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Effects of atrazine on ovarian growth, in the estuarine crab *Neohelice granulata*

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ABSTRACT

Atrazine, a herbicide that is intensively used in Argentina, was assayed to evaluate the alteration of reproduction in a wild species of crustaceans. Adult females of the estuarine crab *Neohelice granulata* were exposed to formulated atrazine during the 3-month pre-reproductive period. Three atrazine concentrations (0.03, 0.3 and 3 mg/L) were assayed, together with a water dilution control. At the end of the exposure period, several variables concerning the energetic status of animals were measured, such as weight gain, glycemia, and energy reserves in both muscle and hepatopancreas. The gonadosomatic index (GSI) was also determined, as well as the proportion and relative area of each oocyte type in histological sections. Besides, the total content of vitellogenin proteins (Vg) in both ovary and hepatopancreas was measured. A significant ($p < 0.05$) decrease of glycogen content was observed in muscle, while a significant ($p < 0.05$) lower area of both previtellogenic and vitellogenic oocytes was verified in the ovary by effect of atrazine, in correspondence with a Vg content significantly ($p < 0.05$) diminished in the ovary and augmented in the hepatopancreas. Besides, a higher proportion of previtellogenic oocytes were seen by effect of atrazine. Taken together, these results indicate a clear reduction and delay in the ovarian growth of the studied species, during the period at which the ovary normally grows up prior to spawning. On the other hand, a decrease of Vg content was observed at 0.3 and 3 mg/L of atrazine after 24-h *in vitro* assays carried out with ovarian explants, providing additional evidence about the inhibitory role of this herbicide on the ovarian growth. These results encourage future research on deleterious effects of atrazine on crustacean reproduction.

1. Introduction

Atrazine is a worldwide spread herbicide, used for inhibiting foliar growth in plants due to its capacity to interfere with the photosystem II. The utilization of this herbicide in South America, particularly in countries such as Argentina (Arancibia, 2013) and Brazil (Dos Santos and Martinez, 2014) has increased over time. In Argentina, this herbicide is applied to corn and sorghum crops, among others, with application doses varying between 1 and 2 kg/ha (Atanor, 2012) through an area of approximately 10 million ha (Arancibia, 2013). In water, atrazine half-life has been reported to be higher than 60 days, ranging its environmental levels 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 was found at concentrations as high as 1 mg/L (Graymore et al., 2001). Although this herbicide is not strongly absorbed to sediments, the fraction associated to this substrate can be very significant (Jablonowski et al., 2011).

The crab *Neohelice* (= *Chasmagnathus*) *granulata* (Decapoda, Grapsidae) is a crustacean species widely distributed through most estuarine environments of Argentina. Along the 150 km of the Samborombón Bay coast (Buenos Aires Province) this species forms dense populations, serving as prey for several fish species that reproduce in the Bay (Sánchez et al., 1991). This estuarine environment receives the discharge of several rivers and channels carrying significant amounts of herbicides applied to the surrounding crops, although the southern edge of Samborombón Bay (Punta Rasa), located at the mouth of the Rio de la Plata estuary and therefore open to the sea, has been considered a relatively clean zone concerning pesticides (Comisión Administradora del Río de la Plata, 1990).

In *N. granulata*, the pre-reproductive period takes place during winter, allowing an active growth and maturation of the ovary. During this period, in addition to the production of vitellogenin by the oocytes themselves (characteristic of the primary vitellogenesis), the ovary enters into the secondary vitellogenesis to significantly grow by uptake of vitellogenin synthetized in the hepatopancreas (Charmantier et al., 1997, Nagaraju, 2011). The reproductive season of *N. granulata* comprises the spring and summer months, involving both spawning and hatching, and eventually the ovarian re-maturation needed to produce

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Table 1
Measured concentrations of atrazine in the experimental series.

Nominal atrazine concentration (mg/L)	Time (h)	Measured concentrations (mg/L)
0 (control)	0	0.0000
	72	0.0000
0.03	0	0.0220
	72	0.0687
0.3	0	0.3540
	72	0.2150
3	0	2.9254
	72	2.8010

further spawns (López Greco and Rodríguez, 1999). After hatching and molting to several larval instars, the megalopa returns to the coast, to reach the reproductive, adult stage after several molts (López Greco and Rodríguez, 1999). Finally, a post-reproductive period is verified during autumn, when molting of the adults occurs, remaining the ovary quiescent.

A delay in the ovarian re-maturation of *N. granulata* ovigerous females has been previously reported by effect of atrazine, during the reproductive period (Álvarez et al., 2015). In *Daphnia sp.*, this herbicide showed to antagonize the effect of juvenoid hormones (Palma et al., 2009), as well as to alter sexual differentiation (Dodson et al., 1999). In fish and other vertebrates, atrazine was able to inhibit gonadal maturation (Tillitt et al., 2010), by interfering with the hypothalamic control of pituitary hormones and also by inducing aromatase activity in gonads (McKinlay et al., 2008). Moreover, in mammalian cell cultures, atrazine has been shown to act as xenoestrogen (Villeneuve et al., 1998; Lascombe et al., 2000).

The objective of this study was to evaluate the effect of atrazine on both the ovarian growth and some metabolic parameters related to the ovarian maturation, during the entire 3-month pre-reproductive period of *N. granulata*. In addition, some *in vitro* assays were carried out with ovarian explants, in order to test the direct effect on atrazine on the ovary.

2. Materials and methods

Adult females of *N. granulata* were randomly collected in June 2014 (body weight = 10.86 ± 0.08 g, N = 60), at the southern edge of Samborombón Bay. All bioassays were conducted in semi-static conditions according to the standard procedures recommended by the American Public Health Association et al. (2005).

2.1. In vivo assays

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. Small aliquots from these stock solutions were added to the test recipients, filled with the appropriate dilution water, in order to obtain the following concentration series of atrazine as active principle: 0.03, 0.3 and 3 mg/L. A water dilution control was also run. Fifteen females were assigned to each atrazine concentration or control. In all experiments, each female was isolated in a glass container filled with 400 mL of saline water prepared by diluting artificial seawater salts (Tetra Marine Salt Pro, US) in dechlorinated tap water (hardness: 80 mg/L as equivalents of $CaCO_3$; final salinity, 12 g/L, pH = 8.0 ± 0.5) provided with constant aeration. The solution of each container was completely replaced twice a week. No changes in water quality parameters were detected just before replacing any test solution. A temperature of 23 ± 1 °C and a photoperiod of 14:10 (L:D) were maintained throughout. All animals were fed twice a week with pellets prepared in the laboratory (according to Chaulet et al., 2012) in an amount equivalent to 5% of body mass, supplemented with *Elodea* sp. fresh leaves *ad libitum*. Females remained exposed to atrazine or control for 90 days, starting by early July.

In order to validate nominal concentrations of atrazine, water samples (15 mL) were taken at 0 and 72 h, *i.e.*, the period for water replacement in all test containers. After filtering samples through 0.45 μm nylon membrane, filtrates were analyzed by high liquid pressure chromatography (HPLC) coupled to mass spectrometry (Agilent®, model VL, quadruple pole). A X-SELECT 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, monitoring the selective ion (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, following its characteristic ions m/z = 221 and 223. An external standard was used for quantification, at the same conditions used for samples.

At the end of the assay, females were weighed to determine body weight (BW); animals were then sacrificed after anesthetizing them in ice water, and both ovaries and hepatopancreas were dissected and weighed in order to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index as GSI or HSI = (GW or HW) / BW) × 100, where GW and HW are the gonad and hepatopancreas wet weight, respectively. Ovaries were then fixed in Bouin solution for 4 h at room temperature, dehydrated in alcohol series and finally embedded in paraplast. Then, 5-μm sections were prepared and stained with haematoxylin and eosin. For each animal, representative sections of the ovary were analyzed to determine both the relative proportion and the area of each kind of oocytes.

Previtellogenic, intermediate and vitellogenic oocytes were characterized according to their size and degree of basophilia. Each type of oocyte, including those abnormal, was systematically counted in at least three ovarian sections from each. For oocyte area, both major and minor diameters of the oocytes showing their nuclei were estimated by means of a micrometric ocular lens, calibrated against a Leitz Wetzlar plate with 1/100 mm spacing, to calculate the oocyte area as (π/4) × major diameter × minor diameter, as in Rodríguez et al. (1994). In order to control biological variability, only the non-ovigerous females were considered for all the estimated variables. In order to express gonadal maturity by means of an integrated index, the percentage content of vitellogenin allocated in vitellogenic oocytes was calculated by multiplying the proportion of vitellogenic oocytes by their mean area, over the sum of the proportion by the mean area of all kind of oocytes measured.

An ELISA assay was employed to determine the total content of vitellogenic proteins (Vg: vitellogenin and vitellins) in ovary, hepatopancreas and hemolymph. 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, conjugated with BIOARS Lab. An anti-rabbit conjugated to peroxidase was used as the secondary antibody. Purified Vg in a 1/500 dilution was used to prepare the standard (0–210 ng). Fifty microliters of either the standard or sample were placed, in triplicate, in a 96-wells plate (Nunc-Immunoplate Polisorp). Samples were previously diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH = 9.6). Both primary

Table 2
Survival, molting and spawninsg of females at the end of the experiment. N: initial number. Nf = final number, considering only the non-molted, non-ovigerous surviving females. No significant differences ($p > 0.05$) with respect to control were observed, in any case.

Atrazine concentration (mg/L)	N	% mortality	% molting	% ovigerous	Nf
0 (control)	15	0	0	20	12
0.03	15	6.67	0	20	11
0.3	15	13.33	6.67	20	9
3	15	33.33	6.67	13.33	7

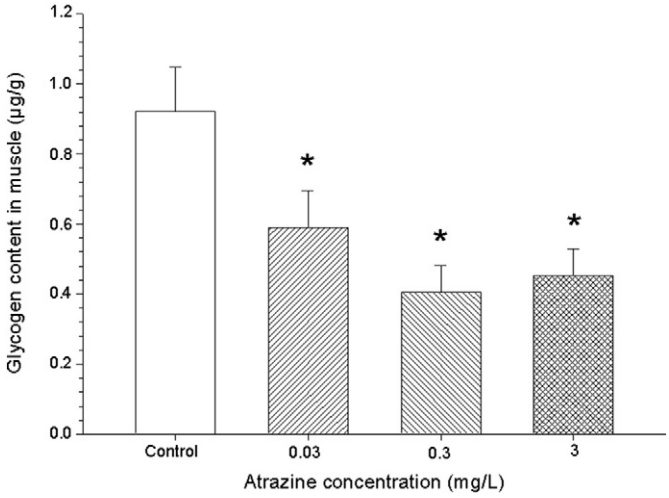

Fig. 1. Glycogen levels in muscle (mean ± SE), at the end of the experiment with formulated atrazine. Asterisks indicate significant differences ($p < 0.05$) with respect to control. Number of females (Nf) is indicated in Table 2.

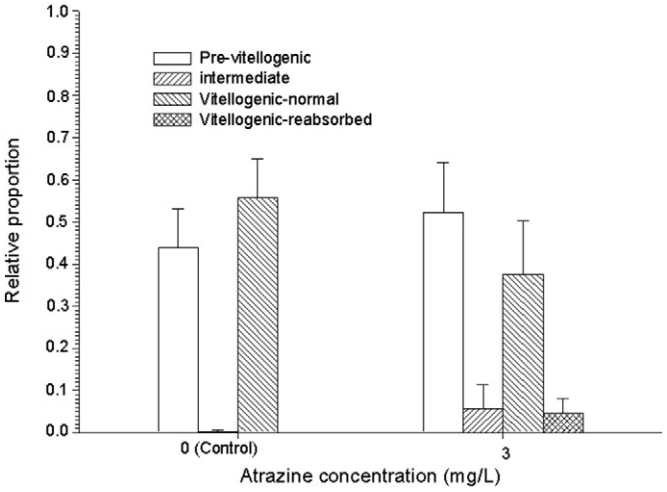

Fig. 3. Proportion of oocyte type (mean ± SE) in the ovary at the end of the experiment with formulated atrazine at 3 mg/L. Asterisk indicates significant differences ($p < 0.05$) with respect to control. Number of females (Nf) is indicated in Table 2.

and secondary antibodies were diluted (1/500) in PBS + 0.05% Tween + 6% powder milk. Absorbance was measured in all wells at 420 nm, by using an ELISA-plates reader (FLUOstar OPTIMA-BMG Labtech., Microplate reader); 2-20-Azino-di-3-ethylbenzthiazoline sulfonic acid was used as chromogen.

Finally, levels of total lipids and glycogen were assessed in both hepatopancreas and chelae muscle, while glucose levels were measured in hemolymph. Total lipids were extracted using the method of Folch et al. (1957) and quantified by the method of Fring and Dunn (1970) by measuring absorbance of the sulphatephosphovainillin complex at wavelength of 530 nm using olive oil as standard. Glycogen was extracted by the method of Van Handel (1965) and quantified as glucose as indicated below, after performing acid hydrolysis with HCl and subsequent neutralization with Na_2CO_3. Protein extraction was performed by the addition of 30% KOH to each sample, followed by 2 h incubation at 100 °C. Protein concentration was assessed according to Lowry et al. (1951) using bovine albumin as a standard and measuring absorbance at 650 nm. Also a small sample of hemolymph from the base of the appendages of each surviving animal was extracted using a 29G needle and a tuberculin syringe. Glucose level was quantified by means of the glucose oxidase method (kit from Wiener Laboratories, Buenos Aires, Argentina).

2.2. In vitro assays

In early June, stock female crabs (maintained under the same conditions used for controls of the *in vivo* assay) were used for the *in vitro* experiments. Ovarian explants (approximately 1 × 0.5 cm each) were incubated for 24 h in CO_2 chambers at 27 °C and in constant darkness. Each female provided a similar piece of ovary to one vial of every group of the same experiment (blocking factor). Medium 199 was prepared using powdered medium with L-glutamine and Earle's salts (Sigma Chemical Co.), dissolved in crustacean saline (Cooke et al., 1977), and modified to compensate for the salts already present in the culture medium. In addition, as in previous studies (Sarojini et al., 1997; Rodríguez et al., 2000), 6 mg of penicillin-G per 100 mL of medium was added to prevent bacterial growth, and the pH was adjusted to 7.4 with 0.5 N NaOH.

Two independent experiments were run, each one at a different atrazine concentration (0.3 and 3 mg/L) together with a paired control with no atrazine added. At the end of the 24 h incubation period, total

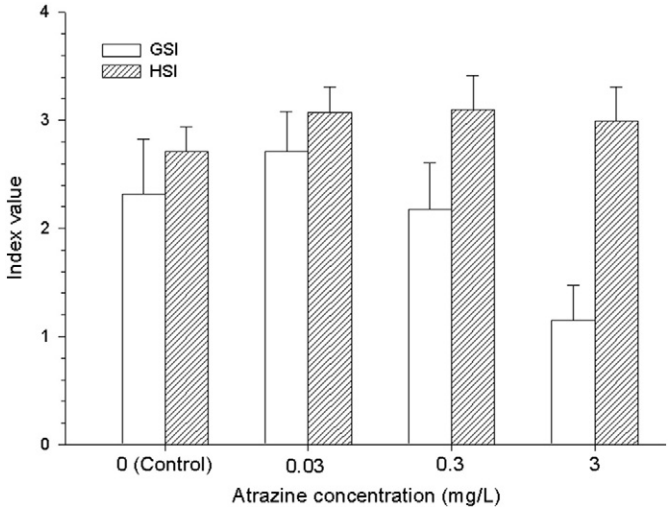

Fig. 2. Gonadosomatic (GSI) and hepatosomatic (HSI) index (mean ± SE) at the end of the experiment with formulated atrazine. Asterisks indicate significant differences ($p < 0.05$) with respect to control. Number of females (Nf) is indicated in Table 2.

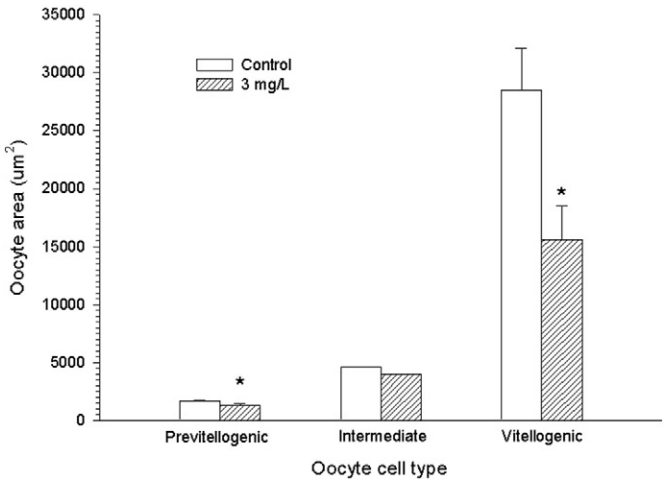

Fig. 4. Oocyte area in the ovary (mean ± SE) at the end of the experiment with formulated atrazine at 3 mg/L. Asterisks indicate significant differences ($p < 0.05$) with respect to control. Number of females (Nf) is indicated in Table 2.

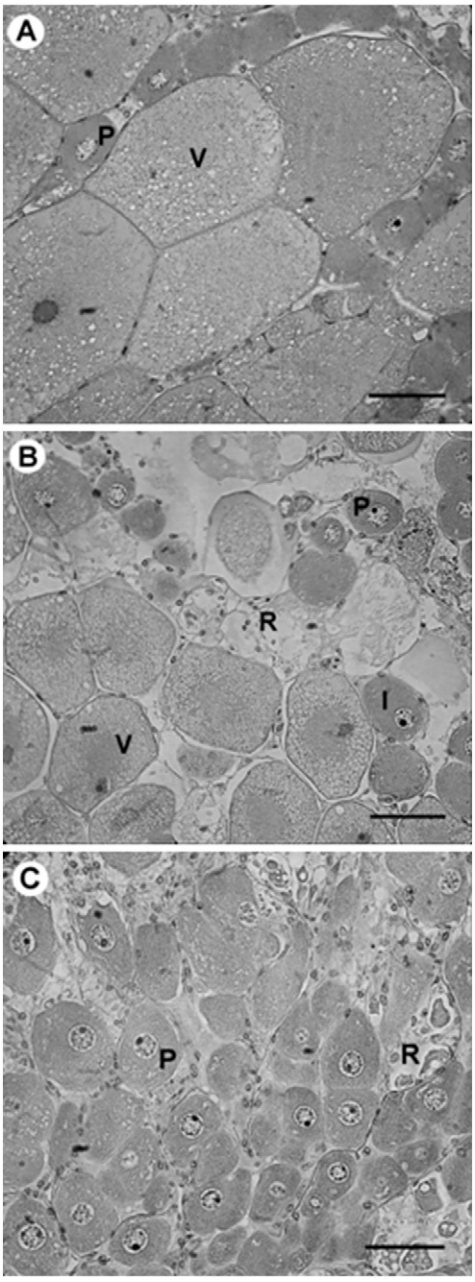

Fig. 5. Histological sections of *N. granulata* ovary. A: control, with normal previtellogenic (P) and vitellogenic (V) oocytes; B: exposed to 3 mg/L, also showing intermediate (I) and reabsorbed (R) oocytes, C: sections from some crabs exposed to atrazine, where only previtellogenic and reabsorbed oocytes could be observed. Scale bar = 50 μm.

content of vitellogenic proteins (Vg) were determined using the same methodology described for the *in vivo* assay.

2.3. Statistical analysis

Percentages of survival, molting and ovigerous females were analyzed by means of a Fischer exact test (Sokal and Rohlf, 1981). Weight gain, glycemia, levels of energy reserves in both hepatopancreas and muscle, GSI, HSI, mean proportion of oocytes, oocyte area, and levels of Vg were analyzed by a one-way ANOVA, followed by LSD multiple comparisons (Sokal and Rohlf, 1981). Normality and variance homogeneity were always confirmed. In all cases, a confidence level of 5% was considered.

3. Results

Validation of nominal concentrations is shown in Table 1. For the two higher concentrations, measured values were very close to the nominal ones; the overall regression coefficient regarding all measured against nominal concentrations was 0.998.

Data on survival, molting and spawning recorded throughout the experiment are shown in Table 2. No significant differences ($p > 0.05$) were found in any atrazine concentration, when compared to control. The same was observed concerning weight gain, glycemia, energy reserves in the hepatopancreas and both lipid and protein content in muscle (data not shown). However, a significant decrease in glycogen levels was observed in muscle for most of the atrazine concentrations (Fig. 1).

A lower GSI could be observed as atrazine concentration increased (Fig. 2); although no significant differences were found, the highest concentration was near to show a significant difference ($p = 0.078$), compared to control. Fig. 3 shows the proportion (P) of each oocyte type,

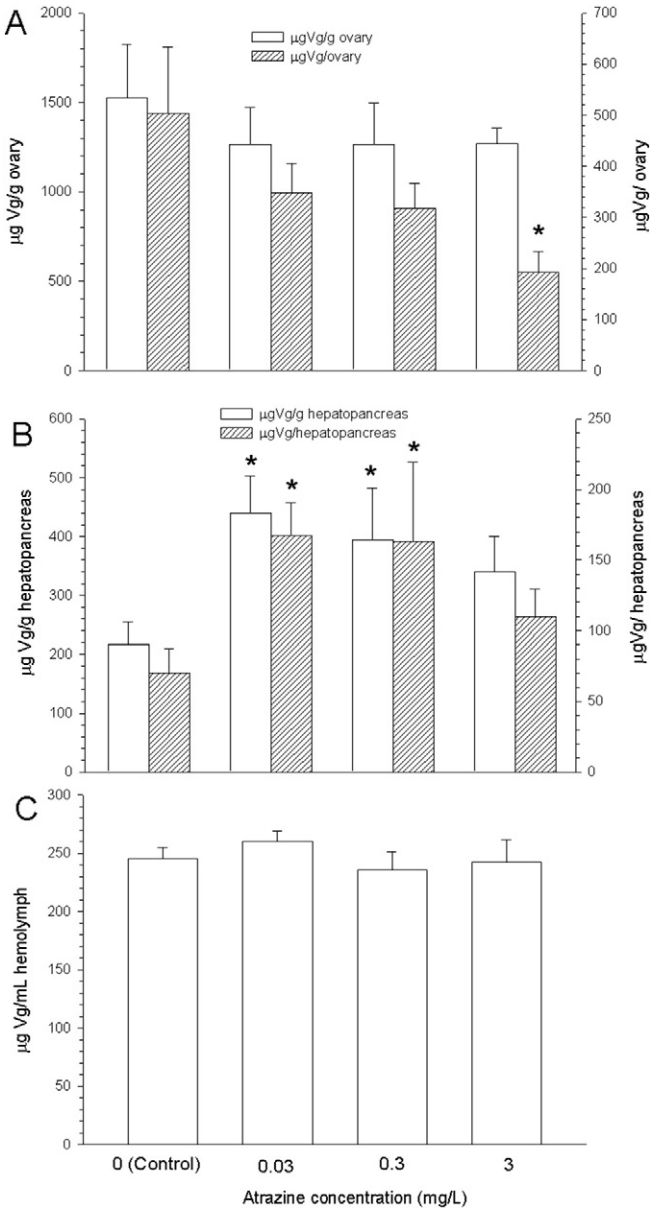

Fig. 6. Total content of vitellogenic proteins (Vg) in ovary (A), hepatopancreas (B) and hemolymph (C) at the end of the experiment with formulated atrazine. Asterisks indicate significant differences ($p < 0.05$) with respect to control. Number of females (Nf) is indicated in Table 2.

measured for both the highest concentration and control. Although no statistically significant ($p > 0.05$), atrazine showed an increase in both the previtellogenic (pre) and intermediate (int) proportion, while decreasing the proportion of normal vitellogenic (vit) oocyte (Fig. 3). The oocyte area (A) is expressed by Fig. 4. A significant ($p < 0.05$) decrease in the area of both previtellogenic and vitellogenic oocytes was detected by the effect of the atrazine at 3 mg/L. Accordingly, the percentage of vitellogenin allocated in vitellogenic oocytes $((P_{vit} \times A_{vit}) / (P_{pre} \times A_{pre} + P_{int} \times A_{int} + P_{vit} \times A_{vit}))$ was 93.29% in controls, *versus* 76.83% in the group exposed to the highest atrazine concentration. Examples of the different types of oocytes mentioned are shown in Fig. 5.

Concerning the vitellogenic protein levels measured after the *in vivo* assay, the ovary of atrazine-exposed groups showed a lower content of Vg than controls, this difference being significant ($p < 0.05$) when the content of Vg of the whole ovary was considered (Fig. 6). On the contrary, Vg levels in the hepatopancreas significantly ($p < 0.05$) increased by effect of atrazine at 0.03 and 0.3 mg/L, compared to control, either per gram of hepatopancreas or considering the whole hepatopancreas. Hemolymph Vg levels were similar ($p > 0.05$) in all treatments (Fig. 6).

Fig. 7 shows the results of the *in vitro* assays. At both atrazine concentration tested, a significant ($p < 0.05$) lower vitellogenin content was noted with respect to controls.

4. Discussion

The lowest concentration used in the current study (0.03 mg/L) was included in the environmental range reported for atrazine (0.01 to 0.1 mg/L, Vonberg et al., 2014, USEPA, 2002). Although the two higher concentrations used in this study (0.3 and 3 mg/L) were higher than those commonly found in the natural environment, they could represent some worst cases, *i.e.*, water pollution near treated crops (up to 1 mg/L, Graymore et al., 2001). Since in the southeast of the Buenos Aires Province, natural habitat of the studied species, an intensive use of atrazine has been recently reported (De Geronimo et al., 2014), the obtained results are also evidencing the potential risk for a wild invertebrate species given by an increased pollution by atrazine.

The observed changes in energy reserves of females exposed to atrazine during 3 months can be interpreted as a compensatory response against the herbicide as an unspecific stressor. However, such changes have been discrete, only involving the utilization of glycogen from muscle, with no significant changes in the long-term energy reserve (*i.e.*, lipids and proteins) whose utilization could strongly affect the weight gain. These results would indicate that the animals would have been in a moderate stress situation, as defined by Sokolova et al. (2012), *i.e.*, a discrete utilization of energy reserve to cover the extra maintenance costs related to damage repair, for instance, with a moderate reduction of the energy that could be allocated for reproductive purposes.

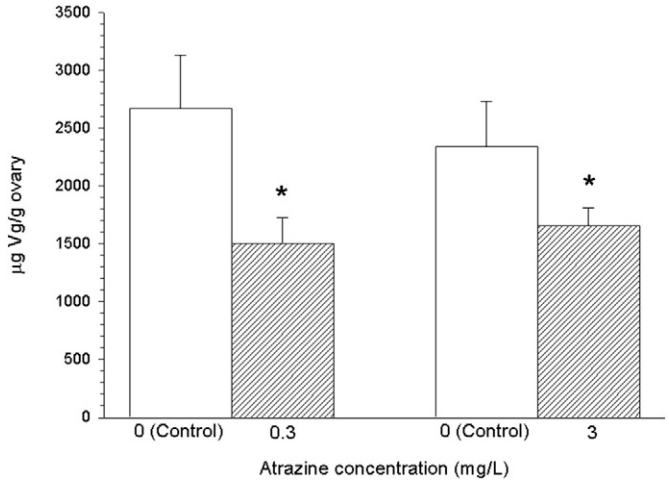

Fig. 7. Total content of vitellogenic proteins (Vg) in ovarian explants incubated for 24 h with formulated atrazine at two different concentrations. Asterisks indicate significant differences ($p < 0.05$) with respect to the respective control. Number of replicates was 12 for 0.3 mg/L of atrazine and its control, and 11 for 3 mg/L and its control.

Although no significant ($p > 0.05$) differences in the gonadosomatic index were detected between the highest atrazine concentration and the control, the histological analysis showed a significant decrease in the oocyte area of both previtellogenic and vitellogenic oocytes at the highest concentration assayed, together with a significant lower content of vitellogenin in the ovary. Taken together, these results indicate that a clear inhibition of the ovarian growth is caused by effect of atrazine at the highest concentration used. In addition, a higher proportion of both previtellogenic and intermediate oocytes was also seen in the ovary of the females exposed to 3 mg/L of atrazine, together with a lower proportion of vitellogenic oocytes; although no significant, these later results were in line with the significant decrease in both oocyte area and vitellogenin content above mentioned.

The results from the *in vitro* experiment were also in accordance with those observed *in vivo*. In fact, the vitellogenin content in ovarian explants was significantly reduced by effect of atrazine at both 0.3 and 3 mg/L in the culture medium, after 24 h of incubation. Since no vitellogenin was available to be imported by oocytes *in vitro*, any change in the vitellogenin content was necessarily related to changes in the endogenous synthesis. An inhibition of oocyte growth, in terms of *in vitro* synthesis of vitellin has been also observed by effect of cadmium, both in the studied species (Medesani et al., 2004) and in the crab *Uca pugilator* (Rodríguez et al., 2000).

One possibility to explain the *in vitro* results is a direct inhibitory effect of atrazine on the synthesis pathway of vitellins, inside the oocytes. However, we were not able to find antecedents of this possible direct effect of atrazine. An alternative hypothesis would be related to the possibility of atrazine acting as an endocrine disruptor on the ovary. As mentioned in the Introduction, there is strong evidence about atrazine playing such role, both in vertebrates (Tillitt et al., 2010, McKinlay et al., 2008) and invertebrates, particularly crustaceans (Palma et al., 2009, Dodson et al., 1999). In *N. granulata*, Álvarez et al. (2015) have reported a delay in ovarian maturation during the early rematuration process that take place in ovigerous females exposed to 5 mg/L of atrazine for 32 days, in terms of a greater proportion of previtellogenic oocytes together with a lower proportion of vitellogenic oocytes, suggesting that any kind of endocrine disrupting took place.

During the *in vitro* incubation of ovarian explants, several hormones could remain acting on the receptors expressed by oocytes, particularly those hormones having a long-term effect, such as steroids and juvenoids. Among them, progesterone and estradiol have been the most frequently reported steroids in crustaceans (Nagaraju, 2011; Fingerman, 1997), being methyl farnesoate the main juvenile hormone described in crustaceans (Nagaruju, 2007; Laufer et al., 2005). Therefore, atrazine could be interfering, during the 24-h incubation period, with any step of the transductional pathway of those hormones. The incidence of several neurohormones, such as the gonad stimulating (GSH) or gonad inhibiting (GIH) hormone (Nagaraju, 2011; Fingerman, 1997) could be also affected during the *in vitro* incubation, although with less probability because of their short-term mode of action.

On the other hand, in the studied species, a significantly accumulation of vitellogenin takes place in the hepatopancreas during the pre-reproductive period, in comparison with other periods (Medesani et al., 2012). As in other crab species (Charmantier et al., 1997, Nagaraju, 2011) this is talking about a central role of the hepatopancreas to synthesize the vitellogenin that will be further taken up by the oocytes during the intense growing they experiment during the mentioned period. Among the hormones stimulating the vitellogenin synthesis in the hepatopancreas, the steroids 17OH-progesterone and 17β-estradiol have been proposed (Meunpol et al., 2007, Warrier et al., 2001) According to our results, atrazine was able to potentiate, at least at 0.03 and

0.3 mg/L, the accumulation of vitellogenin in the hepatopancreas of *N. granulata* during the pre-reproductive period. Therefore, the increase of steroid secretion by effect of atrazine, likely by the ovary, is a plausible hypothesis to be tested further, by means of both *in vivo* and *in vitro* assays.

Acknowledgments

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