP × MP). A total of eight couples were evaluated per treatment. Each pair was placed into an empty polystyrene- (PS-) crystal rearing arena box (11 L × 11 H × 3 W cm) lined with filter paper alongside the standard colony food as described in section 2.1. This was done for a period of 14 days to allow mating and oviposition. After the oviposition period, the number of eggs were recorded and used to determine the females' fertility rates (%) from the individual couples. Similarly, collected egg masses were placed into an empty PS-crystal rearing arena, and bean pods were added to each arena to supplement hatching nymphs. Fecundity rates (%) were determined using the number of eggs that resulted in hatched nymphs.

### *2.4 Reproductive System Morphology*

Analysis of the *E. heros* reproductive system was carried out using unmated adult females and males of different ages. The ages of the males used ranged from 0, 5, 10, and 15 days after emergence (DAE). The ovaries and testes were collected and placed in a saline solution (1.80 g of NaCl; 1.88 g of KCl; 0.16 g of CaCl; 0.004 g of $NaHCO_3$ and distilled water 100 mL, pH 7.4) and immediately fixed in Karnovsky solution (glutaraldehyde 2.5% + paraformaldehyde 4.0% in 0.1 M phosphate buffer and pH 7.2) for four hours. During the fixation period and before further histological processing, the testicular capsule was photographed using a stereoscopic microscope (25×) SZ61 (Olympus®, Tokyo, Japan) coupled with a Moticam® 3.0 MP camera (Motic, Kowloon Bay, China) with a resolution of 1296 × 972 pixels. Photographs were utilized in the morphometric analysis of the reproductive organ. After fixation, the reproductive organs were submitted to the process of inclusion in glycol-methacrylate resin, as follows: the organs were washed in sodium phosphate buffer solution (0.1 M and pH 7.2) three times (five min each), dehydrated in an increasing series of ethyl alcohol (70, 90 and 100% for 20 minutes each),

pre-infiltrated in pure resin solution (hydroxyethyl methacrylate + activator) + 100% alcohol (1:1) for four hours at room temperature (26±1 °C), infiltrated in pure Historesin® resin (Leica Biosynthesis, Wetzlar, Germany) at room temperature for 24 hours, included in polyethylene molds containing a solution of basic resin + activator (dibenzoyl peroxide) + hardener (dimethyl sulfoxide) and kept at room temperature until complete resin polymerization. The blocks with the reproductive organs were cut using an automatic rotating microtome (4 µm), and the produced slides were stained with hematoxylin-eosin and analyzed using a Zeiss® AxioPhot light microscope (Carl Zeiss AG, Oberkochen, Germany).

*2.5 Ovaries*

The most basal oocyte among the ovarian follicles of each organ was analyzed. Measurements of oocyte length and width (µm) were recorded, and the surface area (A, $\mu m^2$) was calculated through the prolate spheroid formula:

$$A = 2\pi b(b + a \arcsin e/e) \quad (1)$$

Where a is the oocyte length semi-axis; b is the oocyte width semi-axis; and e is ellipse eccentricity, given by the formula:

$$e = \sqrt{1 - b^2/a^2} \quad (2)$$

The basal oocyte volume (V, $\mu m^3$) was also calculated using the prolate spheroid formula (Lumbreras et al., 1991), according to the following equation (*a* and *b* remain as described in equations 1 and 2):

$$V = 4/3\ \pi b^2 a \quad (3)$$

*2.6 Testes*

Developmental patterns and possible changes due to the mode of action of pyriproxyfen were evaluated through external and internal morphometric alterations. Observations of orthogonal changes in the shape of the testis were identified, which led to a generalized Procrustes analysis (GPA) based on the contour of the testicular capsules. The demarcation of 100 morphometric landmarks on the perimeter of each organ was digitized using the software tpsDig2 v. 2.31 (Rohlf, 2017), and the estimated average image of the organs was generated with the help of another software, tpsSuper v. 2.05 (Rohlf, 2018). Additionally, to assess internal alterations, the apparent area of Sertoli-like testicular accessory cell (TACs) nuclei was measured using ImageJ v. 1.47, as described by Cremonez et al. (2019).

*2.7 Statistical Analysis*

Fertility and fecundity bioassays of the coupled adults were conducted as a completely randomized block design, with 12 replications per treatment (4) and one pair per replicate (n = 48). Quantitative data were subjected to a Shapiro-Wilk normality analysis and a residual analysis (Fernandez, 1992) to validate an analysis of variance (ANOVA) procedure as the most appropriate statistical test for comparison. In the event of nullities, the values of the variable were transformed ($\sqrt{x + 1}$). Data processing was performed with the help of the software R v. 3.6.2 (R Core Team, 2020).

## 3. Results

*3.1 Biological Parameters*

The fertility and fecundity values represented are the average number of eggs per female, and the average number of hatched nymphs, respectively. Analysis of variance produced no significant observable difference between the treatments evaluated for fertility ($F_{3, 28} = 1.78$, $P = 0.184$) and fecundity ($F_{3, 28} = 2.28$, $P = 0.101$) (Table 1). While a slight decrease in fertility and fecundity was observed when only the male was treated, we observed a more significant reduction of these parameters in treated females. Additionally, the hatching rate was affected more strongly when both insects were treated, causing a 70% reduction in hatched nymphs.

Table 1. Reproductive parameters of *Euschistus heros* couples treated and untreated with a sublethal concentration of pyriproxyfen

| Treatment | Fertility (eggs/female) (avg.±SE) [ns] | Fecundity (hatched nymphs) (avg.±SE) [ns] | Total eggs (n) | Total nymphs (n) | Eclosion (%) | Compared to FC × MC (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Fertility | Fecundity |
| FC × MC | 24.8±2.9 | 23.3±2.6 | 198 | 186 | 94.6 | - | - |
| FC × MP | 24.1±4.7 | 20.4±4.3 | 193 | 163 | 83.5 | 97.5 | 87.6 |
| FP × MC | 13.1±3.8 | 10.9±3.5 | 105 | 87 | 85.6 | 53.0 | 46.8 |
| FP × MP | 16.9±5.4 | 12.9±4.9 | 135 | 103 | 66.2 | 68.2 | 55.4 |

*Note*. FC × MC = check couple = untreated female and male; FC × MP = untreated female and treated male; FP × MC = treated female and untreated male; FP × MP = treated female and male; ns = non-significant difference (analysis of variance). n = 32 couples.

### *3.2 Female Reproductive System*

Ovarioles and their structures in adult *E. heros* females are easily observed through the ventral opening of the abdomen (Figure 1A and B). Although not fertilized, the oogenesis process was complete regardless of treatment, *i.e.*, it was possible to observe postures from older females (10 and 15 days). In newly emerged females (0 days) from the check, the observed oocytes ultimately depend on substrates from the nurse cells because they are not supplied by nutritive material via the follicle (Figure 1C). However, ovarioles from newly emerged females treated with pyriproxyfen already presented previtellogenic oocytes, as evidenced by the presence of vitellogenin in the peripheral region of the ooplasm (Figure 1D). The nurse cells are clearly visible in newly emerged females from the check treatment, being indistinguishable from this moment on in the other evaluated times. Regarding the treatments, it is possible to observe a structural difference between females treated with pyriproxyfen, as a more spaced cell arrangement in the germarium region in relation to females of the control treatment (Figure 2).

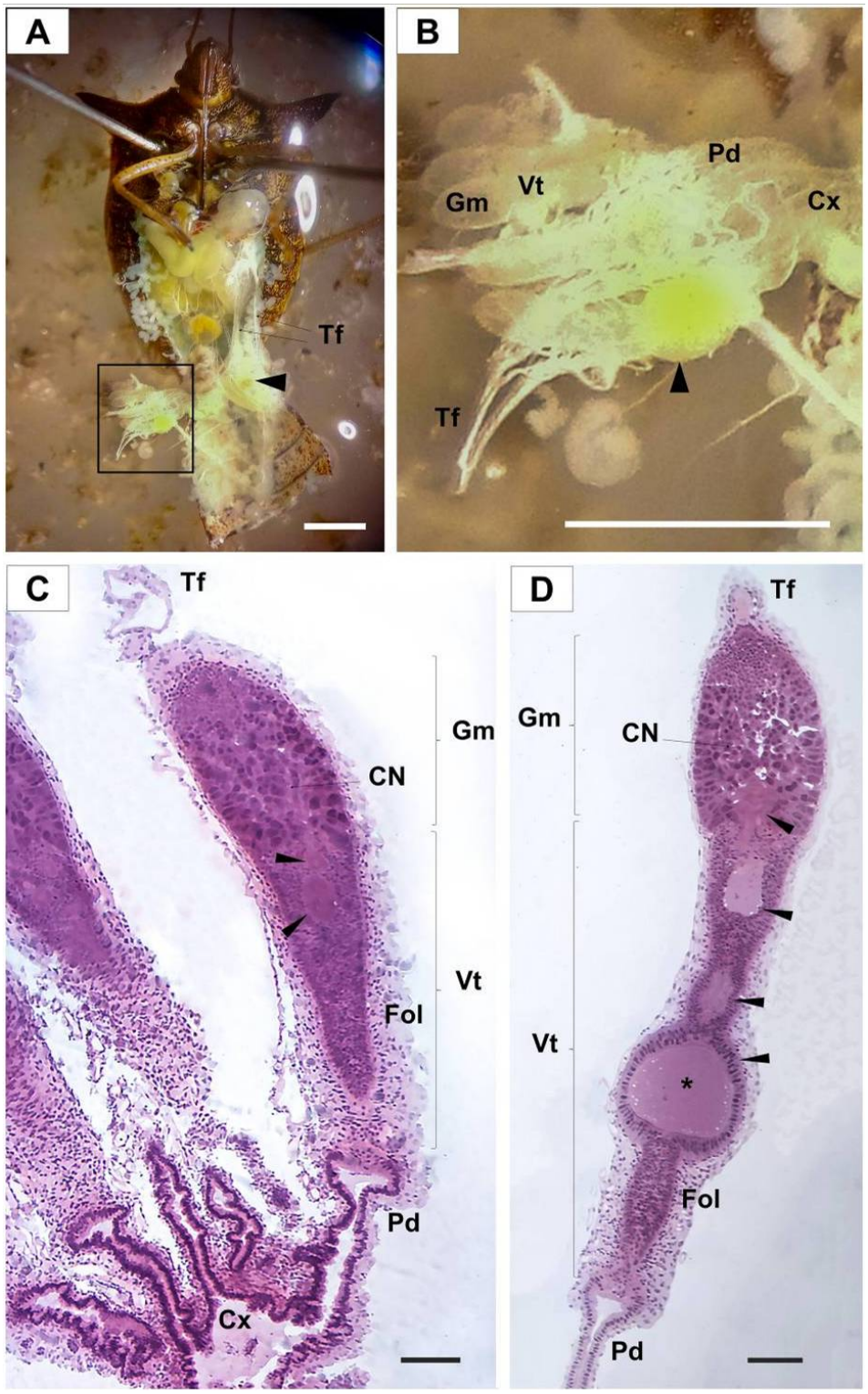


Figure 1. Ovarioles of newly emerged females (0 days after emergence) of *Euschistus heros* treated with a sublethal concentration of pyriproxyfen. A) Female treated with pyriproxyfen with exposed ovaries; B) enlarged detail of the ovary showing the main structures; C) photomicrograph of untreated female ovarioles; D) photomicrograph of ovarioles from a female treated with pyriproxyfen

*Note*. Tf: terminal filaments; Gm: germarium; NC: nurse cells; Vt: vitellarium; Fol: ovarian follicle; Pd: pedicel; Cx: calyx; ►: oocyte.; ✱: previtellogenic oocyte. c-d: Hematoxylin and eosin staining. Bars: a-b = 1 mm; c-d = 100 µm.

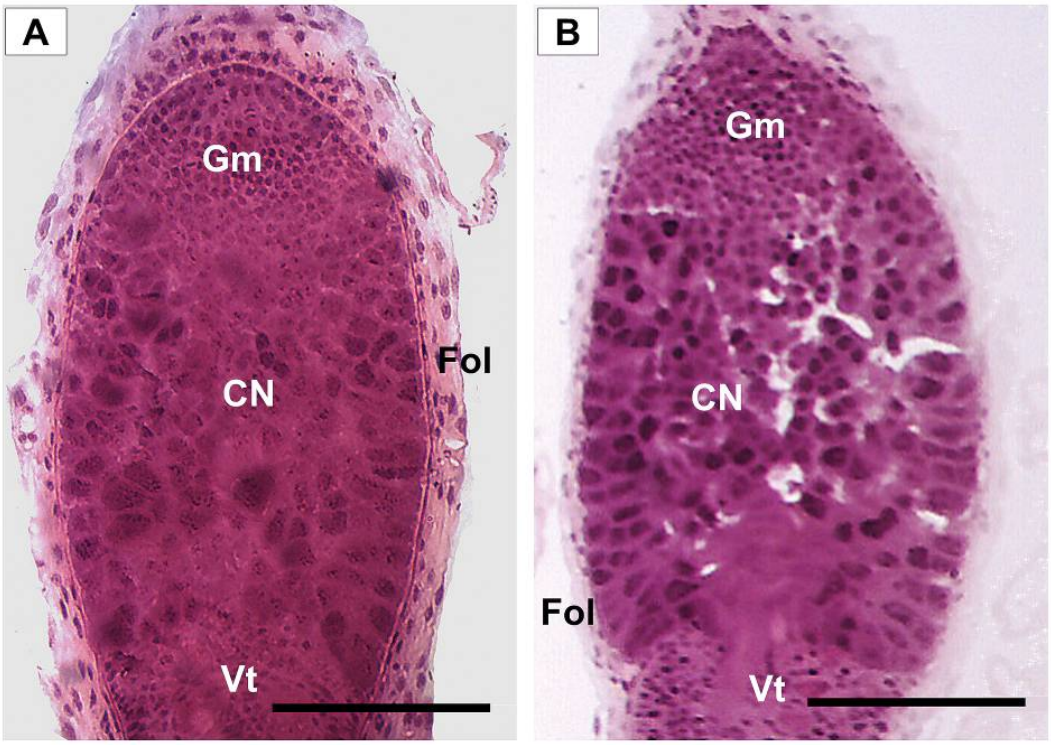


Figure 2. Photomicrographs of the growth region in ovarioles of newly emerged females (0 DAE) of *Euschistus heros* treated with a sublethal concentration of pyriproxyfen. A) Longitudinal section of the germarium region in an untreated female ovariole; B) Longitudinal section of germarium in ovariole of a female treated with pyriproxyfen

*Note*. Gm: germarium; NC: nurse cells; Fol: ovarian follicle; Vt: vitellarium. Hematoxylin and eosin staining. Bars = 100 μm.

From five days onwards, structural, morphological differences are less evident following pyriproxyfen exposure. Females at five days old present oocytes in both the previtellogenic stage, characterized by the presence of unstained granules in the oocyte (Figure 3A), and the vitellogenic stage, in which it is possible to observe intense maturation of the yolk (Figure 3B). At 10 and 15 days of age, it is possible to observe oogenesis in all ovariole follicles at different stages of development. In yolk oocytes, follicular epithelial cells become highly functional, with intense protein synthesis activity (Figure 3C); gradually becoming binucleated cells (Figure 3D). Within this period (10 to 15 days old), basal oocytes are well developed, and choriogenic (Figure 3E), and females already oviposit naturally and without the need for fertilization, however, generating infertile eggs.

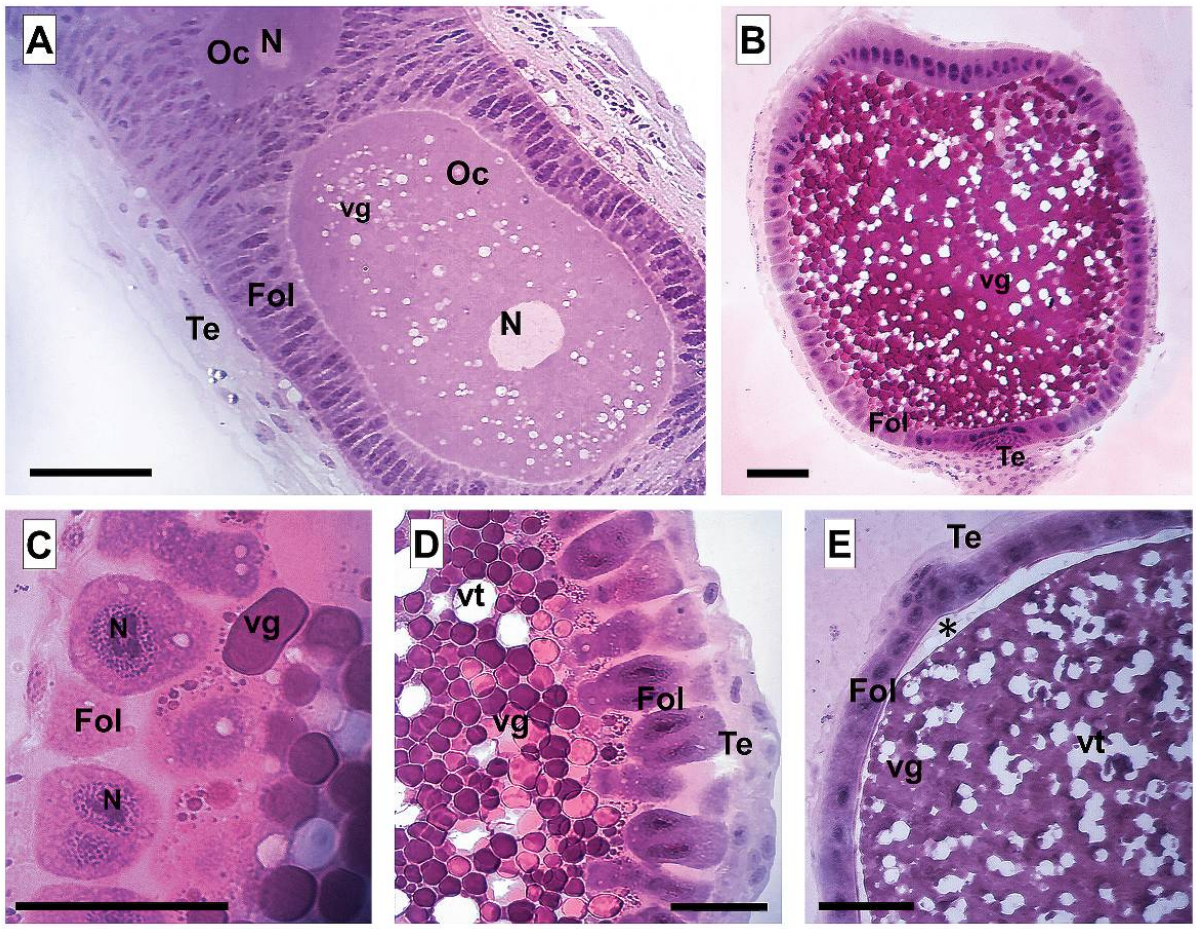


Figure 3. Photomicrographs of basal oocytes at different developmental stages of *Euschistus heros* females treated with a sublethal concentration of pyriproxyfen. A) previtellogenic oocyte in an untreated female (5 days after emergence); B) vitellogenic oocyte in an untreated female (10 days after emergence); C) detail of follicle cells in pyriproxyfen treated females (5 days). Notice nucleus (N) with chromatin clumps indicating high protein synthesis process; D) detail of the follicle region with binucleated cells from untreated female (10 days); E) oocyte in complete choriogenesis stage in untreated female (15 days)

*Note*. Oc: oocyte; N: nucleus; Te: tunica externa; Fol: follicular epithelium; eg: vitellogenin; vt: vitellin; ✱: perioocytic space. Hematoxylin and eosin staining. Bars: a-b = 100 µm; c-e = 50 µm.

Quantitative characteristics of oogenesis were compared based on the morphometric development of the most basal oocyte. From the morphometric variables analyzed (length, width, surface area, and volume), there is a significant difference between newly emerged treated and untreated females (Table 2). Regardless of treatment, a pattern of development was observed; oocytes from newly emerged females have lower proportions compared to other development times, and an expressive difference was also observed in females aged between 5 and 15 days, with oocytes of 10 day-old females showing intermediate values. Regarding the thickness of the follicular epithelium in the basal oocyte region, newly emerged untreated females have an average thickness of 41.1 µm, reducing around 20% every five days, stabilizing at 15 days of age. However, newly emerged females treated with pyriproxyfen showed reduced thickness compared to the control treatment. This reduction in thickness remains constant until the end of the evaluation period, where similarities can be seen with the untreated females (Figure 4). With growth and development, the follicle thickness is inversely proportional to the oocyte area measurement, and all presented significant correlation levels ($R^2 = 0.794 – 0.949$) (Figure 5).

Table 2. Morphometric analysis of metric parameters (mean±SD) of basal oocytes of *Euschistus heros* females treated with a sublethal concentration of pyriproxyfen

| Metric variable | Adult age (days) | Check (distilled water) | Pyriproxyfen $LC_{30}$ (0.688 mL a. i. $L^{-1}$) | p-value[1] |
|---|---|---|---|---|
| Length (µm±SD) | 0 | 78.31±21.83 | 177.87±134.24 | 0.0006* |
| | 5 | 498.14±194.69 | 343.52±143.11 | $0.0560^{ns}$ |
| | 10 | 559.24±242.96 | 517.92±193.98 | $0.6219^{ns}$ |
| | 15 | 594.95±95.30 | 664.82±153.18 | $0.1820^{ns}$ |
| Width (µm±SD) | 0 | 61.85±18.93 | 129.04±93.46 | 0.0004* |
| | 5 | 382.18±144.69 | 276.82±152.67 | $0.1284^{ns}$ |
| | 10 | 464.84±187.95 | 344.05±95.24 | $0.0600^{ns}$ |
| | 15 | 473.50±94.05 | 552.69±184.22 | $0.1998^{ns}$ |
| Superficial area ($\mu m^2$±SD) | 0 | 0.0153±0.0080 | 0.0985±0.1965 | 0.0004* |
| | 5 | 0.6253±0.4043 | 0.3426±0.3249 | $0.0971^{ns}$ |
| | 10 | 0.8937±0.6940 | 0.5344±0.2671 | $0.1145^{ns}$ |
| | 15 | 0.8467±0.3064 | 1.1767±0.6664 | $0.1243^{ns}$ |
| Volume ($\mu m^3$±SD) | 0 | 0.0002±0.0001 | 0.0054±0.0165 | 0.0009* |
| | 5 | 0.0522±0.0464 | 0.0242±0.0320 | $0.1203^{ns}$ |
| | 10 | 0.0943±0.1001 | 0.0382±0.0282 | $0.0785^{ns}$ |
| | 15 | 0.0751±0.0448 | 0.1320±0.1089 | $0.0963^{ns}$ |

*Note*. [1]Student`s t-test: * = significant difference; ns = non-significant difference. n = 56.

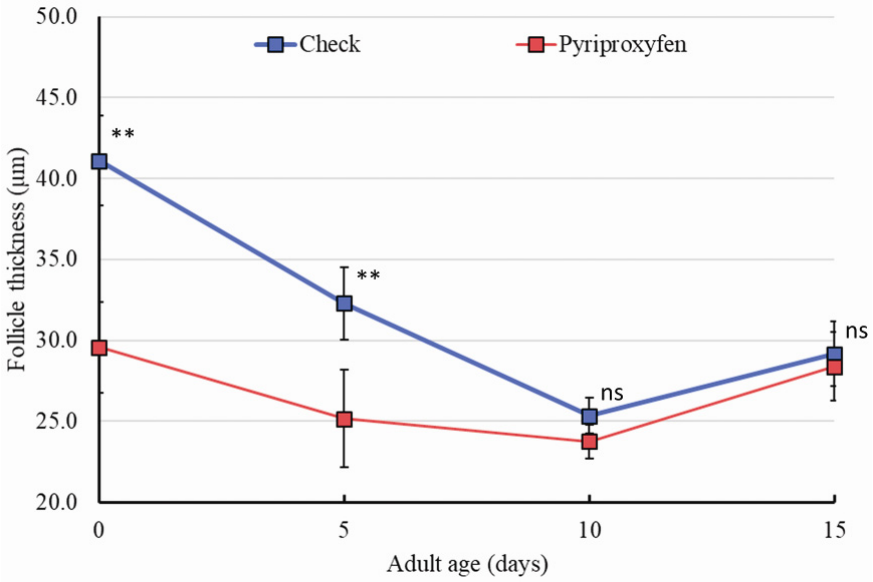


Figure 4. Follicular epithelium thickness in *Euschistus heros* ovarioles treated with a sublethal concentration of pyriproxyfen

*Note*. Differences between treatments by the non-parametric Wilcoxon-Mann-Whitney test: ns = non-significant; * = $p < 0.05$; ** = $p < 0.01$. n = 288.

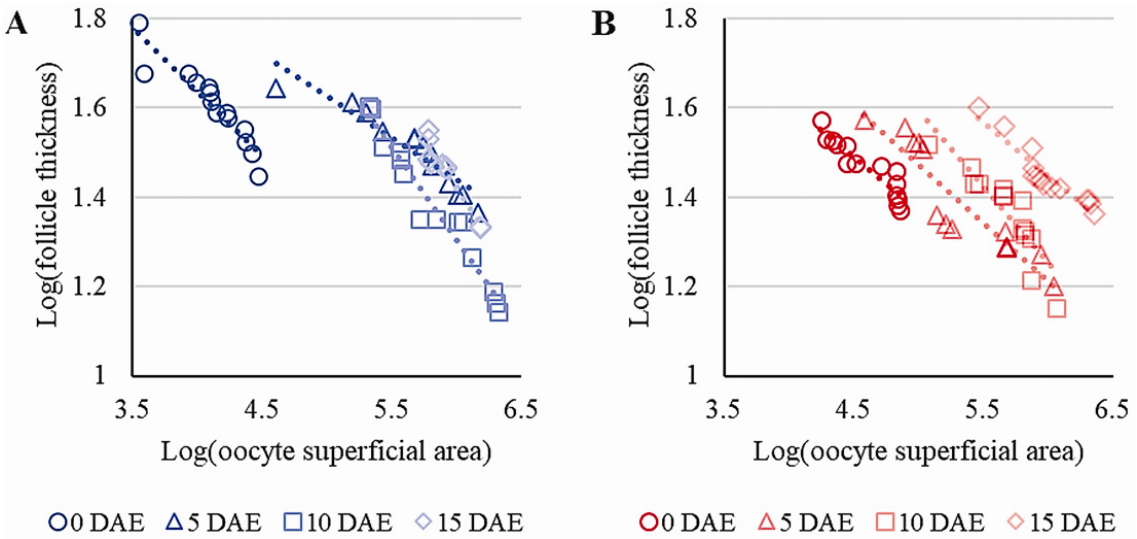


Figure 5. Correlation between basal oocyte surface area measurement and follicular epithelium thickness in *Euschistus heros* females treated with pyriproxyfen. A) correlation in untreated females; B) correlation in treated females; n = 56 oocyte area measurements; 288 epithelial follicle measurements

### *3.3 Male Reproductive System*

The testes of *E. heros* are paired and formed by six follicles each, numbered F1-F6. All follicles follow the same classic spermatogenic configuration described in the literature (Chapman et al., 2013), from the apical to the basal region of a germarium. The growth zone with spermatogonial and spermatocyte cysts also follows this configuration into the maturation (reduction) zone, where several meiotic divisions will give rise to spermatid cysts. Finally, at the transformation zone, the spermiation process takes place, and the transformation of spermatids into sperm occurs. The pattern of development observed throughout GPA was initially oval-shaped (prolate spheroid) in newly emerged males (0 DAE). Throughout the development period, observed changes were characterized by disproportionate hypertrophy of the apical region, specifically in the growth zone (in the spermatogonial and spermatocyte cysts), passing into a final obpiriform configuration (Figure 6).

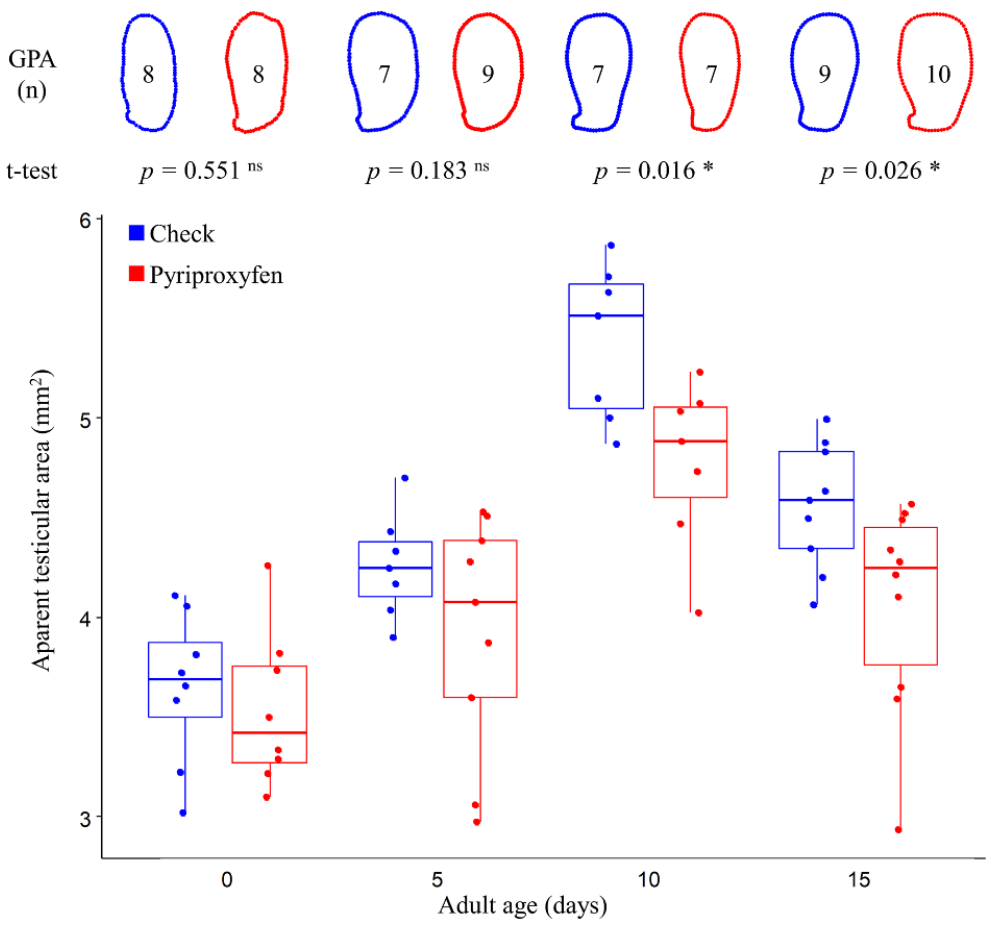


Figure 6. Geometric morphometric analysis and testicular area of *Euschistus heros* treated with a sublethal concentration of pyriproxyfen

*Note*. GPA = generalized Procrustes analysis; n = sample number; ns = non-significant; * = significant at $p \leq 0.05$.

Regarding function, follicles give rise to two morphologically distinct types of germline cells, with smaller cysts being observed in follicles F1, F2, F3, and F5, and a type of cyst with larger cells and more elongated conformation in follicles F4 and F6 (Figure 7A). Throughout the follicle, it is possible to observe all phases of the spermatogenic process, as described by Chapman et al. (2013): it begins with the germarium, where the differentiation of somatic and germ cells occurs, referred to as spermatogonia, that pass through the growth zone and transform into spermatocytes (Figures 7B and 7C). In the maturation zone, spermatogonia undergo the process of meiosis and begin to differentiate into spermatids. The entire sequential process is compartmentalized in cysts with nuclei of somatic TACs adhering to the structure of connective tissue septa (also known as the *tunica interna*) (Figures 7A, 7B, and 7C). These cells are homologous to Sertoli cells in vertebrate organisms (Guraya et al., 1995). Next, the spermatid transformation phase begins (Figure 7C, 7D, and 7E), which initiates the spermiogenesis process, resulting in the obtaining of sperm cysts (Figure 7E). The morphological patterns of the septa structure are variable, and it is possible to observe an increased thickness and differentiated structural cells in older adult insects (Figure 7G). In *E. heros* at ten days of adult age, we observed a narrowing of this tissue in the region of spermatogonia following pyriproxyfen exposure (Figure 7H).

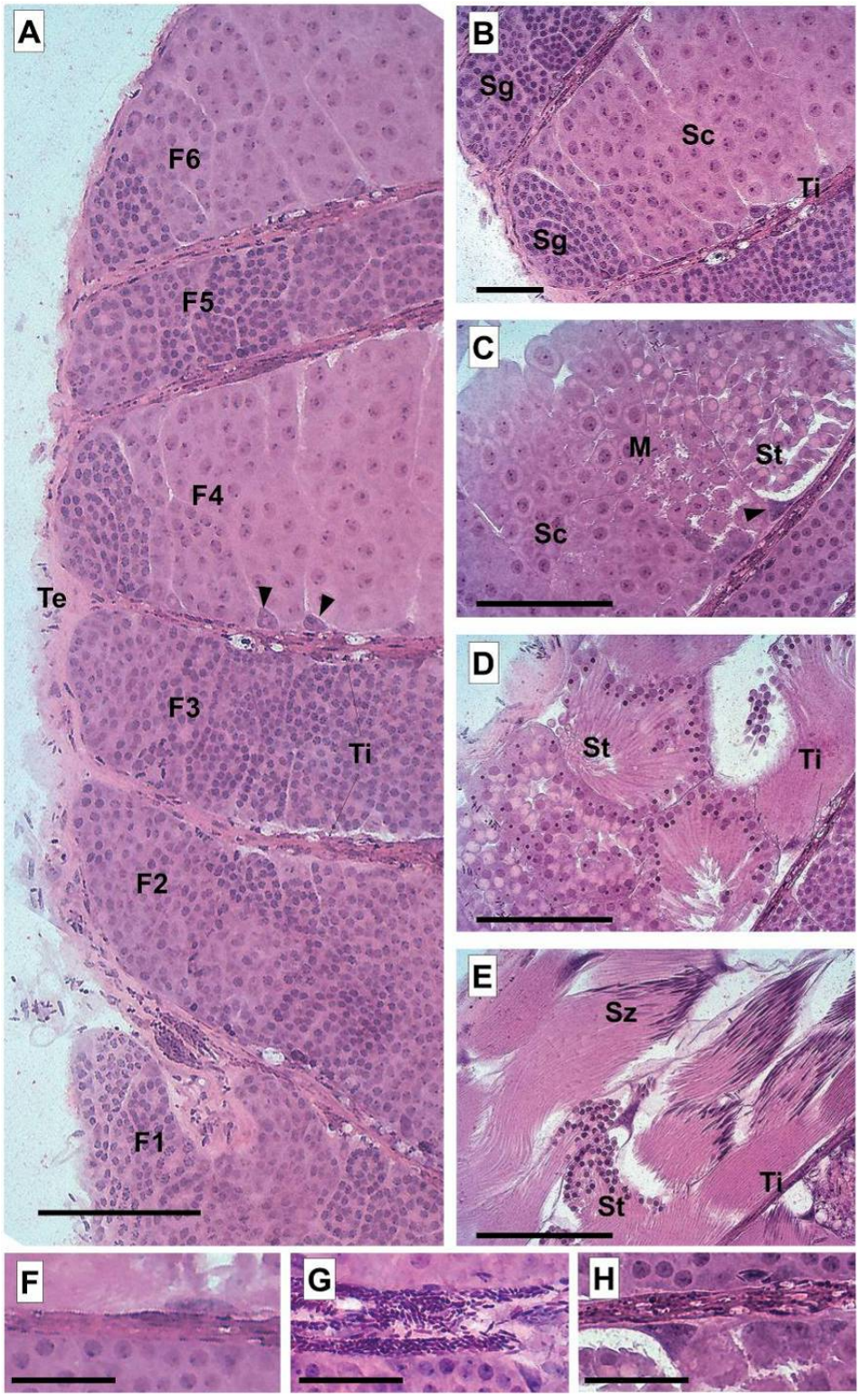


Figure 7. Photomicrographs of spermatogenesis in *Euschistus heros* testis. A) apical portion of the testis, with the differentiation of the six follicles (F1-F6); B) detail of the growth zone; C) detail of the maturation (reduction) zone, region of meiotic division (M); D) detail of the transformation zone; E) detail of spermiogenesis; F) detail of connective tissue septa (Ti) in newly emerged control insects; G) Ti in 10-days-old untreated adult; H) Ti in 10-days-old pyriproxyfen-treated adult

*Note*. Sg = spermatogonia; Sc = spermatocytes; St = spermatids; Sz = spermatozoa; Te = *tunica externa*; Ti = connective tissue septum; ▶ = testicular accessory cell nucleus. Hematoxylin and eosin staining. Bar = 50 µm.

Due to their irregular three-dimensional conformation, it is not possible to clearly define the extent of the TACs in the histological section plane. However, it is possible to observe the nuclei of these cells, attached to the inner connective tissue wall, being present in the growth zones and as reminiscent in the maturation zone. These cells do not change in number, and the apparent nuclear area fluctuates between 50000 and 55000 $\mu m^2$ on average. It was possible to observe a significant reduction of nuclear extension after pyriproxyfen exposure in testes of 10 (t = 2.481; p-value = 0.019) and 15 days old adults (t = 2.458, p-value = 0.020) (Figure 8).

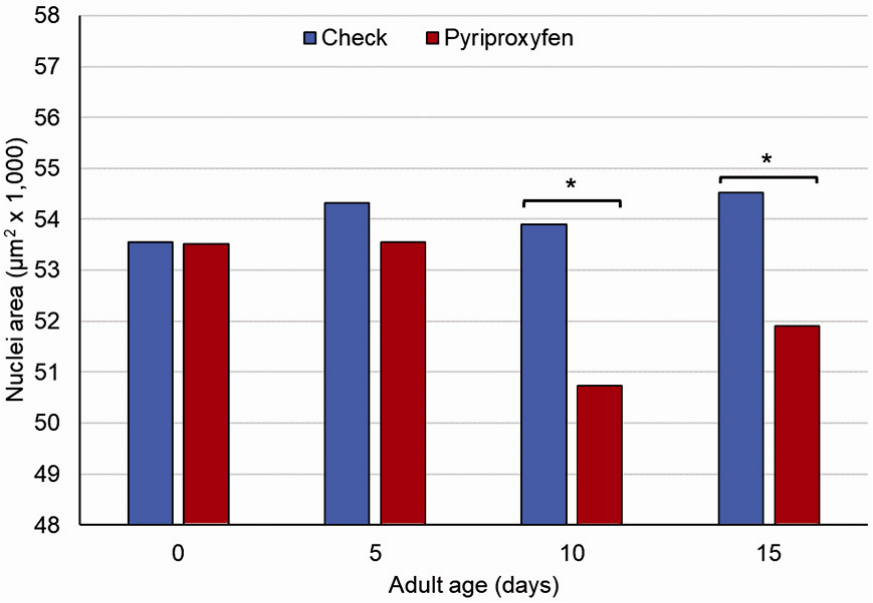


Figure 8. Nuclear area of testicular accessory cells of *Euschistus heros* treated with a sublethal concentration of pyriproxyfen

*Note*. * = significant difference at $p \leq 0.05$, unpaired t-test. n = 240.

## 4. Discussion

### *4.1 Fecundity and Fertility*

The high coefficient of variation observed in all parameters of analysis of fertility and fecundity occurred mainly due to the natural difference in the observed number of eggs laid within each treatment, which consequently corresponds to a variation in the number of hatched nymphs. However, considering the relative proportion between pyriproxyfen-treated and untreated insects, there were alterations in reproductive parameters in couples where at least one individual was treated with pyriproxyfen. The fertility and fecundity decreased when the female was exposed to pyriproxyfen treatment.

The effects of pyriproxyfen on the reproductive parameters of *E. heros* may have occurred due to an imbalance in the homeostasis of JH and other related hormones. Usually, female insects show regulatory patterns dependent on hormonal components. Many are sensitive to JH precursors, such as farnesol, which has a chemical structure very similar to pyriproxyfen, *i.e.*, sesquiterpenoid with a similar mode of action (De Loof, 2015; De Loof & Schoofs, 2019). Furthermore, it is known that pyriproxyfen has a negative effect on reproductive parameters (fecundity, fertility, and nymph viability) in females of the pentatomid *Nezara viridula* (Agüero et al., 2014), as well as decreased fecundity and fertility in *E. heros* observed soybean. It should be acknowledged that these soybean fields were treated with rates recommended for controlling the whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Matsumoto et al., 2021). The interactions are similar and the site of action of JH, as well as its precursors and analogues, are the intracellular receptors of the basic Helix-Loop-Helix/Per-ARNT-SIM (bHLH-PAS) type, such as Met and Gce (Charles et al., 2011; Jindra et al., 2015).

The activity of pyriproxyfen, applied to late nymphs of *E. heros* on the reduction of reproductive parameters of resultant adults may have economic implications, since there are reports of infestations in the vegetative phase of soybean crop when the control of pest caterpillars is also necessary (Timbó et al., 2014). Stinkbugs remain on adjacent host plants during the diapause period (Correa-Ferreira & Panizzi, 1999), physiologically prepared for

dispersal when the crop begins to develop. Thus, management with pyriproxyfen to suppress infesting populations of stinkbugs during the infestation period needs to be evaluated continually, and the anticipation of application should be analyzed in future studies to assess the efficacy in stinkbug and caterpillar IPM.

*4.2 Pyriproxyfen on Female Reproduction*

Pyriproxyfen caused alterations in the morphological distribution pattern of nurse cells in female *E. heros*. A similar result was observed with other insect species, such as in *Monomorium pharaonis* L. (Hymenoptera: Formicidae) (Tay & Lee, 2014), *Diceraeus melacanthus* Dallas (Hemiptera: Pentatomidae) (Cremonez et al., 2017), and in *Aedes aegypti* L. (Diptera: Culicidae) (Ahmed et al., 2020). This occurs because JH is required in different proportions in relation to somatic and germ cells of the germarium. Therefore, a standard gradient is necessary for the correct organization and structure of the ovariole (Luo et al., 2020).

In reproductive terms, the physiology of the oogenesis process is one factor that explains the negative effects of pyriproxyfen on *E. heros*. Oogenesis represents the complete process that goes from the development of the oocyte to oviposition. In Pentatomidae, females can lay eggs even without copulation (Cingolani et al., 2020), a process that requires a large amount of energy. The lack of food, however, can lead to the process of reabsorption of developing oocytes (Kotaki et al., 2016). The ideal rearing conditions and *ad lib* food may have contributed to the oviposition of virgin females of *E. heros*. The developmental patterns of *E. heros* oocytes are similar to those reported in other Heteroptera (Assis et al., 2019; Dittmann & Maier, 1987; Ogorzałek & Trochimczuk, 2009). In *E. heros*, the oocytes are vitellogenic from 5-day-old females onwards, where it was possible to observe binucleated follicular cells with very evident nucleolus in the basal oocytes. A similar result was observed in the ovaries of *Podisus nigrispinus* Say (Hemiptera: Pentatomidae) (Assis et al., 2019). However, in *E. heros* females exposed to pyriproxyfen at N4, vitellogenic oocytes were observed in several newly emerged females (0 days), possibly due to the JH-like activity on vitellogenin incorporation, especially at a regulated sublethal concentration such as $LC_{30}$ (Edwards et al., 1993).

The oocyte's incorporation of vitellogenin depends on the follicle activity, which has a specific permeability and acquires vitellogenin from the hemolymph. Like *E. heros*, the process is evident in 5-day-old females of *Nauphoeta cinerea* Burmeister (Blattodea: Blaberidae) (Buhlmann, 1976). However, the follicular cells of *E. heros* themselves show patterns of protein synthesis, also observed in *P. nigrispinus* (Assis et al., 2019). This explains the reduction in the thickness of this tissue around the oocyte as it develops, as well as why insects treated with pyriproxyfen in early adulthood have thinner follicles since vitellogenesis is anticipated by insecticide activity.

Assuming a central role in the hormonal intermediation of reproduction, JH is responsible for a series of processes. Some models are used to understand the importance of JH on insect reproduction (Santos et al., 2019). In general, there are central receptors that are activated by the hormone and trigger a series of cascading reactions. Thus, through the activation of genes and interactions with other hormones and neuropeptides, they finally act on the synthesis, accumulation, and maturation of lipids and yolk, which are essential for oogenesis. Through interaction with the same receptors (Jindra et al., 2015), pyriproxyfen has a primary activity on reproduction (Edwards et al., 1993; Lenkic et al., 2009). However, in general, enzymes responsible for terminating the action of JH, such as JH esterase and JH epoxy hydrolase, despite being stimulated by JH agonists (El-Sheikh et al., 2016), do not affect their activity (Zhang et al., 1998). The effects of pyriproxyfen on vitellogenin are amply documented. While the product acts as a JH analog, there is a positive correlation between the application of pyriproxyfen and the synthesis of vitellogenin (Edwards et al., 1993), as well as oocyte development (Lenkic et al., 2009). However, high concentrations of pyriproxyfen inhibit vitellogenesis at different stages. This can occur from the synthesis itself (Pinto et al., 2000) or in hormonal imbalances, more specifically in insects with ecdysteroid-dependent vitellogenesis (Barchuk et al., 2002). This may also occur during the process of incorporation of vitellogenin into the ooplasm, mainly due to structural changes in the reproductive system (Xu et al., 2015). Finally, the consequences of hormonal imbalance may ultimately have negative effects on reproduction.

The activity of JH on reproduction also largely depends on the species studied, especially regarding the life cycle. In Pentatomidae, hormonal regulation by JH mediates essential processes, such as the production of pheromone components in precise proportions (Moraes et al., 2008). It may also affect diapause (Penca & Hodges, 2017), resource reallocation, and absorption of developing oocytes (Kotaki et al., 2016). JH also plays an essential role in the functionality of the ovarian follicular structure. The effects of JH application influence the structure to produce more protein, and it is also reported that the natural levels of this hormone increase considerably during vitellogenesis (Dittmann & Maier, 1987). In *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), JH is essential for vitellogenin expression, oocyte maturation, and fecundity (Lu et al., 2016). However, its hormonal functionality can be affected by exposure to pyriproxyfen and the levels of action are usually regulated by

metabolism; however, the insecticide remains active. The implications of pyriproxyfen action on *E. heros* females indicate that the insecticide affects reproductive parameters associated with the functionality of its reproductive system.

*4.3 Pyriproxyfen on Male Reproduction*

The morphometric analysis of the testes of *E. heros* demonstrates a peculiar pattern of development. Mourão and Panizzi (2000) schematically described a similar morphological pattern when analyzing the testis development of *E. heros*. The authors present two degrees of development: immature, with small gonads in an elliptical design, with a greater length:width ratio, and mature, large, with a prolate ellipsoid pattern and well-defined follicles.

The standard development of spermatogenesis in insects occurs from the apical to the basal region towards the *vas deferens*, and the germ line cells are characteristically arranged in cysts (Roose-Runge, 1977). In *E. heros*, the different types of testicular follicles were analyzed in a series of studies (Aguiar et al., 2017; Cremonez et al., 2019; De Souza et al., 2011; Gomes et al., 2013), as well as their characteristic sperm polymorphism. Morphologically, it was possible to distinguish three types of sperm, with type 1 being more common in the follicles of the most proximal half (F1-F3); type 2 sperm, which is morphometrically similar to type 1 and is produced in the characteristically thinner F5; and type 3 sperm, produced in follicles F4 and F6, are naturally more voluminous and up to 4x longer than the others. In the present study, we observed a hypertrophy in the developmental zone, mainly due to the larger area of spermatogonia of the follicles F4 and F6, and that, over time, gives the obpiriform pattern to the testis.

The action of pyriproxyfen on the morphometry of the male sexual organ of *E. heros* is structural and may affect its functionality. It is known that pyriproxyfen acts on the development and internal structure of the testicular capsule in Pentatomidae: the action of pyriproxyfen $LC_{30}$ caused disruption and the presence of material indicative of cell death in sperm cysts of *D. melacanthus*, but no changes could be observed in the nuclei of TACs (Cremonez et al., 2017). Testis fragments of *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) incubated *in vitro* depend on the activity of ecdysteroids and the presence of the internal follicular tissues to complete the cell division processes of spermatogenic lineage (Jacob, 1992), indicating that a paracrine activity of the tissue influenced cell development. This activity might be related to the presence of TACs in the testicular connective tissue. Morphometric changes in TAC nuclei were observed after the application chitin biosynthesis inhibitors, buprofezin and lufenuron, in testes of male *E. heros* at 5 days of adult age (Cremonez et al., 2019). The chitin biosynthesis inhibitors act on intracellular vesicular transport that prevents the activity of the chitin synthetase (CS) enzyme (Matsumura, 2010), a different mechanism from the action of pyriproxyfen. This triggers a cascade of reactions by binding and modulating JH receptors as primary action (Jindra et al., 2015). However, the action of pyriproxyfen produces a generalized reaction towards structural development, especially considering the JH activity on the developmental maturation of the reproductive system itself.

Despite the vast amount of work on the action of JH on the reproduction of females, the understanding of this hormone in males is still scarce. Most studies are associated with hormonal activity in the accessory glands; however, its primary function is related to stimulating the reproduction of females during copulation (Parthasarathy et al., 2009). It is known that JH mediates indirect morphogenetic relationships between different tracts and physiological systems with male sexual development in certain insect species (Contreras-Garduño et al., 2011; Flatt & Kawecki, 2007; Fry, 2006; Pezenti et al., 2021). Additionally, the ingestion of methoprene, a JH analog, promotes faster development of testes in *Bactrocera tryoni* Froggatt (Diptera: Tephritidae) (Adnan et al., 2020). Therefore, the changes observed in the structure and development of the testicular capsule and the nuclei of TACs after exposure to pyriproxyfen may be mainly related to the side effects of *E. heros* late juvenile development.

In Pentatomidae, the reproductive process and offspring viability is a more desired traits than fitness itself, as some processes lead to the emergence of populations resistant to xenobiotics. Regardless, some populations of *E. heros* have an important characteristic of resistance, especially to neurotoxic insecticides such as neonicotinoids. This seems to be a consequence of the emergence of resistant haplotypes from two main lineages in the region of Brazil (Soares et al., 2018). However, it is due to the reproductive potential that the *E. heros* assumes the status of "key-pest" of soybean production systems. Due to this characteristic, the action of pyriproxyfen on the structure of the *E. heros* reproductive system indicates that the insecticide has the potential to control infesting populations of the stinkbug.

In summary, on reproductive terms, adult females of *E. heros* are more sensitive to pyriproxyfen in sublethal concentration applied in the fourth instar compared to males. The insecticide causes morphological and morphometric changes in the reproductive system in both sexes, mainly in the ovarian structure, disarrangement of

the nurse cells, and an increase in basal oocytes size at the beginning of sexual development. This is accompanied by a decrease in the thickness of the follicular epithelium in females, as well as a reduction of the size and shape of the testicular capsule and a drop in the area of testicular accessory cell nuclei in males.

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