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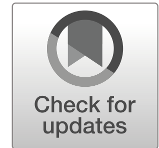
RESEARCH ARTICLE



Interference of an atrazine commercial formulation with the endocrine control of ovarian growth exerted by the eyestalks

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Abstract

Atrazine is currently one of the most used herbicides worldwide. We tested the possible effect of the widely used herbicide atrazine on the endocrine control of ovarian growth exerted by the neurohormones secreted at the eyestalk of the estuarine crab *Neohelice granulata*. For this, both in vivo and in vitro assays were carried out. The in vivo assay comprised the exposure for 1 month to 3 mg/L of a commercial formulation containing 90% of atrazine as active ingredient (Gesaprim 90 WDG®, Syngenta) on three categories of females: intact, ablated of one eyestalk, and ablated of both eyestalks. At the end of the assay, only the intact females showed a significant ($p < 0.05$) decrease in both content of vitellogenic ovarian proteins and proportion of vitellogenic oocytes, compared to a concurrent control. The results of the in vitro incubation of ovarian pieces with the eventual addition to the incubation medium of eyestalk tissue and/or atrazine at 3 mg/L showed a significant ($p < 0.05$) decrease in the proportion of vitellogenic oocytes only when atrazine and eyestalk tissue were added. Taken together, these results strongly suggest that the assayed atrazine formulation may act as an endocrine disruptor at the eyestalk level, by altering the normal secretion of some eyestalk hormone, therefore inhibiting ovarian growth.

Keywords Crustaceans · Eyestalk neurohormones · Ovarian growth · Vitellogenin · Atrazine

Introduction

Atrazine is currently the second most used herbicide in Argentina, after glyphosate (CASAFE 2016), being intensively applied for controlling broadleaf weeds and annual grasses in crops of corn, grain sorghum, and sugarcane (Atanor 2012). Given the processes of runoff and leaching, both atrazine and its metabolites can reach superficial and underground water courses. Although atrazine is a restricted used pesticide in most Western countries (EPA 2017; Pan-Europe 2008), previous studies demonstrate its presence in water bodies at concentrations ranging from 0.1 μg/L, during the period prior to its application (Vonberg et al. 2014, in Germany), to more than 100 μg/L after being applied (USEPA 2002); in water bodies near agricultural areas of the USA and in Australian soil, atrazine concentrations can be as high as 1 mg/L and 100 μg/g, respectively (Graymore et al. 2001). In Argentina, this herbicide is applied at rates that vary between 1 and 2 kg/ha (Atanor 2012), within an area of approximately 10 million ha (Arancibia 2013). Recently, maximum atrazine concentrations ranging from 15.6 to 67.3 μg/L have been reported in rainwater from several Argentine agricultural areas (Alonso et al. 2018; García et al. 2019). Although atrazine is not strongly absorbed to sediments, the fraction associated with this substrate can be very significant and can affect the ecosystem (Jablonowski et al. 2011; Singh et al. 2017).

The crab *Neohelice granulata* (Decapoda, Brachyura) is a crustacean species widely distributed in estuarial environments of Argentina and Brazil, being particularly abundant in the coast of Samborombón Bay (Argentina). Several rivers and channels extending through extensive areas dedicated to agriculture flow to this bay. Due to its wide distribution and its relevant ecological role (both larvae and adult crabs are predated by several fish species), this species has been taken

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as a model species for several ecotoxicological studies (reviewed by Rodríguez et al. 2019). During the pre-reproductive period (winter), a progressive ovarian growth is verified in the females, prior to the reproductive, spawning season (López Greco and Rodríguez 1999). In females of decapod crustaceans, vitellogenesis is a key process for ovarian growth, involving the synthesis of essential lipoproteins for the development of the embryo. The major component of this nutrient material is given by the vitellins derived from a glycoprotein complex lipoprotein known as vitellogenin (Vg) (Tsukimura 2001; Huberman 2000; Charmantier et al. 1997), which constitutes the precursor component of yolk, an essential reserve substance for the subsequent development of embryos (Wilder et al. 2010).

The main endocrine regulatory component of decapod crustaceans is the neuroendocrine complex known as the "X-organ-sinus gland complex" located at the eyestalks (Rodríguez et al. 2007; Fingerman 1997). In the sinus gland, several neurohormones that regulate processes such as molting, dispersion of pigments, and reproduction are stored and secreted; one of these neurohormones, the gonadal-inhibiting hormone (GIH), inhibits gonadal growth (Fingerman 1997). Although one relevant target of the GIH might be the extraovarian sites of Vg synthesis (the hepatopancreas, in decapod crustaceans), there is strong evidence that this hormone also acts directly on the ovary, regulating mechanisms of both endogenous synthesis of yolk and its intake from the hemolymph, which in turn transport the Vg produced and secreted by the hepatopancreas (Wilder et al. 2010; Van Herp 1993; Charniaux Cotton 1985; Lee and Walker 1995).

Previous studies have reported some reproductive alterations due to the exposure to either pure atrazine or its commercial formulations, demonstrating the ability of this herbicide to act as an endocrine disrupter. In fish and other vertebrates, it has been reported that atrazine inhibits gonadal maturation (Tillitt et al. 2010), interferes with the hypothalamic control of pituitary hormones, and induces the activity of the aromatase enzyme (McKinlay et al. 2008). In the particular case of crustaceans, it has been shown that this herbicide antagonizes the effect of juvenoid hormones (Palma et al. 2009) and alters sexual differentiation in species of cladocerans such as *Daphnia* sp. (Dodson et al. 1999) and also in the crayfish *Cherax quadricarinatus* (Mac Loughlin et al. 2016). In the studied species, a delay in ovarian rematuration of ovigerous females exposed to formulated atrazine was observed during the reproductive period, as well as several malformations in the hatched larvae (Álvarez et al. 2015). A further study conducted on *N. granulata* non-ovigerous females exposed to atrazine during the pre-reproductive period also showed a lower area of both previtellogenic and vitellogenic oocytes, together with a diminished Vg content in the ovary (Silveyra et al. 2017), therefore confirming the delay in ovarian growth already seen in ovigerous females, mentioned previously. From these results emerged the general hypothesis about the atrazine acting as an endocrine disruptor, on some hormonal system controlling ovarian growth.

In this context, the present study aimed at specifically testing the possible interference of formulated atrazine with the endocrine control of ovarian growth carried out by the eyestalks. For this purpose, both in vivo and in vitro tests were carried out, using intact and eyestalk-ablated animals.

Materials and methods

In vivo assay

Adult females of *N. granulata* with an average weight of 11.60 ± 0.03 g ($N = 72$) were collected at Punta Rasa, which has been considered a relatively pollution-free area (Comisión Administradora del Río de la Plata 1990). Once in the laboratory, the animals were acclimated for 2 weeks to the same environmental conditions to be used in the in vivo assay, which lasted 1 month (June 16 to July 14), within the pre-reproductive period of the species. Previous assays carried out with the species under study and others related showed a significant ovarian growth during the experimental period considered for the current assay (Canosa 2019; Silveyra et al. 2018).

A nominal concentration of 3 mg/L of atrazine was tested as an active ingredient of the commercial formulation Gesaprim 90 WDG® (Syngenta), which contains 90% (w/w) of the active ingredient. In order to validate the nominal concentration used, water samples (15 mL) were taken at 0 h and 72 h, i.e., the period for water replacement in all test containers, to be further analyzed at the CIMA (La Plata University, Argentina). After filtering samples through a 0.45-μm nylon membrane, filtrates were analyzed by high-pressure liquid chromatography (HPLC) coupled to mass spectrometry (Agilent®, model VL, quadruple pole). A XSelect C18 column was used, using as mobile phase a mixture of acetonitrile:formic acid (0.1%) at 0.5 mL/min. Ions were generated in a ESI source, in positive mode, selective ion monitoring (SIM), for both protonated ions and the fragments derived from the isotopic relation of chlorine m/z = 216, 218, 174, and 176. An isotopic tracer of atrazine (^{5}D) was used as a control of analytical quality, according to Taylor (2005), following its characteristic ions m/z = 221 and 223. An external standard was used for quantification, at the same conditions used for samples. Recovery percentage was > 95%, and the detection limit was 0.12 μg/L.

The experimental design comprised the following treatments:

1. I-Ctrl: intact females, with no herbicide added
2. I-Atz: intact females exposed to 3 mg/L of the commercial atrazine formulation
3. UNI-Ctrl: females with ablation of one eyestalk (uni-ablated crabs), with no herbicide added
4. UNI-Atz: uni-ablated females, exposed to 3 mg/L of the commercial atrazine formulation
5. BI-Ctrl: females with ablation of both eyestalk (bi-ablated crabs), with no herbicide added
6. BI-Atz: bi-ablated females, exposed to 3 mg/L of the commercial atrazine formulation

In addition, ten crabs were sacrificed from the stock, just after the acclimation period and before the ablation, to serve as an initial control for all the treatments mentioned previously. For the ablation procedure, the animals were anesthetized by completely immersing them in an ice bath at 0 °C for 5 min, to further cut the eyestalks at their joint with the cephalothorax, by means of scissors and dissecting forceps. Once the ablation was done, the wound was cauterized by means of a heated metal tip. Two days later, 10 to 12 crabs, intact or eyestalk-ablated, were randomly assigned to each treatment, isolating each animal in a glass recipient of 1 L capacity, containing 400 mL of saline water (12 g/L, pH = 7.8), prepared from artificial salts for seawater (Tetra® Marine Salt Pro) and dechlorinated tap water (hardness 80 mg/L as equivalents of calcium carbonate), under constant aeration. During the 30 days of testing, the photoperiod was maintained at 14:10 (L:D) and the temperature at 25 ± 1 °C. Dissolved oxygen was always > 80% of saturation, while ammonia levels were < 0.2 mg/L; pH varied in 0.5 unit as much. Every 72 h, the animals were fed ad libitum with pellet food prepared in the laboratory (Chaulet et al. 2012), supplemented with fresh leaves of *Elodea* sp.; besides, water from all the containers was completely replaced (semi-static design). The animals that molted or died (absence of any movement after mechanical stimulation) were recorded daily.

At the end of the assay (July 14, 10 a.m.), the fresh weight of each animal was measured (accuracy ± 0.01 g). All animals were in intermolt, according to the criteria used previously for this species (Rodríguez Moreno et al. 2003); they were then (12 a.m.) cold-anesthetized, and the ovary of each female were dissected and weighed in analytical balance (accuracy ± 0.0001 g); a portion of each ovary was stored at − 20 °C for subsequent determination of vitellogenic protein content, while another portion of each ovary was fixed in Bouin solution, for histological analysis.

In vitro assay

In order to provide additional evidence about the possible disruptive effect of atrazine on the endocrine control exerted by eyestalk hormones on the ovarian growth of *N. granulata* and taking also into account that the ovary of crabs is able to synthesize vitellogenic proteins (Lee and Watson 1995; Tsukimura 2001; Li et al. 2006), some ovarian pieces were incubated with or without the addition of eyestalk tissue and/or atrazine. As in previous in vitro studies made with ovarian pieces from *N. granulata* and other crab species, a 24-h incubation period was followed, during which a significant ovarian growth takes place (Eastman-Reks and Fingerman 1985; Rodríguez et al. 2000; Zapata et al. 2003; Medesani et al. 2004; Canosa et al. 2018, among others). Because the histological analysis is giving more detailed information than that provided by the analysis of the total Vg content, the former was employed for this in vitro assay.

The source and the dissection procedure of the animals were the same as those described for the in vivo tests. Once weighed, each ovary was cut into four pieces (weighing from 0.1 to 0.3 g), each female ($N = 12$) providing a similar piece of ovary to each of the treatments (random block design). Each piece of ovary was placed in a well of a 12-well sterile culture plate (Nest Biotech Co., Ltd.®). Each well was previously filled with 2 mL of culture medium M199 (Sigma Chemical Co®), dissolved in a saline solution for crustaceans (Cooke et al. 1977) modified for the species under study (Medesani et al. 2015). The plates were incubated for 24 h in a culture chamber randomizing their position and under controlled conditions of humidity and temperature (25 ± 1 °C), in constant darkness and with a controlled atmosphere of 5% CO_2.

For the in vitro assay, the same commercial formulation used for the in vivo assay was tested; the experimental design comprised the following experimental groups:

1. Control: only ovary added
2. Atz: ovary + 3 mg/L atrazine
3. ET: ovary + eyestalk tissue
4. Atz + ET: ovary + 3 mg/L atrazine + eyestalk tissue

For each female used, group number 3 received one of its eyestalks, while group number 4 received the contralateral eyestalk of the same female, following the aforementioned random block design used for the assignment of the ovarian pieces. At the end of the incubation period, all ovarian pieces were fixed in Bouin solution for histological analysis. Additionally, a fifth portion of ovary from each crab used in this assay was also fixed at the beginning of the assay (time zero) to serve as initial control.

Total content of vitellogenic proteins

The total content of vitellogenic proteins (vitellogenin and vitellins) was determined in ovary samples from the in vivo assay by means of the immunoenzymatic ELISA technique, according to the methodology detailed in previous studies carried out in the species under study (Ferré et al. 2012; Silveyra et al. 2017). Briefly, 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), also in accordance with the code of ethics for animal experiments stated out by the Declaration of Helsinki. Anti-IgG from rabbit was conjugated with BIOARS Lab. An anti-rabbit conjugated to peroxidase was used as the secondary antibody.

Ovaries were cut into small explants (0.1–0.2 g), which were homogenized in sodium phosphate buffer (50 mM, pH 7.4, with 2 μL/mL of protease inhibitor), in a 1:3 (w/v) ratio. Each homogenate was then centrifuged at 10,000*g* for 25 min at 4 °C. Supernatants were further ultracentrifuged (100,000*g* for 50 min, at 4 °C); the resulting supernatants were stored in Eppendorf tubes and frozen at − 70 °C until analysis by ELISA. To this, supernatants were finally diluted (1:3 v/v) in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH = 9.6). On the other hand, Vg purified according to Ferré et al. (2012) was diluted 1/500 (w/v) in coating buffer in order to prepare the standard curve by further dilution in the same buffer (six concentrations between 0 and 1.2 ng/μL). Fifty microliters of either standard or diluted samples was finally placed, in triplicate, in a 96-well plate (Nunc-Immunoplate Polisorp). Both primary and secondary antibodies were diluted (1/500) in PBS 1× + 0.05% Tween + 6% powder milk. Following incubation, absorbance was measured in all wells at 420 nm, by using a plate reader (FLUOstar OPTIMA-BMG Labtech., microplate reader); 2-20-azino-di-3-ethylbenzthiazoline sulfonic acid was used as chromogen. For each animal, Vg content was referred to the whole ovary.

Histological analysis

After performing fixation and dehydration in alcohols of increasing graduation, the pieces were included in Paraplast® and sections of approximately 5 μm to 8 μm in thickness were carried out, using an ultramicrotome rotation RM2125 RTS (Leica®). Finally, the sections were stained with hematoxylin-eosin. For each animal, a representative portion of each section of the ovary was selected in order to determine the relative proportion of the different oocyte types, under an optical microscope. The oocytes were characterized as pre-vitellogenic, intermediate, or vitellogenic according to their size and degree of basophilia (Silveyra et al. 2017). To evaluate the proportions, three sections were analyzed in each cut of the ovary of each female, using a 100-dot grid mounted on an × 8 eyepiece combined with a × 40 objective lens.

Statistical analysis

In order to compare the measured variables among experimental groups, a two-way ANOVA (taking atrazine concentration and kind of ablation as factors) was applied for the in vivo assay, while a randomized block design ANOVA was employed for the in vitro assay, according to the experimental design mentioned previously. ANOVA was always followed by multiple LSD comparisons (Sokal and Rohlf 1981). In case the assumptions of equality of variances were not verified ($p > 0.05$, Levene test), the variances were modeled using the nmle package in R software (version 3.0.5) (Pinheiro et al. 2013), following the maximum likelihood estimation method. The percentages of mortality, molting, and ovigerous females were compared among treatments (as proportions, based on absolute frequencies) by Fisher's exact test (Sokal and Rohlf 1981). In all cases, the confidence level of 95% was considered.

Results

In vivo assay

The nominal atrazine concentration used was very close to that determined by HPLC, which ranged between 2.93 and 2.80 mg/L at 0 h and 72 h, respectively. Regarding mortality, in the case of bi-ablated animals, a significant mortality ($p < 0.05$) was observed in comparison with intact animals, regardless of whether or not they were exposed to the herbicide; the same was observed in relation to the percentage of molting (Table 1). At the end of the assay, no statistically significant differences ($p > 0.05$) were observed among the different treatments, with respect to the gonadosomatic index (Table 1).

With respect to the content of vitellogenic proteins determined at the end of the in vivo experiment, a significant interaction ($p < 0.05$) between factors was noted. Thereby, the intact females that were exposed to the atrazine commercial formulation showed a significant ($p < 0.05$) lower Vg content in the whole ovary compared to the concurrent control group, but a similar ($p > 0.05$) Vg content than that showed by the initial control (Fig. 1). On the other hand, no significant differences ($p > 0.05$) were seen in the content of ovarian vitellogenic proteins between uni- or bi-ablated females exposed to atrazine and their respective concurrent controls. Besides, in the case of bi-eyestalkless crabs, both atrazine-exposed and concurrent controls had a significant ($p < 0.05$) higher Vg level than that of the initial control (Fig. 1).

Figure 2 shows the different types of oocytes found in the histological analysis, from samples taken at the end of the in vivo assay. Pre-vitellogenic oocytes were identified by their smaller size and marked basophilia (stained with hematoxylin), vitellogenic oocytes showed the highest size and a clear eosinophilia (stained with eosin), while intermediate oocyte represents a transitional stage between those two. Besides, some reabsorbed oocytes were seen in most treatments, but not in intact controls. These reabsorbed oocytes implied the

Table 1 Mortality and molting percentages and gonadosomatic index (mean ± standard error) at the end of the in vivo assay

Experimental group	Initial *N*	Mortality (%)	Molting (%)	Gonadosomatic index	Final *N*
I-Ctrl	10	0	0	0.85 ± 0.11	10
I-Atz	10	0	0	0.45 ± 0.05	10
UNI-Ctrl	12	8.3	8.3	0.43 ± 0.06	11
UNI-Atz	12	25	8.3	0.59 ± 0.18	9
BI-Ctrl	12	41.7 *	83.3 *	1.38 ± 0.34	7
BI-Atz	12	41.7 *	83.3 *	0.74 ± 0.12	7

Asterisks indicate significant ($p < 0.05$) differences with respect to the intact (I) control (Ctrl) crabs; Atz = atrazine (3 mg/L); UNI and BI = crabs uni- and bi-ablated of eyestalks, respectively; *N* = number of crabs

participation of follicular cells in order to phagocyte the vitellum.

A significant interaction ($p < 0.05$) between factors was also observed for the histological analysis. In the intact females exposed to the commercial formulation of atrazine, an increase in the proportion of both pre-vitellogenic and intermediate oocytes was observed, together with a significant ($p < 0.05$) decrease of around 60% in the proportion of vitellogenic oocytes, in comparison with the respective concurrent control (Fig. 3). Compared to the initial control, the intact crabs exposed to atrazine showed no significant ($p > 0.05$) differences in the proportion of vitellogenic oocytes; additionally, a slight increase in oocyte reabsorption was also seen by the effect of atrazine (Fig. 3). Although in the uni-ablated females exposed to the commercial formulation, a significant ($p < 0.05$) increase in the proportion of pre-vitellogenic oocytes was observed with respect to the corresponding concurrent control, the concomitant decrease in the vitellogenic oocyte proportion (40% less than that of concurrent control) was not statistically significant ($p > 0.05$). Concerning the bi-ablated females exposed to the atrazine commercial formulation, the proportions of both pre-vitellogenic and vitellogenic oocytes were statistically similar ($p > 0.05$) to those of the respective concurrent control group; in fact, only a decrease of about 20% in the proportion of vitellogenic oocytes was observed in the exposed females, in comparison with the concurrent control group (Fig. 3). In all eyestalkless crabs, though, both the concurrent control and the atrazine-exposed groups had a significantly ($p < 0.05$) higher proportion of vitellogenic oocytes than that showed by the initial control (Fig. 3).

In vitro assay

Figure 4 shows the proportions of the different types of oocytes at the end of the 24-h in vitro incubation. Compared to the initial control, the concurrent control showed a significant ($p < 0.05$) increment in the proportion of vitellogenic oocytes, revealing that an effective growth of the ovarian pieces took place during the 24-h incubation period. On the other hand, only the ovarian pieces incubated in the presence of the formulated herbicide and one eyestalk showed a significantly ($p < 0.05$) lower proportion of vitellogenic oocytes than the concurrent control, while no significant differences with control ($p > 0.05$) were seen in the remaining proportions.

Discussion

In the present study, we tested the possible effect of the widely used herbicide atrazine on the endocrine control of ovarian growth exerted by the neurohormones secreted at the eyestalk of the estuarine crab *Neohelice granulata*. The nominal concentration of atrazine presented in the commercial formulation used was very close to that determined analytically, at either 0 h or 72 h of exposure. Regarding mortality and molting during the assay, only a significant incidence ($p < 0.05$) was detected in the bi-ablated animals. This was a result expected a priori, since the eyestalk ablation involves the removal of several neurohormones, which regulate different aspects of metabolism, as well as molting and reproduction (Fingerman 1997). The elimination of the eyestalk neurohormone molt-inhibiting hormone (MIH) is expected to trigger the molt, as has been observed in previous studies with both females and males of *N. granulata* bi-ablated of eyestalks, at different periods of the reproductive cycle (Stella et al. 2000). On the other hand, the elimination of the GIH source in the bi-ablated animals produced, as expected, an increase in both the vitellogenic protein content and the proportion of vitellogenic oocytes, in comparison with intact animals.

According to Silveyra et al. (2017), formulated atrazine (Gesaprim 90®) assayed in vivo was able to reduce the ovarian growth of *N. granulata* females at 3 mg/L, but not at lower concentrations (0.03 mg/L and 0.3 mg/L), even after an exposure period of 3 months. Similarly, in the current study, the ovary of intact females exposed in vivo for 1 month to 3 mg/L of atrazine as an active ingredient showed a significantly lower content of vitellogenic ovarian proteins, in comparison with non-exposed intact females serving as concurrent control; moreover, the vitellogenic protein level of atrazine-exposed crabs was similar to that of the initial control. These results had a correlation in the histological analysis, since a significant decrease of around 60% was observed in the proportion

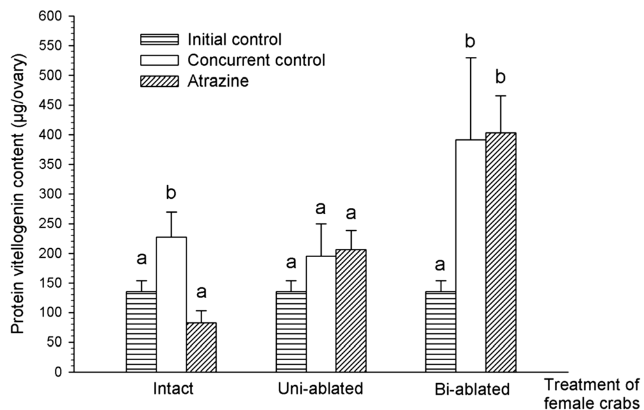

Fig. 1 Vitellogenic protein content (µg/ovary) in the ovary of females non-exposed (concurrent control) or exposed to a commercial formulation containing 3 mg/L of atrazine, at the end of the in vivo 30-day assay. The values are expressed as the mean ± standard error. For each treatment, different letters indicate significant differences ($p < 0.05$)

of vitellogenic oocytes in intact females exposed to atrazine, concomitantly with an increase in the proportion of pre-vitellogenic and intermediate oocytes. At the same time, no differences were noted in the proportion of vitellogenic oocytes between the atrazine-exposed intact females and the initial control, therefore highlighting that the herbicide is arresting the ovarian growth of *N. granulata*. Although we are unable to state out that these effects were caused exclusively by atrazine as the active ingredient of the formulation used, several preliminary assays showed that pure atrazine caused similar results that the formulation Gesaprim 90® (containing 90% w/w of atrazine), on the ovary of the studied species (Silveyra 2019).

On the other hand, females with ablation of one or both eyestalks and exposed to the 3 mg/L of formulated atrazine for 1 month did not present differences in the vitellogenic protein content, with respect to their respective concurrent control groups. Concerning the proportion of vitellogenic oocytes, females ablated of one or both eyestalks and exposed to the atrazine commercial formulation neither showed differences with respect to their respective concurrent controls. Besides, the concurrent control group of either uni- or bi-eyestalkless crabs significantly increased the proportion of vitellogenic oocytes as compared to the initial control, and also did the bi-eyestalkless crabs exposed to atrazine. Taken together, the results obtained with both intact and eyestalkless crabs are indicating that the effect of atrazine on the ovary may be caused through imbalances at the eyestalk level.

According to Charmantier et al. (1997), the growth of oocytes in the ovary is mainly determined by the gradual decrease in GIH, synthesized and secreted by the sinus gland located at the eyestalks; therefore, if the secretion of this

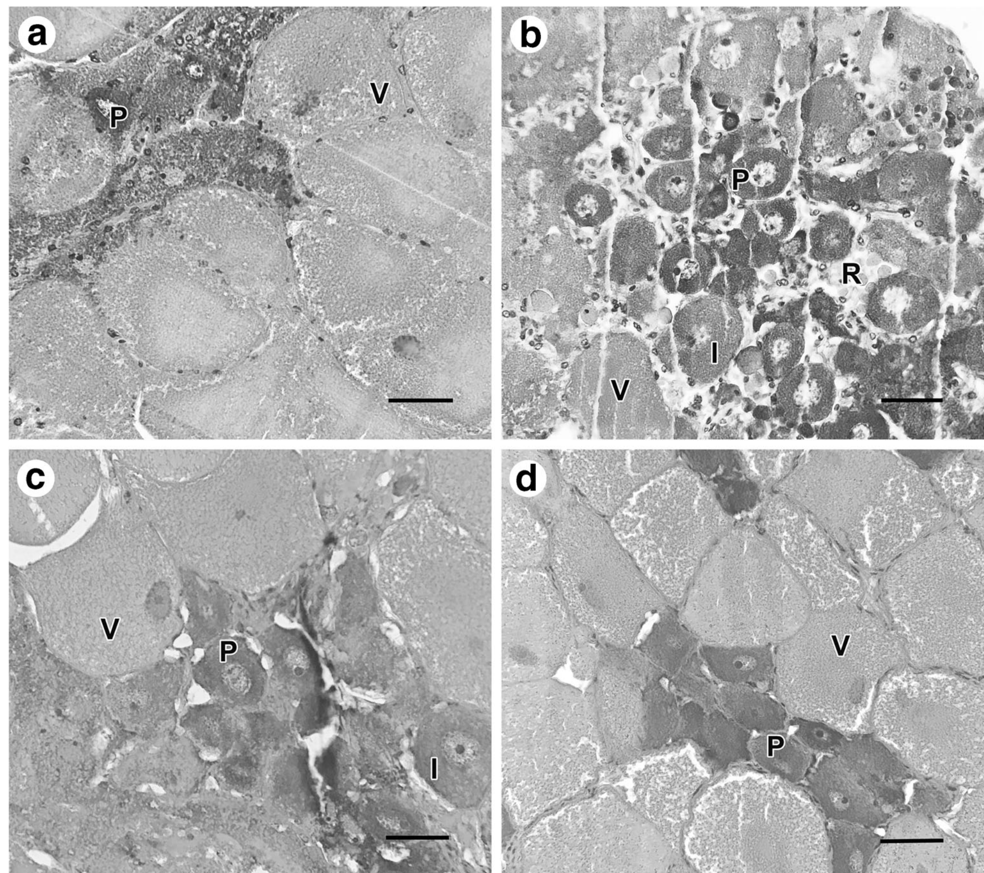

Fig. 2 Histological sections of the ovary, showing the different kinds of oocytes observed in each treatment. **a** Intact control. **b** Intact crabs exposed to a commercial formulation containing 3 mg/L of atrazine. **c**, **d** Uni- and bi-eyestalkless control crabs. P, previtellogenic oocytes; I, intermediate oocytes; V, vitellogenic oocytes; R, reabsorbed oocytes. Scale bars = 50 µm

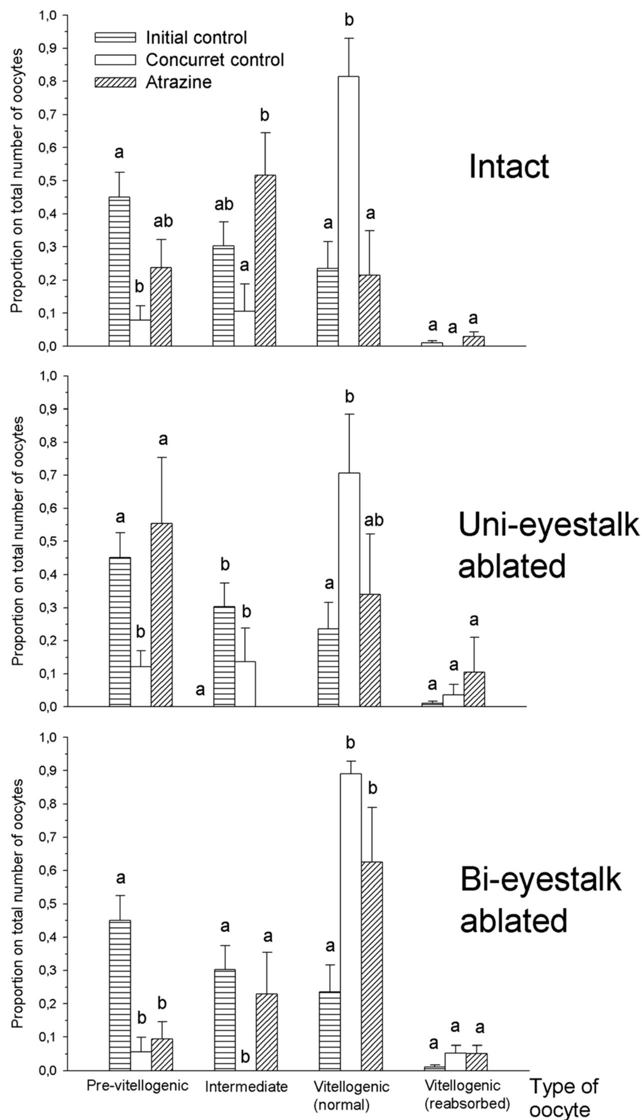

Fig. 3 Relative proportion of the different types of oocytes in the ovary of females non-exposed (control) or exposed to a commercial formulation containing 3 mg/L of atrazine, at the end of the in vivo assay. The values are expressed as the mean ± standard error. For each type of oocyte, different letters indicate significant differences ($p < 0.05$). The absence of any histogram indicates a proportion of zero

hormone increases when the ovary is in the process of vitellogenesis, the reduction in the proportion of vitellogenic oocytes in intact females could be explained and possibly also the increase in the proportion of vitellogenic oocytes in reabsorption, as suggested by Avigliano et al. (2018). Oocyte reabsorption may also be caused by CHH (hyperglycemic hormone of crustaceans), which is synthesized and secreted by the eyestalks and the retina (Fanjul-Moles 2006), and which has been considered by several authors as the “stress hormone” of crustaceans, a physiological equivalent to cortisol of vertebrates (Manfrin et al. 2016; Chang 2005).

In an in vivo context, the possible abnormal increase of GIH, besides inhibiting directly the ovarian growth of intact

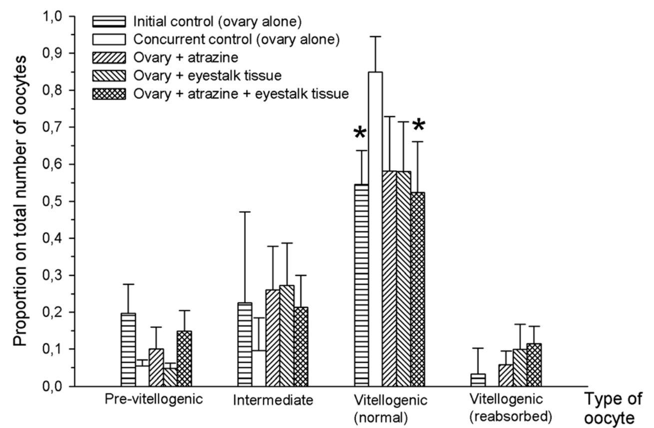

Fig. 4 Relative proportion of the different types of oocytes in ovarian pieces incubated in vitro and exposed for 24 h to a commercial formulation containing 3 mg/L of atrazine. The values are expressed as the mean ± standard error. The asterisks indicate significant differences ($p < 0.05$) with respect to the concurrent control group. The absence of any histogram indicates a proportion of zero

animals exposed to the herbicide, could also decrease the secretion of other hormones stimulating gonadal growth, such as the *gonad-stimulating hormone* (GSH, secreted by the thoracic ganglion and brain) or sex steroids. In this regard, it has been proposed that GIH could inhibit the secretion of GSH from the nervous system and also the secretion of sex steroids by the ovary (Subramoniam 2016; Nagaraju 2011; Charmantier et al. 1997). On the other hand, the secretion of methyl farnesoate is inhibited by the eyestalk hormone mandibular organ–inhibiting hormone (MOIH), another neuropeptide from the same family as GIH and CHH (Fanjul-Moles 2006; Van Herp 1998), whose secretion could also be enhanced by atrazine, thereby decreasing the circulating levels of methyl farnesoate, a juvenoid hormone that has been shown to stimulate ovarian growth (Nagaraju et al. 2006; Rodríguez et al. 2002). Finally, it should be considered that not only the hormonal secretion pathways are affected, but eventually, the degradation and/or transduction pathways of the hormones involved in the vitellogenesis process could also be altered by exposure to the atrazine formulation.

The results of the in vitro assay showed that the proportion of vitellogenic oocytes was significantly reduced only in those ovarian pieces that were incubated in the presence of eyestalk and atrazine. These results support the hypothesis that formulated atrazine, instead producing a direct effect on the ovary, is acting on the eyestalks to increase the secretion of GIH or any other related eyestalk peptide with inhibitory effects on the ovarian growth; alternatively, a lower secretion of some eyestalk peptide with stimulatory effects on the ovary, such as a particular CHH isoform (De Kleijn and Van Herp 1998; Fanjul-Moles 2006) may be occurring. Although a relevant target of the GIH or other related peptides might be the extraovarian sites of Vg synthesis (in decapod crustaceans, mainly the hepatopancreas), there is strong evidence

indicating a direct effect of those eyestalk peptides on the ovary, inhibiting the uptake of extraovarian Vg by oocytes and also the synthesis of Vg by the ovary itself (Wilder et al. 2010; Tsukimura 2001). The results obtained in vitro were also consistent with those observed in vivo in the intact animals exposed to atrazine, which showed a lower ovarian growth, an effect that was not observed in the animals without eyestalks.

From the results obtained both in vivo and in vitro, we conclude that commercial formulation of atrazine assayed may be acting as an endocrine disruptor, by interfering with the normal secretion of eyestalk hormones during the pre-reproductive period, therefore inhibiting the ovarian growth needed to warrant a successful spawning during the reproductive period of the studied species.

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