Ecotoxicology and Environmental Safety 131 (2016) 96–103

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of atrazine on growth and sex differentiation, in juveniles of the freshwater crayfish *Cherax quadricarinatus*

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ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form
9 May 2016
Accepted 12 May 2016

Keywords:
Atrazine
Crayfish
Sex differentiation
Growth

ABSTRACT

The effect of the herbicide atrazine was assayed in early juveniles of the redclaw crayfish *Cherax quadricarinatus*. Four cohorts of juveniles (a total of 280 animals) were exposed for 4 wk to each one of three atrazine concentrations (0.1, 0.5 and 2.5 mg/L) or a control (0 mg/L), from a commercial formulation having 90% of active principle. At the end of the exposure, no significant ($p > 0.05$) differences in either mortality or molting were noted. However, the weight gain and the protein content of abdominal muscle decreased significantly ($p < 0.05$) in the highest atrazine concentration as compared to control, indicating that atrazine acted as a relevant stressor, although at a concentration higher than those reported in the environment. Besides, the proportion of females increased progressively as the atrazine concentration increases, being significantly ($p < 0.05$) higher than that of controls at the highest concentration assayed. Both macroscopic and histological analysis revealed a normal architecture of gonopores and gonads in both control and exposed animals. The obtained results strongly suggest that atrazine could be causing an endocrine disruption on the hormonal system responsible for the sexual differentiation of the studied species, increasing the proportion of female proportion without disturbing the gonad structure.

1. Introduction

Atrazine ($C_8H_{14}ClN_5$) is a systemic selective herbicide belonging to the chlorotriazines group (Kogan, 1992). It acts as a photosynthesis inhibitor, impairing the electron transport at photosystem II site (Hill reaction), therefore avoiding the normal production of ATP, NADPH and H^+ (Phyu et al., 2011). Atrazine is currently one of the most intensively used herbicides worldwide (Jablonowski et al., 2011), mainly applied on corn crops (USEPA, 2006). In Argentina, this herbicide is used to control broadleaf weeds and grasses in corn, sorghum and sugar cane crops (Atanor, 2012; Costa, 2004) with application doses varying between 1 and 2 kg/ha (Atanor, 2012), through an extension of approximately 10 million ha (Arancibia, 2013). Atrazine has been reported in water bodies at very variable concentrations, mostly ranging from 0.1 μg/L in Germany (Vonberg et al., 2014) to 100 μg/L in rivers of North America (USEPA, 2002). In waters adjacent to treated field, atrazine was found at a concentration as high as 1 mg/L Graymore, 2001). Since the application in agricultural areas occurs in spring, the highest environmental concentrations have been found during late spring and early summer (Solomon et al., 2008; Schottler et al., 1994). Although a half life of 50 d has been reported in laboratory conditions, some authors have estimated that atrazine could persist in soil for 2–6 months (Stephenson and Solomon, 1993).

The possibility of atrazine acting as a xenoestrogen, both *in vivo* and *in vitro*, has been widely reported (Tillitt et al., 2010; McKinlay et al., 2008; Lascombe et al., 2000; Villeneuve et al., 1998). Dramatic changes in the sexual differentiation of amphibians have been reported at concentrations as low as 0.1 μg/L (Tavera Mendoza et al., 2002; Hayes et al., 2002), while consistent effects have been observed on growth, morphology and functionality of both fish and amphibians gonads, at concentrations not higher than 0.5 mg/L (Rohr and McCoy, 2010; Orton et al., 2006). Although invertebrates have been studied in a less extent, atrazine has been shown to decrease the male offspring of *Daphnia sp*, probably by interfering with methyl farnesoate, the sex determinant hormone in daphnids (Palma et al., 2009). Besides, a decrease in total *nauplii* production *per* female was seen in successive generations of estuarine copepods exposed to environmentally relevant atrazine

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http://dx.doi.org/10.1016/j.ecoenv.2016.05.009
0147-6513/

concentrations (Bejarano and Chandler, 2003). A reduced abundance of cladocerans exposed in natural conditions to 0.5 mg/L of atrazine has been noted, in correlation with a decreased in the phytoplankton biodiversity (Dewey, 1986). LC50 values of atrazine for some crustaceans such as copepods and shrimps have been reported ranging from 8 to 20 mg/L (Stringer et al., 2012; Griboff et al., 2014).

Cherax quadricarinatus von Martens 1868 (Decapoda, Parastacidae), native from northern Australia and commonly known as the redclaw crayfish, is a crustacean species particularly suitable for aquaculture (Edgerton, 2005; Jones, 1997). It is intensively or semi-intensively cultured in several countries of Central and South America, such as México, Cuba and Ecuador (Palafox et al., 1999). In Argentina, a production of 30 t/year has been reported (Luchini and Pann,é Huidobro, 2008). *C. quadricarinatus* presents direct development, hatching a first juvenile instar that becomes independent from his mother at the juvenile III stage (Jones, 1997). At optimum environmental conditions (27 °C, freshwater and a good provision of zooplankton) juveniles growth rapidly, attaining the sexual maturity between 6 and 12 months (Wingfield, 2001; Levi et al., 1999; Jones, 1997). Growth rate of male juveniles has been reported as significantly higher than that of female juveniles (Sánchez De Bock and López Greco, 2010; Rodgers et al., 2006).

C. quadricarinatus presents a sexual dimorphism, although some cases of intersexuality have been reported (Bugnot and López Greco, 2009, Sagi et al., 2001). Females have gonopores at the base of the third pair of pereiopods, while those of males are placed at the base of the fifth pair, together with the presence of soft projections, named as *appendices masculinae*, specialized for transferring the spermatophores to the female abdomen; males are also characterized by the red patch placed laterally in their chelae (Vázquez and López Greco, 2007, 2010). Sexual differentiation, both concerning the primary (ovary and testes) and secondary (gonopores and *appendices masculinae*) characters, synchronously starts at a very early developmental stage, i.e., juvenile VI or VII, weighing 100 mg, to fully develop at a body weight ranging from 1 to 5 g (Vázquez and López Greco, 2007; Vázquez et al., 2004). The androgenic gland (AG) adhered to the distal part of the *vas deferens*, produce a peptidic hormone (AGH) responsible for the sexual differentiation of males. This hormone inhibits the sexual differentiation to female, maintains the spermatogenic activity of testes and the development of the secondary sexual characters of males (Chang and Sagi, 2008; Sagi and Khalaila, 2001; Nagaraju, 2011).

This study is aimed at determining the effect of atrazine on survival, growth rate, energy reserves levels and sexual differentiation, in early juveniles of the redclaw crayfish *C. quadricarinatus*.

2. Materials and methods

Juvenile redclaw were reared under laboratory conditions from a reproductive stock supplied by the Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. After mating in the laboratory, four ovigerous females were selected for the assays. At the juvenile III stage, young crayfish were isolated in small recipients provided with refuges and continuous aeration; other environmental conditions were the same used in previous studies (Vázquez and López Greco, 2007, Tropea et al., 2010). Once attained a body weight of 135 ± 3 mg (stage VI-VII, at the beginning of sexual differentiation), each juvenile was transferred to a plastic recipient filled with 200 mL of filtered and dechlorinated freshwater (hardness=80 mg/L as $CaCO_3$ equivalents, pH=8.0 ± 0.8), providing a plastic net as refuge and continuous aeration. After 3–5 d of acclimation, each juvenile was weighed at a precision of ± 0.1 mg; 15–20 juveniles were then randomly assigned to each treatment (atrazine concentration or control).

Toxicological bioassays were conducted according to the guidelines recommended by American Public Health Association et al. (2005). Four assays were done, each one with a cohort of juveniles hatching from a different ovigerous female. For all the assays, three atrazine concentrations were run: 0.1, 0.5 and 2.5 mg/L of active principle, from a commercial formulation (Gesaprim 90%, Syngenta). A water dilution control, with no toxic added, was also run. All test solutions (atrazine concentrations and control) were renewed every 72 h, while a temperature of 27 ± 1 °C and a photoperiod of 14:10 (L: D) were maintained throughout. During the assays, juveniles were daily fed *ad libitum* with pelleted fish food (Tetra Diskus®, 50% protein), supplemented with *Elodea sp.* fresh leaves, according to previous studies (Avigliano et al., 2014; Chaulet et al., 2012, 2008; Tropea et al., 2010). All the assays lasted 4 wk, within the period of December to July.

Some water samples were taken at 0 and 72 h from different recipients, in order to validate nominal concentrations. Samples were filtered through 0.45 μm nylon membrane, and filtrates were analyzed by high liquid pressure chromatography (HPLC) coupled to mass spectrometry (AgilentR, model VL). A X-SELECT C18 column was used, using as mobile phase a mixture of acetonitrile: formic acid (0.1%) at 0.5 mL/min. An isotopic tracer of atrazine (^{5}D) was used as a control of analytical quality, while an external standard was used for quantification, at the same conditions used for samples.

Mortality was daily monitored. All animals were weighed weekly, in order to determine the percent weight gain (WG) as (Wt-Wi/Wi) x100, where Wi= initial and Wt= body weigh at any time of measurement. At the end of the 4th week of exposure, all animals were sacrificed and abdominal muscle was dissected and immediately freezed at −20 °C to further determine the level of energy reserves. Due to the small size of juveniles, abdominal muscle was pooled every 2–3 animals, from the same treatment and cohort. Glycogen was extracted and quantified by the method of Lo et al. (1970), suitable for small samples. Total proteins were quantified according to Bradford (1976), adapted to juveniles of the studied species (Stumpf et al., 2014; Calvo et al., 2013). To quantify lipids, the protocol proposed by Frings and Dunn (1970) was followed.

Just after dissection of abdominal muscle, the cephalotorax from each animal was fixed in Bouin´s solution for 4 h at room temperature, being later transferred to ethanol 70 °C. The tissues were finally dehydrated and embedded in paraffin, seriated Sections, 5–7 μm thick, were stained with hematoxylin-eosin, for histological analysis of both gonads and hepatopancreas. To determine sex, the placement of gonopores was identified (at the third pair or pereiopods for females, at the fifth pair for males). In addition, males were identified by the *appendices masculinae* (at the coxa of the fifth pair of pereiopods). Such juveniles presenting at least one gonopore characteristic of each sex, as well as a differentiated *appendices masculinae* were identified as intersex, according to previous studies (Vázquez and López Greco, 2007; Vázquez et al., 2010).

Percentages of survival and molting were analyzed by means of a X^2 test (Sokal and Rohlf, 1981). WG, as well as the levels of energy reserves in abdominal muscle, were analyzed by a factorial ANOVA test, taking the atrazine concentration as a fixed factor and the cohort as a random factor; *a posteriori* LSD comparisons were also made (Sokal and Rohlf, 1981). Normality and variance homogeneity were always confirmed. The Fischer exact test was used for comparing the sex proportion between control and every atrazine concentration. In all cases, a confidence level of 5% was considered.

3. Results

The validation of atrazine nominal concentrations is shown in Table 1. Measured concentrations were close to the nominal ones. Therefore, the latter will referred hereafter. Table 2 shows the results of survival and molting. No significant differences ($p > 0.05$) were noted in survival between control and any atrazine concentration, therefore indicating that all concentrations assayed were sublethal. Overall grand mean of survival was near 83%. Because juvenile crayfish often eat their molts (Jones, 1997), those eventually observed throughout the experiments were not quantified for statistical analysis.

From the 3rd wk of the assays, WG of all juveniles exposed to the highest atrazine concentration was significantly ($p < 0.05$) lower than that of control (data not shown); Fig. 1 shows the results corresponding to the end of the assays (4th wk), either considering the whole data set (Fig. 1(A)) or discriminating by sex (Fig. 1(B)). Again, WG of entire set of juveniles exposed to the highest atrazine concentration was significantly ($p < 0.05$) lower with respect to control (Fig. 1(A)). Nevertheless, while significant ($p < 0.05$) differences were still observed considering only juvenile females, no differences ($p > 0.05$) in WG were observed between control and exposed juvenile males (Fig. 1(B)).

No significant ($p > 0.05$) differences were detected for both glycogen and lipid content of muscle (Fig. 2(A),(B)). In addition, protein content of the abdominal muscle was significantly ($p < 0.05$) lower at the highest atrazine concentration, with respect to control (Fig. 3(C), whole data set). No significant ($p > 0.05$) differences were noted among cohorts, for either WG or energy reserves.

The results of sex determination are shown in Fig. 3. Compared to control (33% of females, 66% of males), a gradual shift to a relative increment in the proportion of females can be seen as the atrazine concentration increases, being such increment statistically significant ($p < 0.05$) at the highest atrazine concentration (61% of females, 39% of males). Only 4 intersex juveniles (3 at 0.1 mg/L, 1 at 0.5 mg/L) were detected in the total of 233 juveniles analyzed. Fig. 4(A) shows an example of a juvenile female (gonopores placed at the base of the third pair of periopods), while Fig. 4 (B) shows an example of a male (gonopores placed at the base of the fifth pair of periopods, with *appendices masculinae* developed), Fig. 5 shows a representative section, at the histological level, of both testes and ovaries of *C. quadricarinatus* juveniles. Concerning females, normal ovaries containing oogonias and primary oocytes were observed, in both control atrazine-exposed juveniles. A normal architecture of testis was also seen in male juveniles, both control and exposed. Intersex juveniles also showed normal testes. Finally, the hepatopancreas of all juveniles analyzed showed a preserved structure, with no evident signs of pathologic injuries, considering previous studies (Calvo et al., 2011, 2012) as a reference of the normal hepatopancreatic architecture in the studied species.

Table 1.
Validation of nominal atrazine concentrations used in the experiments. Detection limit.

Nominal concentrations (mg/L)	Measured concentrations (mg/L)	
	0 h	72 h
0 (control)	0.000	0.000
0.1	0.059	0.060
0.5	0.354	0.480
2.5	2.39	3.17

Table 2.
Survival for each assayed cohort (C) of *Cherax quadricarinatus* juveniles, at the end of the 4-wk assay. N= number of animals. No significant differences ($p > 0.05$) were found in any case.

Atrazine concentration - tration (mg/L)	Cohort	Initial N	N of survivals	Overall mean (%)
0 (control)	1	20	16	85.71
	2	15	15	
	3	15	14	
	4	20	15	
0.1	1	20	17	90.00
	2	15	15	
	3	15	15	
	4	20	16	
0.5	1	20	14	78.57
	2	15	15	
	3	15	12	
	4	20	14	
2.5	1	20	14	78.57
	2	15	15	
	3	15	13	
	4	20	13	

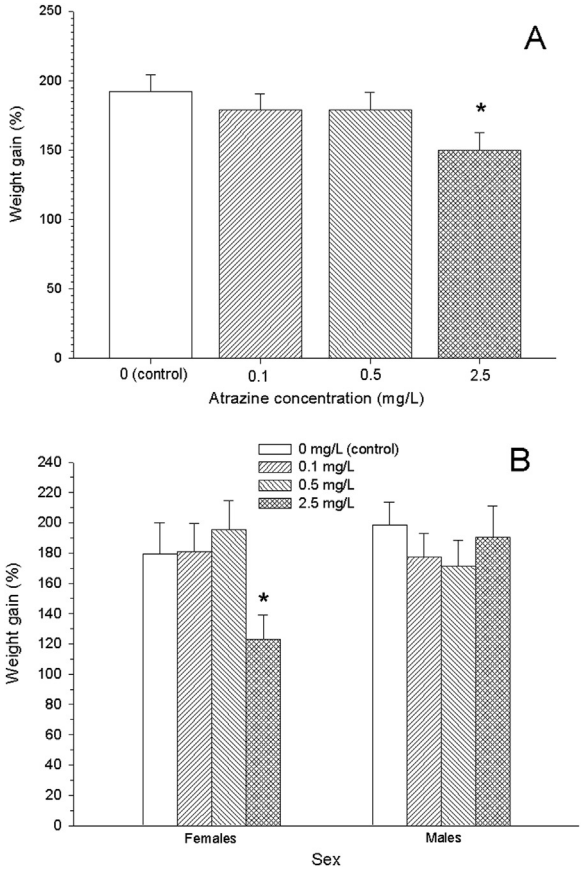

Fig. 1. Weight gain of *Cherax quadricarinatus* juveniles at the end of the 4-wk assays. A: whole data, B: discriminated by sex. Asterisk indicates significant differences ($p < 0.05$) with respect to control.

3.1. Discussion

The mortality observed during the exposure to atrazine was similar to that registered in controls, clearly indicating the lack of lethal effects at the assayed concentrations. Although the highest concentration used in this study was higher than those found in the natural environment (Graymore et al., 2001), it should be considered that in the southeast of the Buenos Aires Province, natural habitat of the studied species, an intensive use of atrazine

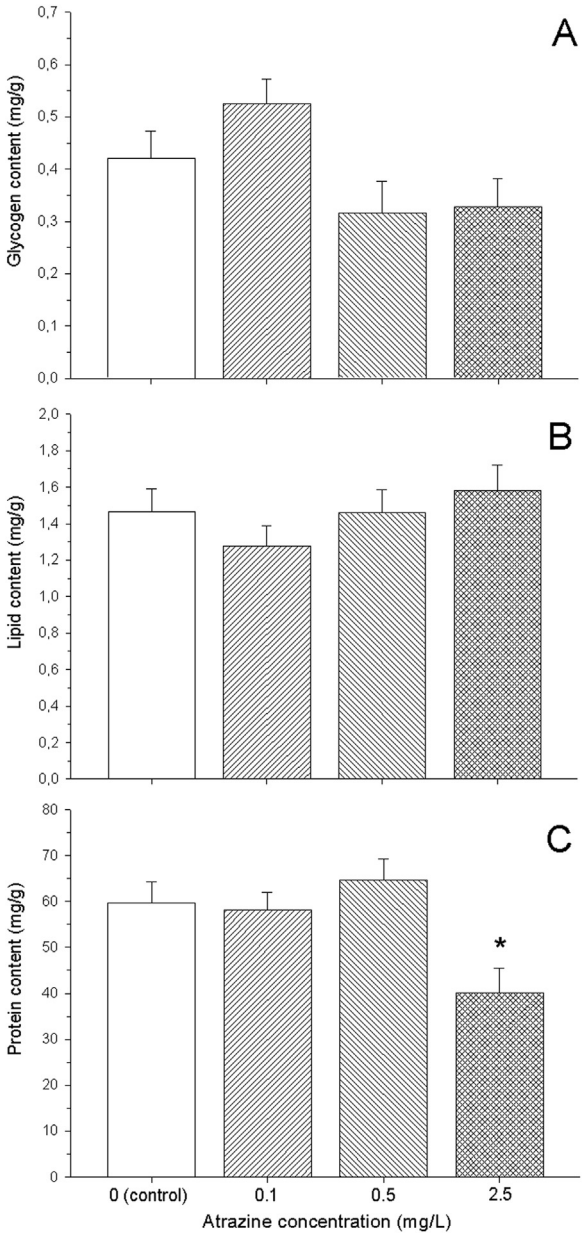

Fig. 2. Energy reserves in muscle at the end of the 4-wk assays (whole data). Asterisk indicates significant differences ($p < 0.05$) with respect to control.

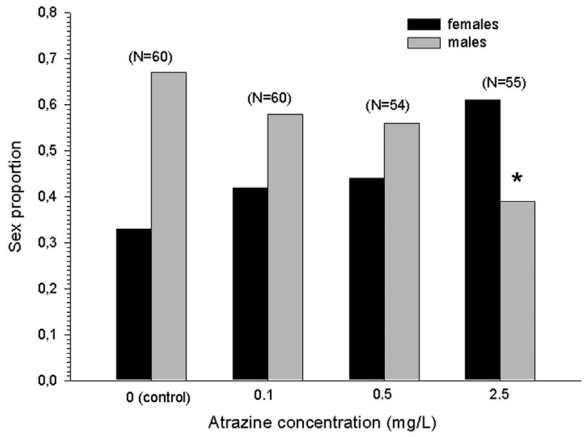

Fig. 3. Sex proportion at the end of the 4-wk assays. Asterisk indicates significant differences ($p < 0.05$) with respect to control. Intersex animals ($N=4$) are not included.

has been recently reported (Gerónimo et al., 2014). In a wider context, atrazine utilization in South American countries such as Argentina (Arancibia, 2013) and Brazil (Kelly and Martinez, 2014) tends to increase over time.

The effects of some herbicides other than atrazine, particularly glyphosate, have been previously reported for juveniles of the studies species. For instance, in advanced juveniles of *C. quadricarinatus* (body weight > 5 g), a significant decrease of weight gain was observed after 50 days of exposure to a mixture of 30 mg/L of glyphosate and polyoxyethylenamine (Frontera et al., 2011). Moreover, in early juveniles of *C. quadricarinatus*, whose body weight was similar to that of the juveniles used in the current study, a reduction of weight gain of 35% was observed after 40 days of exposure to 40 mg/L of glyphosate (Avigliano et al., 2014). Despite both glyphosate and atrazine are efficient herbicides, they have quite different mode of actions (Lydon and Duke, 1989; Phyu et al., 2011). It is remarkable that the significant reduction in the weight gain caused by atrazine (current study), was detected at a concentration one order of magnitude below the above mentioned concentrations of glyphosate, stressing the environmental risk of the former herbicide.

As pointed out by Sokolova et al. (2012) energy allocation for growth and/or reproduction of invertebrates can be compromised due to the cost of maintenance in stress situations. As discussed later, an increased utilization of energy reserve nonspecifically caused by atrazine as stressor seems to have occurred, therefore explaining the decrease in weight gain caused by atrazine. Discriminating by sex, though, we have verified that the observed drop in weight gain was true for female juveniles, but not for male juveniles. This result could be related to the different energetic investment made by each sex during the ovarian growth following gonad differentiation, more relevant in the case of females. Tropea et al. (2011), in an annual study on sexual differentiation and growth of *C. quadricarinatus* juveniles, suggest that the higher somatic growth rate of males, compared to that of females, would be the result of an indirect effect of the androgenic gland, which is inhibiting the development of gonads as ovaries, and consequently reducing the energy demand for gonad differentiation. The higher potency of males for somatic growth could be then explaining why they were less affected by atrazine than females, at least in relation to this process.

In fact, a correlation between a lower weight gain and a lower content of energy reserves was previously reported in *C. quadricarinatus* juveniles exposed to glyphosate. This loss of energy reserves comprised a lower content of both glycogen and muscle protein (Frontera et al., 2011, for advanced juveniles), as well as a lower content of muscle lipids, and both muscle and hepatopancreatic proteins (Avigliano et al., 2014, for early juveniles). It is well known that energy reserves, whether carbohydrates, lipids or proteins are used to face the exposure to several stressors, both in fish and crustaceans (Hutchinson, 2002; Morris et al., 2005; Glusczak et al., 2006, 2007; Frontera et al., 2011; Calvo et al., 2012, 2013; Avigliano et al., 2014; Stumpf et al., 2014). In juveniles of the studies species, a decreased growth rate, together with a utilization of energy reserves, has been reported under several unfavorable conditions acting as stressors (Jones, 1997; Calvo et al., 2013; Stumpf et al., 2014).

The results obtained in the current study indicate a significant decrease of protein content in muscle, in juveniles of *C. quadricarinatus* exposed to 2.5 mg/L of atrazine, in correlation with a reduced weight gain. Since, as mentioned before, a similar effect was seen in the same experimental model by effect of other pesticides and environmental agents, we can conclude that an incremented utilization of muscle protein as energy reserve seems

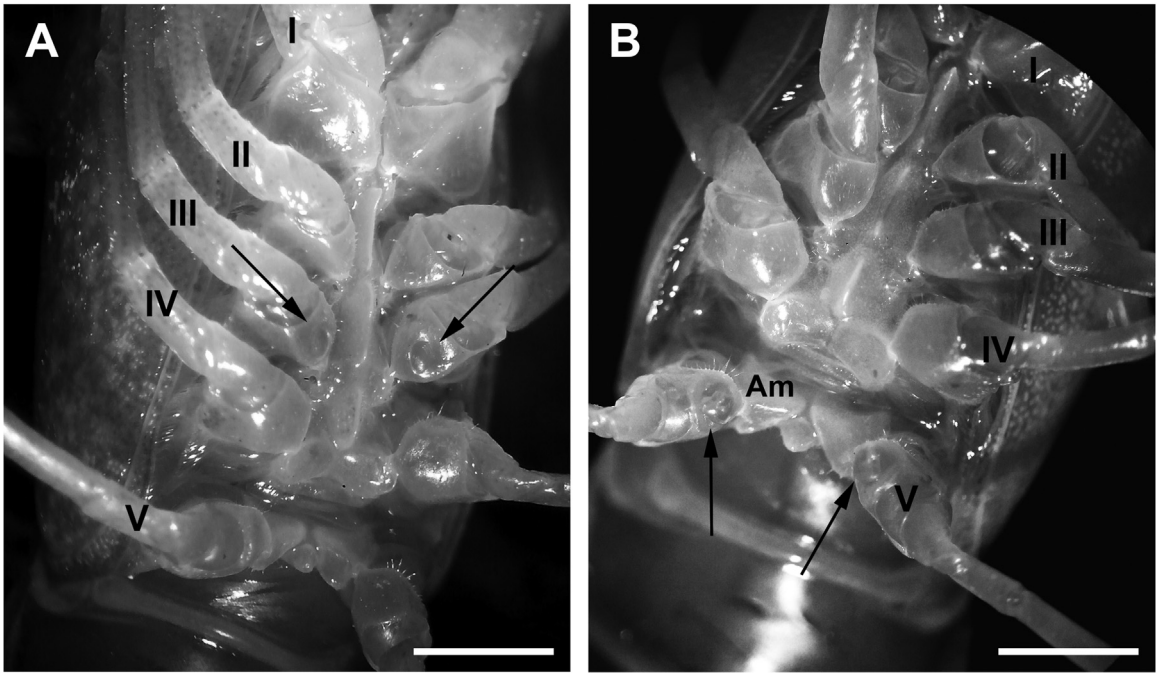

Fig. 4. Secondary sexual characters used for sex determination. (A) female, (B) male. Roman numbers indicate the pairs of pereiopods. Arrows indicate the position of gonopores. for both sexes. Am: *appendix masculina.* Scale bar: 1 mm.

to have occurred as an unspecific response to a chronic stress, in this case represented by the exposure to atrazine. Some authors have proposed that crustaceans have the ability to utilize proteins as energy source for growing, due to their high protein requirement in the diet, together with their limited capacity to store carbohydrates and lipids (Dall and Smith, 1986; Rosas et al., 2000; Sánchez-Paz et al., 2006). The crustacean hepatopancreas is able to carry out gluconeogenesis from muscle protein as substrate, to elevate glycemia for supporting an increased metabolic demand (Sánchez-Paz et al., 2006). In *C. quadricarinatus*, proteins have been identified as one of the main energy reserves for either growth or reproduction (Ghanawi and Saoud, 2012).

The effect of several pollutants on sexual differentiation of crustaceans has been reported in some extent. For instance, the incidence of intersex forms in marine amphipods increased in contaminated waters of Scotland, compared to reference sites; at the same time, the proportion of males decreased, suggesting that a de-masculinization took place, probably involving an interference with the androgenic gland, which is essential for sexual differentiation of crustacean males (Ford et al., 2004). Further studies, especially in amphipods, have supported this hypothesis, stating that intersexuality is enhanced by endocrine disruptors (revised by Ford et al., 2008). In this context, a direct effect of atrazine on the androgenic gland of crustaceans is therefore a very plausible hypothesis to be tested.

Although atrazine has been designed to interfere with photosynthesis, several evidences obtained from laboratory vertebrate animals indicate that this herbicide can act as a xenoestrogen (Villeneuve et al., 1998; Lascombe et al., 2000). In amphibians, the effect of atrazine on metamorphosis has been broadly documented (Freeman et al., 2005; Brodeur et al., 2013), as well as its role as a potent endocrine disruptor, causing castration and feminization of males, among other effects (Carr et al., 2003; Hayes et al., 2002). A meta-analysis made on the literature published on both amphibians and fish, showed consistent effect of atrazine on the gonadal function (Rohr and McCoy, 2010). In fact, the inhibition of gonadal maturation was also reported for several vertebrate groups (Tillitt et al., 2010). The interference of atrazine with the hypothalamic control of pituitary hormones, as well as the induction of the enzyme aromasate (which catalyze the conversion of androgens in estrogens) could be the main causes of the effects mentioned above (McKinlay et al., 2008). However, although a significant change in the sex ratio was seen in *Xenopus laevis* tadpoles exposed to 10 or 100 μg/L of atrazine, no relationship with estrogen or aromatase activity was seen (Oka et al., 2008).

In crustaceans, atrazine has been also described as an endocrine-disrupting compound (Rodríguez et al., 2007). Under this approach, several possibilities can be considered in order to explain the increased proportion of juveniles differentiated as females by effect of atrazine, observed in the current study. Since no aromatase activity has been reported in crustaceans (Swevers et al., 1991), the possible interference with this enzymatic pathway should be *a priori* discarded. Instead, the androgenic gland of malacostracan crustaceans should be seriously considered as a possible target of the herbicide under study. As mentioned before, this AG has shown to have a crucial role in the development and maintenance of both primary and secondary sexual characters of crustaceans, including some special cases such as intersex and protandric hermafroditism (Barki et al., 2003; Manor et al., 2004; Okumura et al., 2005).

In the studied species, both Barki et al. (2003), Karplus et al. (2003) have reported that the implantation of AG in females, induces both the sexual and aggressive behavior of males; moreover, the administration of AG extracts had a clear masculinizing effects on both primary and secondary sexual characters (Khalaila et al., 2001; Manor et al., 2004). On the contrary, the ablation of AG in *C. quadricarinatus* intersex resulted in a clear feminization, including the induction of vitelogenin synthesis (Khalaila et al., 1999; Barki et al., 2006). In the same species, Tropea et al. (2011) found that when AG ablation was made on advanced juvenile males (near 2 g of body weight) the testes could differentiate, but some abnormalities were evident in the distal portion of *vas deferens*, together with the absence of male gonopores and *appendices masculinae* in the fifth pair or pereiopods. In the current study, the exposure to atrazine began on very early juveniles (near 100 mg of body weight), at which sexual differentiation initiates. During this

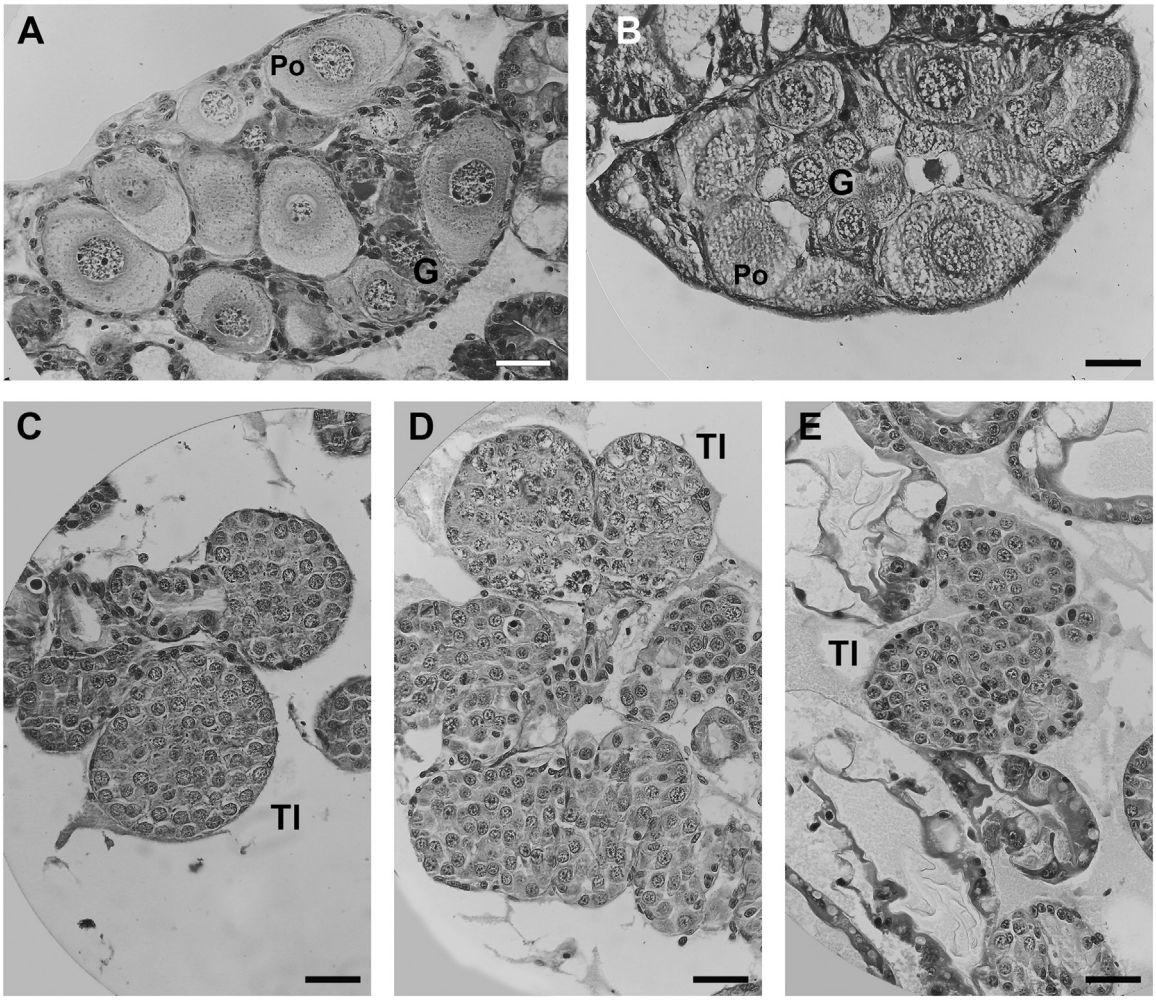

Fig. 5. Representative histological sections of both ovaries and testes of juvenile crayfish at the end of the experiment. A and B: ovary of control and exposed to 2.5 mg/L of atrazine, respectively; C and D: testis of control and exposed to 2.5 mg/L of atrazine, respectively. E: gonads (normal testes) of intersex crayfish. Po: primary oocytes, G: gonias, Tl: testicular lobes. Scale bar: 200 μm.

critical exposure period, AG could be affected by atrazine, therefore increasing the normal ratio of females: males. However, some other possibilities other than the effect of atrazine on AG should not be discarded, such as the possible interference of the herbicide with the eyestalk neurohormones controlling AG hormone production (Khalaila et al., 2002), or with the reproductive functions of methyl farnesoate, whose role on sexual differentiation and/or testicular development has been reported in *Daphnia magna* (Palma et al., 2009) and other crustaceans (Nagaraju and Borst, 2008).

Finally, all the juveniles differentiated as males showed a normal appearance in both their *appendices masculinae* (which presented a symmetric lobulation) and the opening of their gonopores (placed apically). Concerning the histological aspect of gonads we have also observed a normal appearance of either ovaries or testes, in both control and atrazine-exposed juveniles. Even the hepatopancreas, a tissue particularly susceptible to be altered by pollutants and other stressors, showed no evident pathologies.

4. Conclusions

We can conclude that, after an exposure of *C. quadricarinatus* early juveniles to 2.5 mg/L of atrazine during the critical period of sexual differentiation, the effects mainly consisted of a reduction in somatic growth (lower weight gain and protein content in muscle) together with an increased proportion of exposed juveniles differentiate in females, presumably due to an endocrine disruption.

Acknowlegments

This study was supported by grants from the University of Buenos Aires (UBACYT 2012–2015 program, code 20020110100044, UBACYT 2014–2017 program code 20020130100186BA), ANPCYT (PICT 2012, project 1333) and CONICET (PIP 2012–2014, number 219).

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