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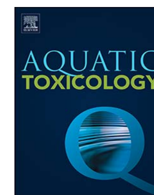

Effects of atrazine on vitellogenesis, steroid levels and lipid peroxidation, in female red swamp crayfish *Procambarus clarkii*

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Animals

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

2.2. Atrazine solution

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

2.3. Atrazine concentrations and experimental conditions

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

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

2.4. Tissue collection

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

2.5. Vitellogenin expression

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

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

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