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Environmentally-relevant concentrations of atrazine induce non-monotonic acceleration of developmental rate and increased size at metamorphosis in *Rhinella arenarum* tadpoles

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ABSTRACT

Despite of the various studies reporting on the subject, anticipating the impacts of the widely-used herbicide atrazine on anuran tadpoles metamorphosis remains complex as increases or decreases of larval period duration are almost as frequently reported as an absence of effect. The aim of the present study was to examine the effects of environmentally-relevant concentrations of atrazine (0.1, 1, 10, 100, and 1000 μg/L) on the timings of metamorphosis and body size at metamorphosis in the common South American toad, *Rhinella arenarum* (Anura: bufonidae). None of the atrazine concentrations tested significantly altered survival. Low atrazine concentrations in the range of 1–100 μg/L were found to accelerate developmental rate in a non-monotonic U-shaped concentration–response relationship. This observed acceleration of the metamorphic process occurred entirely between stages 25 and 39; treated tadpoles proceeding through metamorphosis as control animals beyond this point. Together with proceeding through metamorphosis at a faster rate, tadpoles exposed to atrazine concentrations in the range of 1–100 μg/L furthermore transformed into significantly larger metamorphs than controls, the concentration–response curve taking the form of an inverted U in this case. The no observed effect concentration (NOEC) was 0.1 μg atrazine/L for both size at metamorphosis and timings of metamorphosis. Tadpoles exposed to 100 μg/L 17β-estradiol presented the exact same alterations of developmental rate and body size as those treated with 1, 10 and 100 μg/L of atrazine. Elements of the experimental design that facilitated the detection of alterations of metamorphosis at low concentrations of atrazine are discussed, together with the ecological significance of those findings.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most widely used pesticides in the world. Initially registered in 1958, it rapidly became the main herbicide used on corn, sorghum and sugarcane due to its relative affordability and its effectiveness at selectively controlling weeds without damaging the crop. Over the last 15 years, however, the safety of atrazine has generated much controversy as growing evidences demonstrated both the widespread contamination of surface and ground waters and the potential for atrazine to cause endocrine disruption and immunotoxicity (Rohr and McCoy, 2010; Bishop et al., 2010).

Atrazine is, indeed, one of the most frequently detected pesticide contaminant in ground, surface and drinking water (Fisher et al., 1995; Senseman et al., 1997; Giroux, 2002; Kolpin et al., 2002; Lerch and Blanchard, 2003; Andriulo et al., 2004; Gilliom et al., 2006; Byer et al., 2011). In agricultural areas, rates of detection are often near 100 percent with concentrations in rivers and streams in the parts per billion range (usually below 20 μg/L). Being relatively persistent in soils and soluble in water, atrazine commonly enters water bodies through runoff, and concentrations in surface waters often peak after rainfalls (Solomon et al., 1996; Giddings et al., 2005), sometimes reaching the parts per million level in shallow ponds (Kadoum and Mock, 1978; Klaine et al., 1988; Eisler, 1989; Huber, 1993; Battaglin et al., 2000).

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Atrazine can furthermore be transported aerially and has been detected great distances from where it is used in rainwater, fog, ambient air, arctic ice and seawater (Glotfelty et al., 1987; Chernyak et al., 1996; Thurman and Cromwell, 2000; Mast et al., 2007; Vogel et al., 2008). In 2004, the E.U. banned the use of atrazine due to its ubiquitous and unpreventable presence in water (E.U., 2004).

Additionally, atrazine has been found to cause immunotoxicity (Brodkin et al., 2007; Rowe et al., 2008) and endocrine disruption in mammals, fish, and amphibians (Cooper et al., 2000; U.S. EPA, 2005; Fan et al., 2007; Langlois et al., 2010; Rohr and McCoy, 2010). Demonstrated endocrine activities of atrazine include estrogenic and anti-estrogenic effects (Sanderson et al., 2001; Seung et al., 2003; Holloway et al., 2008) and alterations of the hypothalamo-pituitairy-adrenal axis (Bisson and Hontela, 2002; Goulet and Hontela, 2003; Cericato et al., 2008). These endocrine activities of atrazine have been shown to cause alterations of gonad development, morphology and function (Hayes et al., 2002, 2011; Tavera-Mendoza et al. 2002a,b; Carr et al., 2003), shifts of the sex ratio towards the female phenotype (Oka et al., 2008; Langlois et al., 2010), decreased reproductive success (Bringolf et al., 2004; Tillitt et al., 2010) and alterations of growth and metamorphosis in tadpoles (Diana et al., 2000; Brown Sullivan and Spence, 2003; Brodeur et al., 2009).

With respect to the metamorphosis of tadpoles, although more than twenty studies have now presented data regarding the effects of atrazine in anurans, reaching a conclusion remains difficult as reports of increase (Briston and Threlkeld, 1998; Diana et al., 2000; Freeman et al., 2005; Brodeur et al., 2009) or decrease (Brown Sullivan and Spence, 2003; Coady et al., 2004; Freeman and Rayburn, 2005; Zaya et al., 2011) of the time to metamorphosis are almost as frequent as reports of an absence of effect (Allran and Karasov, 2000; Carr et al., 2003; Boone and James, 2003; Bridges et al., 2004; Orton et al., 2006; Hayes et al., 2006; Storrs and Semlitsch, 2008; Oka et al., 2008; Kloas et al., 2009; Williams and Semlitsch, 2010; Spolyarich et al., 2010; Choung et al., 2011). Although there are probably various reasons for these conflicting findings, the apparent frequentness of non-monotonic concentration–response curves is likely an important factor (Brodeur et al., 2009; Rohr and McCoy, 2010).

The aim of the present study was to examine the effects of environmentally-relevant concentrations of atrazine on the timings of metamorphosis and body size at metamorphosis in the common South American toad, *Rhinella arenarum*. The experimental design executed in the present study is a follow-up of an earlier study in which we demonstrated a non-monotonic acceleration of the time to climax and delayed tail resorption (Brodeur et al., 2009). Exposures conducted in the current study are longer (from stage 25 instead of stage 38) and tested concentrations are lower and more similar to levels commonly detected in the environment.

2. Materials and methods

2.1. Tadpoles

Adults of the common South American toad, *R. arenarum*, weighing approximately 200–250 g were captured in pasture fields of Buenos Aires Province, Argentina. Ovulation of female toad was induced by means of an intraperitoneal injection of homologous hypophysis suspended in 1 ml of AMPHITOX solution (AS) (Herkovits and Perez-Coll, 2003). Oocytes were fertilized *in vitro* using fresh sperm suspended in AS. The resulting embryos were maintained in AS at 20 ± 2 °C until reaching stage 25 (Gosner, 1960). Tadpoles were offered boiled swiss chard ad libitum when they began feeding at stage 24–25.

2.2. Experimental protocol

Nominal concentrations of atrazine tested were 0.1, 1, 10, 100 and 1000 μg/L. The experimental design also included a control group exposed to AS only, a solvent control group exposed to AS containing 0.02 percent (v/v) of acetone, and a positive control group exposed to 100 μg/L of 17β-estradiol (E2). Tadpoles used in the experiment were pooled from four different clutches. Twenty replicates were performed for every concentration tested. In every replicate, ten tadpoles having recently reached stage 25 were placed in 40 mL of AS with or without (controls) atrazine. To avoid evaporation of test solution, experimental tanks consisted of two superposed 10 cm-diameter glass Petri dishes. Test solutions were entirely replaced every 48 h, and temperature was maintained between 20 ± 2 °C throughout the experiment. A piece of boiled swiss chard of approximately 2 cm^2 was added to the test vessels after changing the solutions so as to provide tadpoles with ad libitum food. Dead tadpoles were removed and Gosner stage and survival were evaluated every other day when renewing the solutions. After 40 days of exposure, tadpoles were transferred to two-superposed 15 cm-diameter glass Petri dishes containing 100 mL of test solution to provide space to the growing tadpoles. Frogs completing metamorphosis were examined for malformations and their body mass and snout-vent length were measured.

2.3. Preparation of test solutions

Technical-grade atrazine (CAS no. 1912-24-9) with a purity of 98 percent was obtained from Chem Service (West Chester, PA, USA). Test solutions of 1000, 100, 10, 1 and 0.1 μg/L of atrazine were prepared by sequentially diluting a 10 mg/L stock solution of atrazine with AS. The stock solution was prepared in AS using acetone as a carrier solvent to insure homogenous dissolution of atrazine. New stock solution was made up every 2 weeks and was conserved in darkness at 4 °C. Atrazine concentration of stock solutions was verified through high-performance liquid chromatography using C18 columns (15 cm × 4.6 mm) and acetonitrile:0.1 percent acetic acid (80:20) as the mobile phase. Detection was realized using quadrupole mass spectrometry and atmospheric pressure ionization electrospray. The detection limit for this method was 0.02 mg atrazine/L. Actual concentrations of the stock solutions did not deviate from the nominal concentration and averaged ($\pm$ standard error, $n=4$), 10.22 ± 0.414 mg atrazine/L. 17β-estradiol (E2) with a purity of 98 percent was obtained from Sigma-Aldrich, St-Louis, MO, USA. A stock solution of 5 mg/mL was prepared in acetone. The test solution of 100 μg/L was made up by diluting 20 μl of this stock solution into 1 L of AS.

2.4. Determination of body condition

Body condition was determined based on the residuals from the regression of body mass on snout-vent length, as described in Schulte-Hostedde et al. (2005). In this method, the average body weight for a given length is established through a regression line so that an individual with a positive residual is considered to be in a good condition whereas an individual with a negative residual is regarded as having a reduced body condition. In the current study, only data from control groups were used to perform the regression between body mass and snout-vent length, so that the resulting regression line would be representative of healthy subjects. The theoretical body weight value of frogs from both control and treated groups (obtained by introducing the length of the animal in the equation of the regression line) was then subtracted from the measured body weights so as to obtain a so-called "residual" value. Residuals therefore represent an expression of the difference existing between the measured weight of the metamorph and that of an average healthy subject with the same snout-vent length. The term "residual" is maintained here for consistency with other studies using a similar approach even though values from treated groups are not true residuals as these data points were not used to compute the regression line.

2.5. Data analysis

For every parameter examined (survival of tadpoles, T39, T42 and TCM, proportion of tadpoles reaching the different stages, intervals between stages, body length and weight, body condition), the control and solvent control groups were first compared by a *t*-test or by a rank sum test, if normality and equal variance could not be obtained. As differences were never observed between the two control groups, data from both groups were combined for further analyses. A four-parameter logistic regression equation was fitted for every treatment to the cumulative numbers of animals reaching stage 39, stage 42 or completing metamorphosis in function of exposure duration using GraphPad Prism software version 3.02. With each curve fitted, the software calculates the time for 50 percent of the individuals to reach the stage being examined (stage 39 (T39), stage 42 (T42) or completion of metamorphosis (TCM)). The duration of the intervals between stage 39 and stage 42, and between stage 42 and completion of metamorphosis were calculated by subtracting, for each replicate of every treatment, the value of T39 from the value of T42 and the value of T42 from the value of TCM.

The values of T39, T42 and TCM, the snout-vent lengths of the metamorphs, and the intervals between stage 42 and completion of metamorphosis, were compared amongst treatments using a one-way analysis of variance (ANOVA) followed by a Holm–Sidak test for multiple comparisons. For their part, the