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*

Camila Mac Loughlin [a], Ivana. S. Canosa [a], Gabriela R. Silveyra [a], Laura S. López Greco [b], Enrique M. Rodríguez [a,*]

[a] Lab. of Crustacean Physiology, Dept. of Biodiversity and Experimental Biology, FCEN, University of Buenos Aires, IBBEA, CONICET-UBA, Ciudad Universitaria, Pab. II, C1428EHA, Buenos Aires, Argentina
[b] Lab. of Biology of Reproduction and Growth of Crustaceans, Dept. of Biodiversity and Experimental Biology, FCEN, University of Buenos Aires, IBBEA, CONICET-UBA, Ciudad Universitaria, Pab. II, C1428EHA, Buenos Aires, Argentina

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

* Corresponding author.
E-mail address: enrique@bg.fcen.uba.ar (E.M. Rodríguez).

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.