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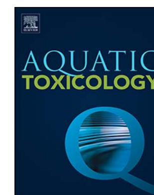

Effects of atrazine on vitellogenesis, steroid levels and lipid peroxidation, in female red swamp crayfish *Procambarus clarkii*

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ABSTRACT

Atrazine, a widely use herbicide, has been classified as a potential endocrine disruptor, especially for freshwater species. In this study, we tested the hypothesis that atrazine can affect reproduction in crayfish through dysregulation of vitellogenin expression and hormone synthesis. Adult female crayfish (*Procambarus clarkii*) were exposed during one month to atrazine at concentrations of either 1 or 5 mg/L. At the end of the exposure, ovaries, hepatopancreas, and hemolymph samples were harvested for analysis of vitellogenin expression and steroid hormone levels. Ovarian tissue was also sampled for both biochemical and histological analyses. Our results show that atrazine-exposed crayfish had a lower expression of vitellogenin in the ovary and hepatopancreas, as well as smaller oocytes, and reduced vitellogenin content in the ovary. Despite these effects, circulating levels of estradiol increased in females exposed to 5 mg/L of atrazine, showing that the inhibiting effect of atrazine on vitellogenin production was not related to a lower secretion of sexual steroids. Instead, some early stimulating effects of estradiol on vitellogenesis could have occurred, particularly in the hepatopancreas. On the other hand, atrazine caused a higher metabolic effort, in terms of lactate production, presumably triggered to provide the energy needed to face the unspecific stress produced by the herbicide. Lipid peroxidation was not affected by atrazine, but glutathione levels were significantly increased.

1. Introduction

Atrazine, a widely used herbicide applied to inhibit weed by interfering with the photosystem II, is one of the most widely used herbicides in the United Stated with over 24,000 tons applied yearly (Moore et al., 2007). The half-life of atrazine in water is longer than 60 d, and its environmental levels range from 0.1 to 100 μg/L (Vonberg et al., 2014; USEPA, 2002). In waters adjacent to treated fields, as well as in groundwater, atrazine concentrations were as high as 1 mg/L (Graymore et al., 2001). Although this herbicide is not commonly absorbed in sediments, the fraction associated to this substrate can be very significant (Jablonowski et al., 2011).

The red swamp crayfish *Procambarus clarkii* is an introduced species inhabiting extensive areas in the US, especially in Louisiana, where the culture of this species is integrated with rice farming (Huner and Barr, 1991).Therefore, *P. clarkii* is likely to be exposed to several herbicides and other pesticides. Several studies have been published concerning the effects of some pollutants on *P. clarkii*, which has been also taken as a sentinel species for the study of biomarkers (Goretti et al., 2016; Alcorlo et al., 2006). However, no reports have addressed the effect of atrazine on this widespread species. In the Mississippi basin, the natural habitat where *P. clarkii* grows and reproduces to be exploited as a renewable economic resource, atrazine was found in 97% of all water samples (Rebich et al., 2004); atrazine amounts carried by the Mississippi river were the highest among all herbicides detected (Clark and Goolsby, 2000).

Moreover, *P. clarkii* has been considered a model species for a great variety of studies, since it is representative of the biology of most decapods crustaceans. For this reason, the reproductive biology of this species has been extensively studied. For instance, the ovarian cycle of this crayfish has been fully characterized by Kulkarni et al. (1991), while the roles of neurotransmitters and hormones involved in gonadal growth have been reviewed by Fingerman (1995). During primary vitellogenesis, the oocytes synthesize vitellogenin by themselves, but

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during the secondary vitellogenesis, crustacean ovaries grow significantly by uptaking the vitellogenin synthesized in the hepatopancreas (Charmantier et al., 1997; Nagaraju, 2011).

Some vertebrate-like sexual steroids, such as 17-hydroxyprogesterone and 17β-estradiol, have been reported to be involved in the regulation of crustacean vitellogenesis, presumably secreted by the ovarian follicular cells and eventually by the hepatopancreas (Fingerman et al., 1993; Warrier et al., 2001; Lafont and Mathieu, 2007). The expression of several steroidogenesis enzymes leading to the synthesis of estradiol and progesterone has been reported in the freshwater prawn *Macrobrachium rosenbergii* (Thongbuakaew et al., 2016). In addition, the Pm-p23 progesterone receptor has been isolated and sequenced from the ovary of the shrimp *Penaeus monodon*, and its expression studied throughout the ovarian cycle (Preechaphol et al., 2010).

A decrement in both the oocyte area and vitellogenin ovarian content has been observed in females of the crab *Neohelice granulata* following exposure to atrazine during the pre-reproductive period, when the ovary is intensively growing (Silveyra et al., 2017). Moreover, atrazine caused a delay in ovarian re-maturation of *N. granulata* ovigerous females, during the reproductive period (Álvarez et al., 2015). Atrazine was shown to antagonize the effect of juvenoid hormones in *Daphnia sp* (Palma et al., 2009), altering sexual differentiation (Dodson et al., 1999). This herbicide also inhibited gonadal maturation in fish and other vertebrates (Tillitt et al., 2010) by interfering with the hypothalamic control of pituitary hormones secretion, and by inducing aromatase activity (McKinlay et al., 2008). Atrazine has also been shown to act as xenoestrogen in mammalian cell cultures (Villeneuve et al., 1998; Lascombe et al., 2000).

Based on previous evidence, we hypothesized that atrazine reduces the ovarian growth of *P. clarkii*, presumably by interfering with the hormonal regulation of gonadal growth in females. To test this hypothesis, in this study we evaluated the effect of atrazine on vitellogenesis in *P. clarkii* adult females, by measuring vitellogenin gene expression and vitellogenin protein content in both ovary and hepatopancreas, ocyte development in the ovary, and hemolymphatic sex steroid levels. In addition, the effect of atrazine exposure on biomarkers of metabolic stress and lipid peroxidation was also evaluated.

2. Materials and methods

2.1. Animals

Adult *P. clarkii* crayfish (N = 46) were obtained from a local dealer (Carolina Biological Supply) in April 2017. Upon arrival, crayfish were immediately sexed and separated into large aquaria and allowed to acclimate for two weeks. Following this acclimation period, female crayfish (N = 34) were then transferred to individual round plastic containers (4 ¾" × 4 ¼") filled with 400 ml of dechlorinated aged tap water (pH = 7.6 ± 0.2; hardness = 125 mg/L, as $CaCO_3$ equivalents). The toxicological bioassay was conducted in semi-static conditions according to the standard procedures recommended by the American Public Health Association et al. (2005). The code of ethics for animal experiments stated in the Declaration of Helsinki was always followed.

2.2. Atrazine solution

The commercial formulation Gesaprim90® from Syngenta (90% of atrazine as active principle, in granules) was used. A stock solution of atrazine was prepared weekly, by dissolving the appropriate amount of the formulation in distilled water.

2.3. Atrazine concentrations and experimental conditions

Small aliquots from the atrazine stock solutions were added to the individual containers, in order to obtain the following concentrations: 0 (aged tap water), 1 mg/L (4.6×10^{-6} M) and 5 mg/L (2.3×10^{-5} M) of atrazine as the active ingredient. Aged tap water without addition of atrazine served as control. The test solution in each container was completely replaced twice a week. Animals were randomly assigned to the different treatments and kept in room at temperature of 22 ± 1 °C and a photoperiod of 14:10 (L:D) throughout the experiment. All animals were fed twice a week with Tetra Color granules (TETRA®). Animals remained exposed to atrazine or control for 30 days, starting in early May.

In order to validate nominal concentrations of atrazine, water samples (15 ml) were taken at 0 and 72 h every week, i.e., the period for water replacement in all test containers. The atrazine concentration in these samples was measured using theatrazine ELISA microtiter plate kit (Abraxis®).

2.4. Tissue collection

At the end of the experiment, females body weight (BW) was determined by using a Mettler Toledo electronic balance (0.01 g precision). Carapace length (from the rostral tip to the posterior median end of the cephalothorax) was measured with a Vernier digital caliper (VINCA DCLA-0805, 0.03 mm precision). Samples of hemolymph (200 to 300 µl) were withdrawn from the pre-branchial sinus at the base of the fifth pereiopod of each surviving animal, with a tuberculin syringe fitted with a 29G needle, and stored at −80 °C until analysis. Animals were then sacrificed after anesthetizing them in ice water, and ovaries and hepatopancreas were quickly dissected and weighed at 0.0001 g precision. The gonadosomatic and hepatosomatic indexes were calculated as the weight of ovary or hepatopancreas/body weight x 100. After separating a portion for histological analysis, hepatopancreas and ovaries were stored at −80 °C until they were processed.

2.5. Vitellogenin expression

Gonads and hepatopancreas were processed to determine the expression of vitellogenin mRNA. At the time of harvest, tissues were snap frozen in liquid nitrogen and stored at −80 °C. To extract RNA, all tissues were pulverized and homogenized in TRIzol (Life Technologies, Carlsbad, CA), and passed through a series of 18G, 21G, and 23G needles using a syringe. Homogenates were briefly centrifuged, and the Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) kit was used to obtain DNA-free total RNA through an in-column DNAse I digestion. Purified RNA was quantified by Nanodrop, and RNA quality and absence of genomic DNA was verified with a Bioanalyzer 2100 at the Penn State Hershey Genome Sciences Core Facility.

A total of 200 ng of RNA were retro-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in the presence of RNA inhibitor, following the manufacturer's protocol. cDNA was then diluted with ultra-pure water for quantification of *P. clarkii* vitellogenin (Vg) transcripts (Accession: KR135171.1), and 18S rRNA (control, validated by Jiang et al., 2015, for *P. clarkii*) by Real-Time PCR. Briefly, every10 µl reaction contained 10 ng (18S) or 40 ng (Vg) of cDNA mixed with 5 µl of the Power Up SYBR Green Master Mix (Life Technologies), and 10 mM of the following primers: Vg forward: 5′-CCAGAAGACGCCACAAGAA-3′, Vg rev: 5′-CAGAAGGCATCAGCCAATC-3′, 18S for: 5′-TCCGCATCACACTCACGT-3′, 18S: 5′-TGGAACCCTCTCCACAGG-3′. PCR reactions were run in triplicate in 384 well plates using a 7900 HT Real-Time PCR instrument following standard conditions: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Following amplification, a dissociation curve was generated by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments to check for specificity of amplification. The PCR product size was verified by running an aliquot of the amplification reaction on an agarose gel, and the fragments were purified for sequence confirmation. Each sample was amplified in triplicate.

2.6. Vitellogenic protein content

Tissue processing for vitellogenic proteins determination: Ovaries were cut in small fragments (0.1–0.2 g), and homogenized in sodium phosphate buffer (50 mM, pH = 7.4, with 2 µl/mL of protease inhibitor), at a 1:3 (w/v) ratio. Each homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and supernatants were further ultra-centrifuged at 100,000 *g* for 50 min at 4 °C, using an Optima XPN series Beckman ultracentrifuge. The resulting supernatants were stored in Eppendorf tubes at −80 °C until further analysis by ELISA.

2.7. Determination of vitellogenic protein levels

The total content of vitellogenic proteins (Vg: vitellogenin and vitellins) in ovary, hepatopancreas, and hemolymph was determined by ELISA. A primary antibody against native Vg was obtained by inoculating rabbits with purified Vg, following the procedure used in previous studies (Dreon et al., 2003; García et al., 2008). An anti-rabbit antibody conjugated to horseradish peroxidase was used as secondary antibody. The Vg standard curve (0–270 ng) was prepared with a purified Vg solution diluted 1/5000. For the assay, 50 µl of either standard or sample were loaded in triplicate in a 96-well plate (Corning). Samples were diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH = 9.6) at a 1/3 dilution for ovary and hepatopancreas, and at 1/10 dilution for hemolymph. Both primary and secondary antibodies were diluted (1/500) in PBS + 0.05% Tween + 2% BSA. Absorbance was measured at 415 nm wavelength with an ELISA plate reader (Spectra ax M190) using 2–20-Azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) as chromogen.

2.8. Ovarian histology

A portion of each ovary was fixed in 10% paraformaldehyde solution at room temperature, dehydrated in alcohol series, and embedded in paraplast. Blocks were cut in 5-µm sections and stained with hematoxylin and eosin. Tissues were processed in an automated Tissue-Tek VIP processor (Sakura Finetek USA, Torrance, CA). Sections were cut at 5 µm for routine hematoxylin and eosin staining. Images were captured with an Olympus BX51 microscope (Olympus America, Center Valley, PA) and DP71 digital camera using the Cell Sens Standard 1.12 imaging software.

For each animal, representative sections of the ovary were analyzed by light microscopy to determine the relative area of each the three oocytes maturation stages. Previtellogenic, intermediate, and vitellogenic oocytes were characterized according to their size and degree of basophilia. For oocyte area, both major and minor diameters of the oocytes showing their nuclei were estimated by means of a micrometric ocular lens. These were calibrated against a Leitz Wetzlar plate with 1/100 mm spacing, to calculate the oocyte area as $(\pi/4) \times$ major diameter $\times$ minor diameter, as in Rodríguez and Medesani (1994).

2.9. Steroid hormone and metabolite determination in hemolymph

Previous to any determination, hemolymph samples were homogenized and centrifuged at 13,000*g* for 20 min, at 4 °C. Then, each sample was diluted 1/10 and aliquoted for further determinations. The circulating levels of estradiol and testosterone were measured using the estradiol and testosterone ELISA kits (Cayman®), respectively, in the same ELISA plate reader above mentioned, at 412 nm wavelength. Additionally, the hemolymph from a few stock control males (N = 12) was withdrawn at the end of the assay, to measure both estradiol and testosterone.

Glucose content was determined by using the colorimetric assay kit from Cayman®, and measured at 515 nm in a Soft Max Pro spectrophotometer. Lactate levels were measured by means of the L-Lactate assay kit (Cayman®), in a Spectra Max M5 fluorescence spectrophotometer, at 535 and 590 nm of excitation and emission wavelengths, respectively.

2.10. GSH and TBARS determination

Levels of both glutathione (GSH) and TBARS (thiobarbituric acid reactive substances, indicative of lipid peroxidation) were measured in the hepatopancreas. GSH was determined by using glutathione assay kit (Cayman®), at 410 nm, after homogenizing 100 mg of tissue in 100 µl of Cold Buffer and centrifuged at 10,000*g* for 15 min, at 4 °C. TBARS were measured using a colorimetric assay kit (TCA method, Cayman®), at 530 nm; hepatopancreas samples weighting 25 mg of tissue were previously homogenized in 250 µl of RIPA buffer and centrifuged at 1600*g* for 10 min, at 4 °C.

2.11. Statistical analysis

Results were analyzed by means of a one-way ANOVA, followed by Tukey multiple comparisons (Sokal and Rohlf, 1981), once the assumptions for this test were confirmed. A minimum confidence level of 5% was considered for statistical significance.

3. Results

3.1. Validation of used concentrations, survival and morphometry

Fig. 1 shows the validation of the nominal concentrations used (0 = control, 1 and 5 mg/L of atrazine). Measured concentrations were very close to the nominal ones, their differences ranging in average between 7.8 and 12%. Number of surviving animals, as well as body weight and length are shown in Table 1. Only in the case of the hepatosomatic index, a significant increment (29% in average, $p < 0.01$) was observed in either of the atrazine concentrations assayed, with respect to control. No significant ($p > 0.05$) differences were found for any other variables listed in Table 1.

3.2. Vitellogenin expression and vitellogenic protein content

The relative expression of vitellogenin in both ovary and hepatopancreas of the group exposed to the highest atrazine concentration was significantly lower (4.4 and 3.4 folds lower for the ovary and hepatopancreas respectively, $p < 0.01$) than that of the control (Fig. 2). Although it was not statistically significant ($p > 0.05$), a marked decrease of the vitellogenin content was observed in the ovary, at either atrazine concentrations assayed (Fig. 3), in accordance with the lower

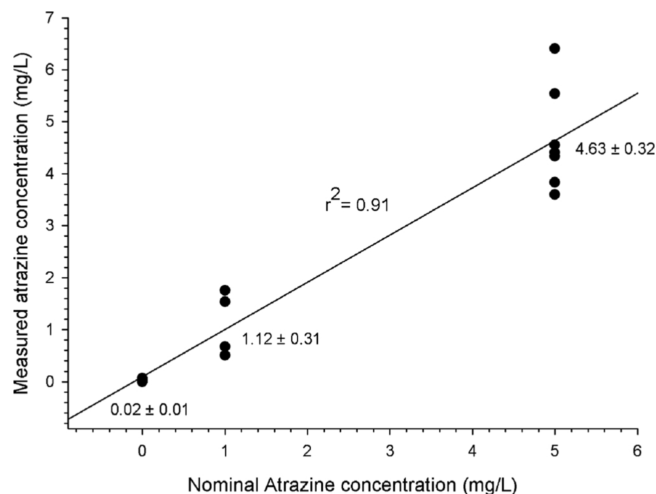

Fig. 1. Measured versus nominal atrazine concentrations used in the assay. The regression line is shown. Mean ± standard error is indicated for each measured concentration.

Table 1
Morphometric measurements of females in each experimental group.

Atrazine concentration (mg/L)	Initial N	Final N	Weight (g)	Length (mm)	GSI	HSI
0 (control)	12	10	15.96 ± 0.81	42.57 ± 0.86	1.75 ± 0.43	5.05 ± 0.35
1	8	8	14.80 ± 0.47	41.72 ± 0.48	2.01 ± 0.37	6.19 ± 0.23 *
5	12	12	15.67 ± 0.46	42.39 ± 0.48	2.59 ± 0.47	6.12 ± 0.21 *

Means ± standard errors are shown. Number of females (N) is also indicated. GSI: gonasomatic index, HSI: hepatosomatic index. Asterisks indicate significant differences ($p < 0.05$) with respect to control.

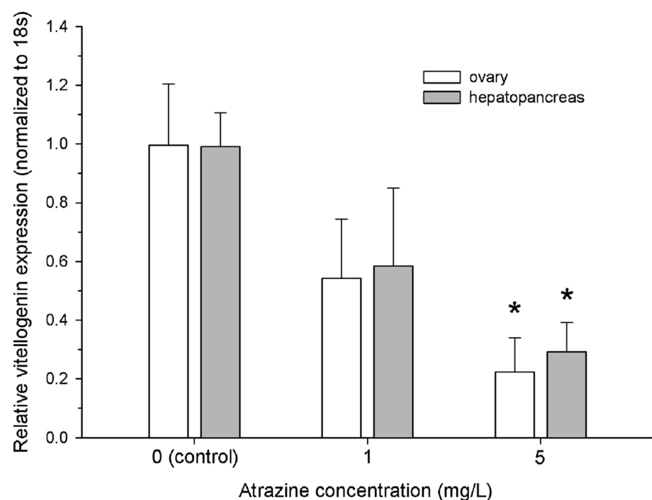

Fig. 2. Relative expression of *P. clarkii* vitellogenin (Vg) transcripts, in ovary and hepatopancreas at the end of the assay. Values normalized to 18S rRNA are shown. Means ± standard errors are shown. Asterisks indicate significant differences ($p < 0.05$) with respect to control.

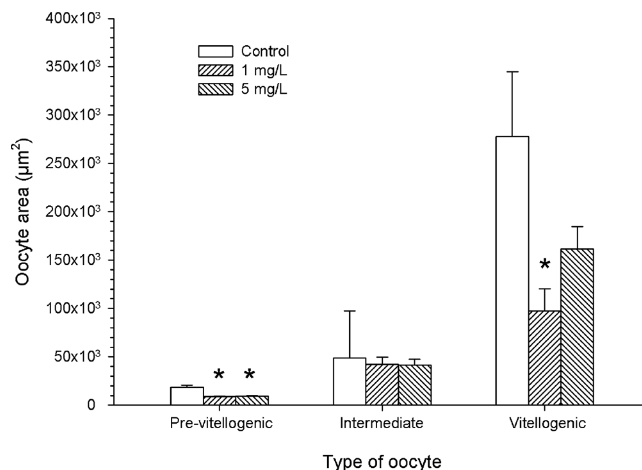

Fig. 4. Oocyte area, for each of the three oocyte types recognized in the ovary of *P. clarkii,* at the end of the assay: Pre-vitellogenic, whose vitellum is completely synthesized by the oocyte; Vitellogenic, which grows faster via uptake of vitellogenin produced by the hepatopancreas and secreted to the hemolymph; and Intermediate, corresponding to a transition stage. Means ± standard errors are shown. Asterisks indicate significant differences ($p < 0.05$) with respect to control.

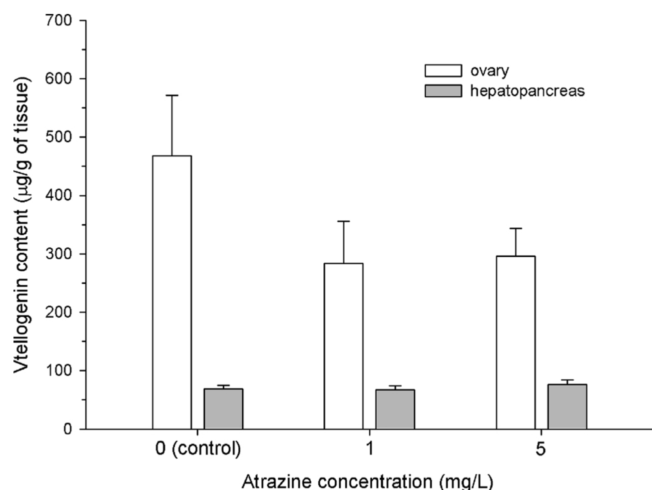

Fig. 3. Vitellogenin content in ovary and hepatopancreas of *P. clarkii*, at the end of the assay. Means ± standard errors are shown.

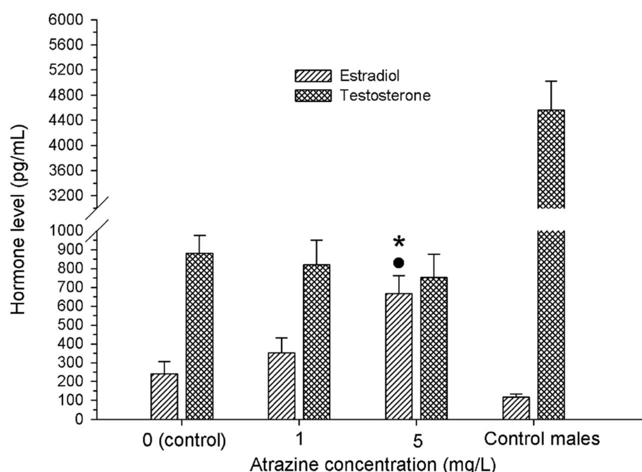

Fig. 5. Estradiol and testosterone levels in hemolymph of *P. clarkii* females, at the end of the assay. Means ± standard errors are shown. Asterisk and dot indicate significant differences ($p < 0.05$) with respect to control and the lowest concentration, respectively. Control male data (N = 12) are included for comparison purposes.

expression above mentioned.

3.3. Ovarian histology

The histological analysis of the ovary showed a significant ($p < 0.05$) decrease of 2.8-fold in the area of vitellogenic oocytes at 1 mg/L of atrazine, with respect to control; moreover, a significantly ($p < 0.01$) lower area (2-fold in average) was observed in pre-vitellogenic oocytes, at either atrazine concentration assayed vs. control (Fig. 4).

3.4. Steroid hormone and metabolite determination in hemolymph

The circulating levels of both estradiol and testosterone are shown by Fig. 5. While no changes ($p > 0.05$) were noted in testosterone levels, a significant ($p < 0.01$) increase of estradiol (2.8-fold) was seen in the hemolymph of females exposed to 5 mg/L of atrazine, with respect to either control or the lowest concentration. The ratio testosterone/estradiol ranged from 3.7 in control females to 1.1 in those females exposed to the highest atrazine concentration. As a reference, the ratio corresponding to control males was estimated in 38.5 (Fig. 5).

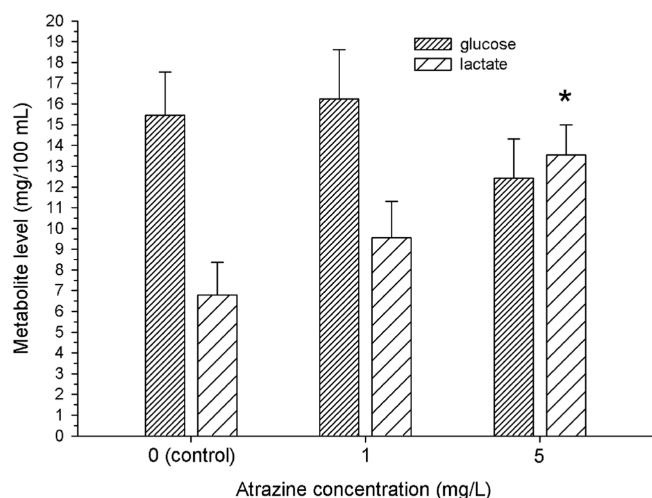

Fig. 6. Glucose and lactate levels in hemolymph of *P. clarkii*, at the end of the assay. Means ± standard errors are shown. Asterisk indicates significant differences ($p < 0.05$) with respect to control.

Although glucose hemolymphatic levels did not show statistically significant differences ($p > 0.05$) among treatments, lactate levels were significantly ($p < 0.05$) increased (2-fold) at the highest atrazine concentration (Fig. 6).

3.5. GSH and TBARS determination

A marked increase in GSH levels (17-fold, $p < 0.01$) was observed in the hepatopancreas of females exposed to 5 mg/L of atrazine, compared to either control or the lowest atrazine concentration. No significant differences ($p > 0.05$) were observed in the lipid peroxidation, estimated by TBARS, at any atrazine concentration (Fig. 7).

4. Discussion

Crayfish farming is a major industry in Louisiana, with a production of about 150 million pounds per year, which implies an annual injection of $300 million into the statés economy (Crayfish production, 2018). Over 125,000 acres are devoted to crayfish culture in the lower basin of Mississippi, being 90% of harvested crayfish represented by *P. clarkii* (Huner and Barr, 1991). Culture of *P. clarkii* in the Mississippi basin is associated with rice crops; rice represents the main source of organic matter to support the detrital-based food-web for crayfish (McClain and Romaire, 2009).

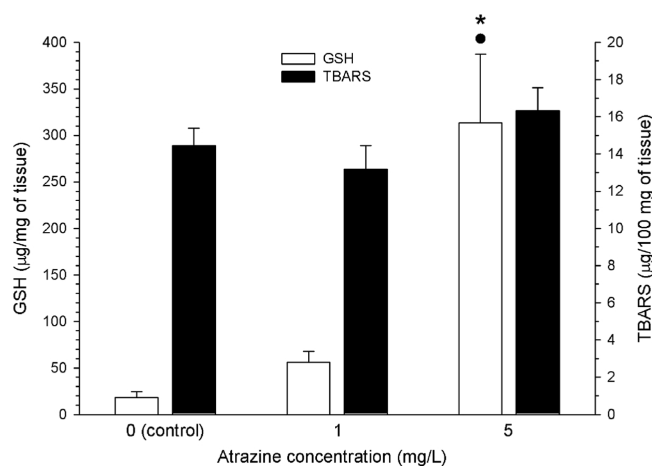

Fig. 7. Glutathione (GSH) and lipid peroxidation (TBARS levels) in hepatopancreas tissue of *P. clarkii*, at the end of the assay. Means ± standard errors are shown. Asterisk and dot indicates significant differences ($p < 0.05$) with respect to control and the lowest concentration, respectively.

Several herbicides, including atrazine, are locally applied to rice crops throughout the Mississippi basin (Coupe et al., 1998). Moreover, atrazine is intensively applied to extensive crop fields having affluents draining to the Mississippi River (Coupe et al., 1998; Moore et al., 2007; Rebich et al., 2004). A two-decade study on the amount of atrazine annually discharged by these affluents into the Mississippi, yielded an atrazine load averaging near 400 tons/year, the highest among all the pesticides detected, peaking this load between May and August (Clark and Goolsby, 2000). This period coincides with the maximum reproductive effort timing of *P. clarkii* (Huner and Barr, 1991). Therefore, studying the effect of atrazine exposure on these crayfish is both ecologically and economically relevant.

Validation of the atrazine concentrations used in our studies indicated a close correspondence between the nominal and measured concentrations. The lowest concentration used represents a possible environmental concentration, in the upper limit of the environmental range reported for this herbicide, i.e., water pollution near treated crops (up to 1 mg/L, Graymore et al., 2001). Although assayed concentrations of atrazine would represent worst cases, determination of their chronic effects to reproductive parameters is not only of interest to the protection of *P. clarkii*, a species of high economic value, but is also of interest for further studies aimed at establishing biomarkers in any other decapods crustacean species, also deepening in the knowledge of how this herbicide can affect crustacean reproduction.

At least two different variables indicated a net decrease in the production of vitellogenin due to atrazine exposure: 1) a significantly lower expression of vitellogenin transcripts in both ovary and hepatopancreas, and 2) a significant decrease in the oocyte area of both previtellogenic and vitellogenic oocytes. Taken together, these results indicate a clear inhibition of ovarian growth in crayfish exposed to atrazine, especially at the highest concentration used. In a previous study using the estuarine crab *N. granulata*, a significant decrease in both ovarian vitellogenin and oocyte area was observed following atrazine exposure (Silveyra et al., 2017). In that study, females were exposed up to 3 mg/L of atrazine during the entire three months pre-reproductive period; in the current study, females were exposed to as much as 5 mg/L, but only during one month. A delay in ovarian rematuration of *N. granulata* with atrazine exposure, during the reproductive period, has also been reported (Álvarez et al., 2015).

In the current study, the presence of both estradiol and testosterone has been noted in the hemolymph ofcontrol animals, either females or males. However, the ratio testosterone/estradiol was quite different between sexes: 3.7 for females compared to 38.5 for males. Similar ratios have been reported in other crayfish species (Mirheydari et al., 2013, 2014). In *P. clarkii* females exposed to 5 mg/L of atrazine, estradiol levels increased up to 2.8-fold over control. Although atrazine has been reported to induce aromatase activity in several vertebrate species, leading to increased estrogen levels (Hayes et al., 2006), to date, no expression of aromatase has been reported in crustaceans (Swevers et al., 1991). Nevertheless, it is possible that some other enzymatic pathways involved in the synthesis of estrogens could be enhanced by the herbicide under study

In early *Cherax quadricarinatus* juveniles crayfish, exposure to atrazine at 2.5 mg/L brought about an increase in the proportion of females undergoing sexual differentiation (Mac Loughlin et al., 2016), possibly due to the increase in circulating estradiol level observed. In this regard, a positive correlation has been observed between circulating levels of progesterone, estradiol, and vitellogenin, in both shrimp and crabs (Coccia et al., 2010; Warrier et al., 2001; Shih and Tseng, 1999; Quinitio et al., 1994). A stimulating effect of 17-hydroxyprogesterone on ovarian growth was also observed *in vitro*, in the estuarine crab *N. granulata* (Zapata et al., 2003). An increment of both estrogen and vitellogenin was observed in females of the shrimp *Neocaridina denticulate* exposed to chlorinated insecticides (Huang et al.,

2004). However, no positive correlation between increased levels of estradiol and vitellogenin was evident in the current study. In fact, while estradiol significantly increased at the highest atrazine concentration, an inhibition in the vitellogenin expression was observed in ovaries and hepatopancreas at the end of the assay.

A possible explanation to such apparent paradox could be that during the first weeks of the experiment, the augmented level of estradiol induced by atrazine was effectively able to stimulate the vitellogenin synthesis (mainly in the hepatopancreas) and its accumulation in the ovary, but towards the end of the assay other inhibiting effect of atrazine on vitellogenin expression could have been manifested. Evidence of these possible counteracting effects are the following: 1) the hepatosomatic index (HI) was significantly higher at either atrazine concentration tested, possibly due to the early stimulating effect of estradiol on the hepatopancreas; 2) even when the vitellogenin expression at the end of the experiment was inhibited, no significant decrease in the gonadosomatic index (GI) and ovarian vitellogenin content was noted, suggesting that an early compensatory accumulation of vitellogenin took place in the ovary; 3) the significant increase in estradiol observed at 5 mg/L correlated with the absence of significant differences in the oocyte area noted at the same concentration.

According to Silveyra et al. (2017), atrazine exposure potentiate the accumulation of vitellogenin in the hepatopancreas of *N. granulata* during the pre-reproductive period, at atrazine concentrations of 0.03 and 0.3 mg/L, but this hormonal induction was lost at 3 mg/L, a concentration very close to the highest assayed in the current study. A similar pattern, but in a temporal scale, appears to have taken place in the current experiment. In other terms, although we have not made any determination at intermediate times, it is quite possible that atrazine could have induced the vitellogenin accumulation in the hepatopancreas and ovary at shorter times of exposure, either by elevating estradiol or by other mechanism, to inhibit vitellogenin synthesis at longer times of exposure, as was verified at the end of the experiment, by a mode of action not related to the estradiol regulation.

In fact, atrazine could have affected the vitellogenin production by several mechanisms. One possibility could be the interference of atrazine with endocrine systems other than that related to sexual steroids; in this sense, methyl farnesoate and several stimulating neurohormones (Nagaraju, 2011; Fingerman, 1997) should be taken into account. Another issue to be considered is the deleterious effects caused by atrazine as a general stressor. This possibility is consisted with our previous hypothesis about the late inhibitory effect of the herbicide, i.e., the more prolonged the exposure to the stressor, the more marked effect on energy reserves depletion. Sokolova et al. (2012) found that chronic stress could lead to the consumption of energy reserves in aquatic invertebrates, namely glycogen, lipids and protein as ultimate resource. In this respect, vitellogenin represents the main protein in mature oocytes, and therefore it may play a role during chronic stress.

It is also interesting to note the significant increase seen in the hemolymph lactate level. This increase indicates that a metabolic effort is taking place, presumably to obtain extra energy required during the exposure to the atrazine. According to Santos and Keller (1993), lactate stimulates the secretion of the crustacean hyperglycemic hormone, proposed as the “stress hormone” of crustaceans (Chang et al., 1999), which leads to a higher delivery of glucose to the hemolymph as a substrate for an increased anaerobiosis. Therefore, although a relative steady level in the glucose hemolymphatic level was observed among treatments, in those animals exposed to atrazine, such balance was probably the net result of increased glucose exportation from the hepatopancreas and an augmented metabolic rate evidenced by the higher lactate production.

Lipid peroxidation (estimated through TBARS levels) seemed to have not been affected. However, GSH levels were strongly increased during the exposure to atrazine. Such increased levels of GSH seemed to have been enough for protecting lipids against the reactive oxygen species typically produced during the oxidative stress. Similarly, several antioxidant enzymes induced in the freshwater prawn *Palaemonetes argentinus* exposed to atrazine, were able to prevent lipid peroxidation (Griboff et al., 2014). Nevertheless, the mixture of atrazine with other pesticides (such as glyphosate) caused both lipid peroxidation and DNA damage in several tissues of clams (Santos and Martínez, 2014).

In summary, our results show that atrazine was able to inhibit vitellogenin production in *Procambarus clarkii* females, via inhibition of its expression in both ovary and hepatopancreas, therefore reducing ovarian growth. On the other hand, atrazine produced higher titters of estradiol, which could have counteracted some of the inhibiting effects mentioned above, particularly in the hepatopancreas. Finally, a metabolic effort (evidenced by the augmented lactate production), together with the increase of glutathione levels were observed, evidencing a clearly stressful effect of the herbicide. The current study represents a contribution to deepen the knowledge of the complex effects that take place by effect of atrazine in a representative decapod crustacean species, which also has a high commercial value as a renewable natural resource.

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