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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

Julie C. Brodeur [a,*,1], Alina Sassone [b], Gladys N. Hermida [b], Nadia Codugnello [c,1]

[a] *Instituto de Recursos Biológicos, Centro Nacional de Investigaciones Agropecuarias (CNIA), Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Buenos Aires, Argentina*

[b] *Amphibian Biology Laboratory - Animal Histology, Biodiversity and Experimental Biology Department, Faculty of Exact Sciences, University of Buenos Aires, Buenos Aires, Argentina*

[c] *Ecology Laboratory, University National of Lujan, Ruta 5 y Avenida Constitución, Luján, Buenos Aires, Argentina*

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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).

* Corresponding author at: Instituto de Recursos Biologicos, Centro Nacional de Investigaciones Agropecuarias (CNIA), Instituto Nacional de Tecnologia Agropecuaria (INTA), 1686, Hurlingham, Buenos Aires, Argentina.
E-mail addresses: jbrodeur@cnia.inta.gov.ar, julbrodeur@hotmail.com (J.C. Brodeur).
[1] Members of the "Consejo Nacional de Investigaciones Científicas y Técnicas" (CONICET), Argentina.

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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

survival of tadpole, the intervals between stage 39 and stage 42, the proportions of individuals reaching stage 39, stage 42 or completing metamorphosis, the body mass of the metamorphs and the residuals describing body condition were compared amongst treatments using a non-parametric Kruskal–Wallis one-way ANOVA on Ranks, as normality and equal variance could not be obtained for these data. All *t*-tests, ANOVAs and multiple comparison tests were conducted using SigmaStat 3.11 statistical software (SPSS, Chicago, IL, USA). The criterion for significance was set at $p < 0.05$ in all cases.

3. Results

3.1. Survival

Survival of tadpoles (mean ± confidence interval) exposed to 0.1, 1, 10 and 100 μg atrazine/L ranged between 75.7 percent and 82.5 percent at the end of the experiment, and was not significantly different from that of the control group (74.2 ± 6.5 percent) or the estradiol-treated group (78.0 ± 8.5 percent). The only concentration of atrazine that generated significant mortality was 1000 μg/L, which reduced survival down to 52.0 ± 10.0 percent. Irrespectively of the atrazine treatment, most of the mortalities occurred during the first 20 days of exposure; the number of animals present in each groups varying little afterwards.

3.2. Timings of metamorphosis

The first individuals to reach stage 39 did so after 38 days of exposure. Of the tadpoles that were alive on this day, between 84 percent and 95 percent eventually completed metamorphosis, no significant difference existing in this proportion amongst the various treatment groups. Curves describing the progression of the metamorphosing tadpoles until stage 39, stage 42 and completion of metamorphosis are illustrated in Figs. 1a–3a, respectively. The presence of treatment-induced differences in the progression of metamorphosis was evaluated statistically by comparing the time necessary for 50 percent of the tadpoles to reach the three stages of development examined (T39, T42, and TCM). Data obtained demonstrate that tadpoles exposed to 1000 μg atrazine/L needed more time than controls in order to reach stage 39 (increased T39) whereas, oppositely, exposition to 1, 10 and 100 μg atrazine/L reduced the duration of T39 (Fig. 1b). This atrazine-induced acceleration of development exhibited a U-shaped concentration–response relationship; 10 μg atrazine/L causing a greater reduction of T39 than 1 and 100 μg/L, these last two concentrations generating a comparable effect (Fig. 1b).

A similar U-shaped pattern of acceleration of development in function of atrazine concentration was observed when considering T42 (Fig. 2b) or TCM (Fig. 3b). This observation, together with the absence of between-treatment difference in the duration of the intervals between stage 39 and stage 42, and between stage 42 and completion of metamorphosis (Fig. 4), indicate that the impact of atrazine on metamorphosis occurred entirely between stage 25 and stage 39 for concentrations between 1 and 100 μg/L. Interestingly, tadpoles treated with 100 μg/L of 17β-estradiol presented an acceleration of development of the same amplitude and characteristics as the one observed in tadpoles treated with 1 and 100 μg/L of atrazine (Figs. 1–3).

The effect on metamorphosis generated by the exposure to 1000 μg atrazine/L differed from that caused by an exposure to lower concentrations of atrazine. Indeed, the initial deceleration of development observed between stage 25 and stage 39 (Fig. 1b) was followed by a decrease in the duration of the interval between stage 39 and stage 42, which caused the overall duration of metamorphosis to be similar to controls (Fig. 3b). For its part, 0.1 μg atrazine/L was the only concentration of atrazine tested

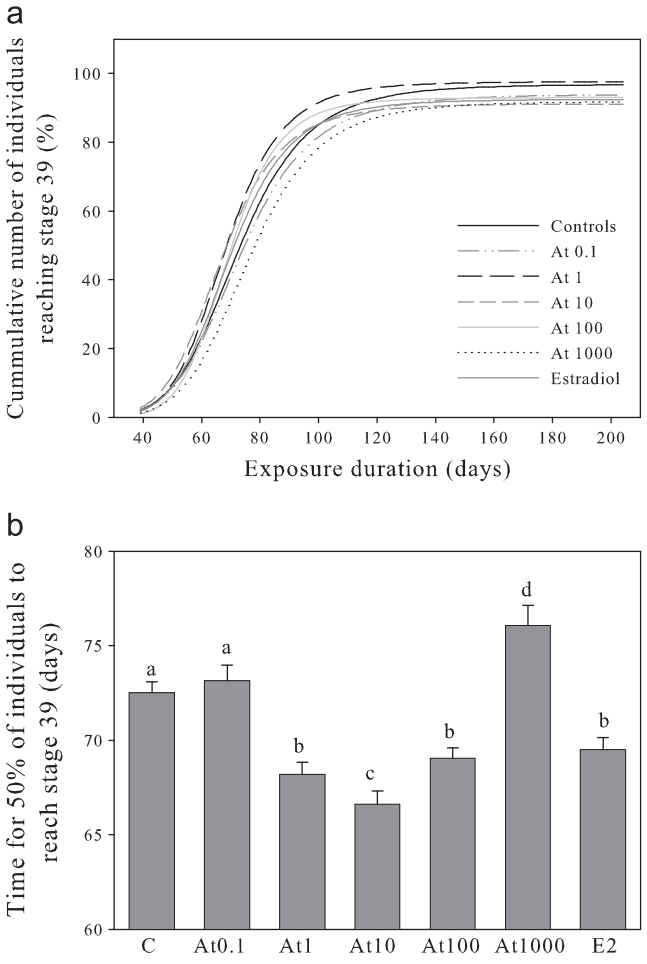

Fig. 1. (a) Cumulative number of individuals reaching stage 39 over time for control larvae of *Rhinella arenarum* and larvae exposed to atrazine and 17β-estradiol. The lines represent the sigmoidal curve models calculated for every treatment. (b) Time required (mean ± S.E.) for 50 percent of the larvae of *Rhinella arenarum* to reach stage 39 for controls and larvae exposed to atrazine and estradiol. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2 = tadpoles exposed to 100 μg/L of 17β-estradiol. Bars with the same letter are not significantly different ($P < 0.05$).

that did not affect the timings of metamorphosis, this concentration therefore representing the no observed effect concentration (NOEC).

3.3. Body size and condition at completion of metamorphosis

Tadpoles exposed to 17β-estradiol and to 1 and 10 μg/L of atrazine transformed into significantly longer and heavier metamorphs than controls (Fig. 5). A similar result was obtained when body size data were examined in terms of body condition, with the addition that a significant difference was also found for tadpoles exposed to 100 μg/L of atrazine, the concentration–response curve clearly taking the form of an inverted U in this case (Fig. 6). Again, the NOEC for size and condition at metamorphosis was 0.1 μg/L.

4. Discussion

Past studies regarding the effects of atrazine on developmental rate have reported all three possible types of effect: acceleration, deceleration, and the absence of effect. However, with the

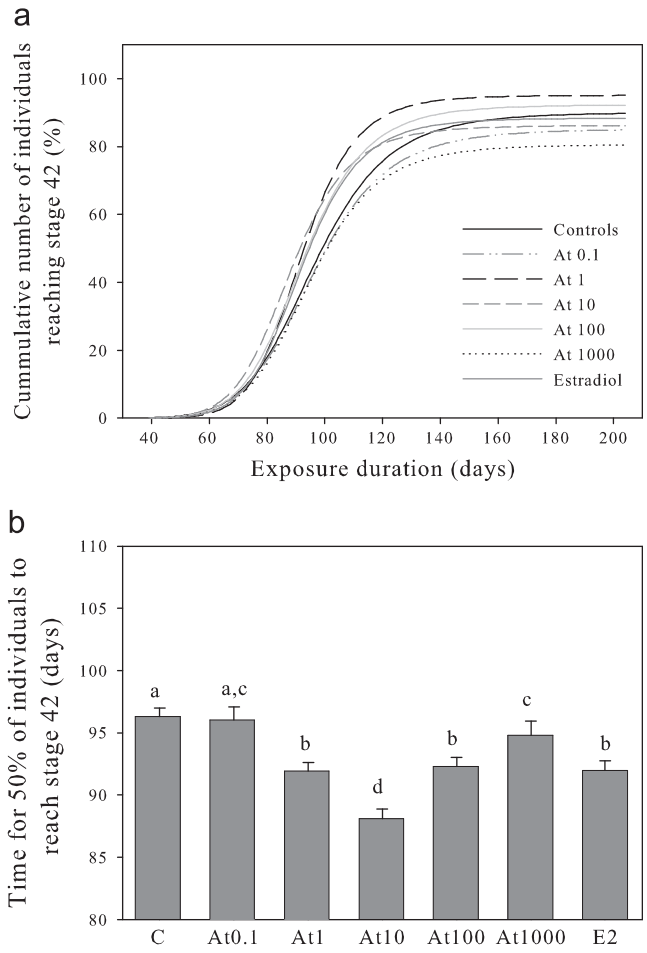

Fig. 2. (a) Cumulative number of individuals reaching stage 42 over time for control larvae of *Rhinella arenarum* and larvae exposed to atrazine and 17β-estradiol. The lines represent the sigmoidal curve models calculated for every treatment. (b) Time required (mean ± S.E.) for 50 percent of the larvae of *Rhinella arenarum* to reach stage 42 for controls and larvae exposed to atrazine and estradiol. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2=tadpoles exposed to 100 μg/L of 17β-estradiol. Bars with the same letter are not significantly different ($P < 0.05$).

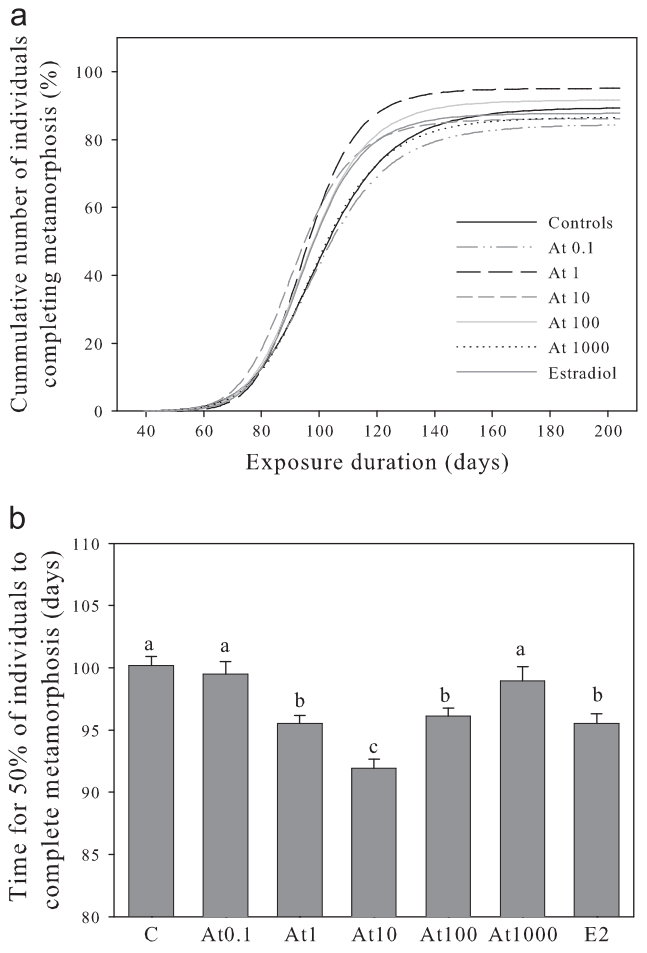

Fig. 3. (a) Cumulative number of individuals completing metamorphosis over time for control larvae of *Rhinella arenarum* and larvae exposed to atrazine and 17β-estradiol. The lines represent the sigmoidal curve models calculated for every treatment. (b) Time required (mean ± S.E.) for 50 percent of the larvae of *Rhinella arenarum* to complete metamorphosis for controls and larvae exposed to atrazine and estradiol. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2=tadpoles exposed to 100 μg/L of 17β-estradiol. Bars with the same letter are not significantly different ($P < 0.05$).

exception of one study that detected a deceleration of metamorphosis at 10 μg/L of atrazine (Coady et al., 2004), all seven other studies to have reported alterations of metamorphic rate did so for concentrations of atrazine between 100 and 1000 μg/L (Briston and Threlkeld, 1998; Diana et al., 2000; Freeman et al., 2005; Brodeur et al., 2009; Brown Sullivan and Spence, 2003; Freeman and Rayburn, 2005; Zaya et al., 2011). The current study therefore constitutes one of the first reports of an alteration of developmental rate at low atrazine concentrations; an acceleration of metamorphosis being observed in tadpoles exposed to atrazine concentrations in the range of 1–100 μg/L.

Moreover, the observed acceleration of metamorphosis followed a non-monotonic U-shaped concentration–response relationship; 10 μg/L of atrazine having a significantly greater effect than 1 and 100 μg/L; these two concentrations producing an equivalent effect. Similar non-monotonic accelerations or decelerations of the time needed to complete metamorphosis have previously been observed in *R. arenarum*, *Bufo americanus*, *Hyla versicolor* and *Rana clamitans* (Diana et al., 2000; Coady et al., 2004; Freeman et al., 2005; Brodeur et al., 2009). In our previous study with *R. arenarum* (Brodeur et al., 2009), exposures to atrazine started from stage 38 and continued until completion of metamorphosis. Under this experimental design, the U-shaped acceleration of metamorphosis was found to occur between stages 38 and 42 at 100 and 1000 μg/L of atrazine. Comparatively, in the current study, the U-shaped acceleration of the metamorphic process occurred entirely between stages 25 and 39 for concentrations from 1 to 100 μg/L; treated tadpoles proceeding through metamorphosis as control animals beyond this point. Only tadpoles exposed to 1000 μg/L of atrazine did present the acceleration of metamorphosis between stage 39 and 42 in both studies. However, in contrast to previous results (Brodeur et al., 2009), the overall duration of metamorphosis of this treatment group did not differ from controls as the acceleration of metamorphosis was preceded by an important deceleration of developmental rate (between stages 25 and 39).

Another difference between our two studies lie in the fact that, in the present study, the time needed for tail resorption did not significantly differ between controls and treated groups whereas a delay in tail resorption had previously been noted with 100 and 1000 μg/L of atrazine (Brodeur et al., 2009). Overall, the series of differences and similarities observed between the two studies we realized with *R. arenarum* illustrates how variations in the way tadpoles are exposed may alter the response to atrazine, even though some aspects of this response may be maintained. Furthermore, evidences gathered in this and previous studies

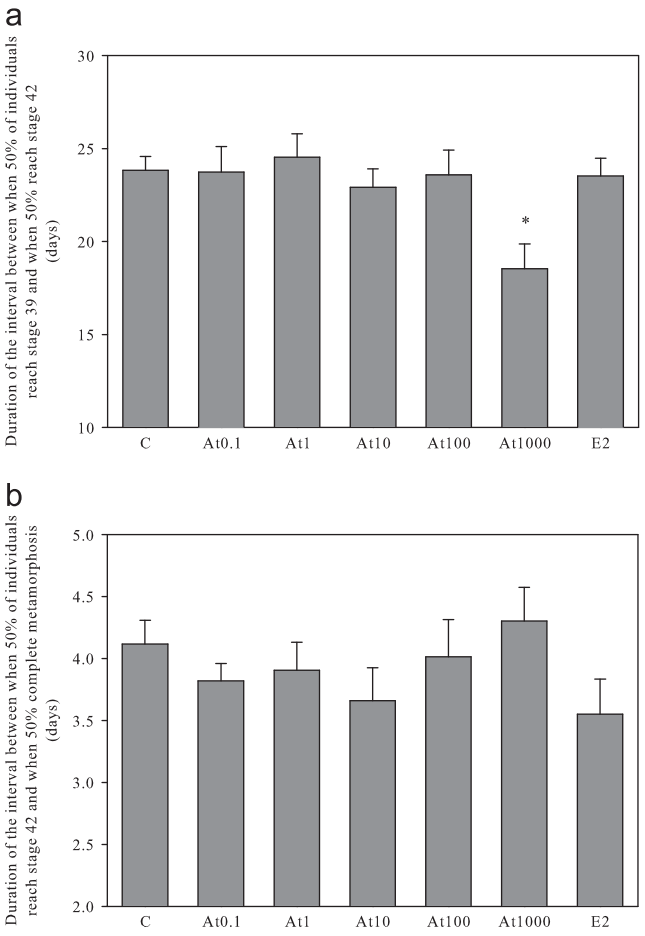

Fig. 4. (a) Duration of the interval (mean ± S.E.) between when 50% of *Rhinella arenarum* larvae reach stage 39 and when 50% reach stage 42. (b) Duration of the interval (mean ± S.E.) between when 50% of *Rhinella arenarum* larvae reach stage 42 and when 50% complete metamorphosis. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2 = tadpoles exposed to 100 μg/L of 17ß-estradiol. * = significantly different from controls ($P < 0.05$).

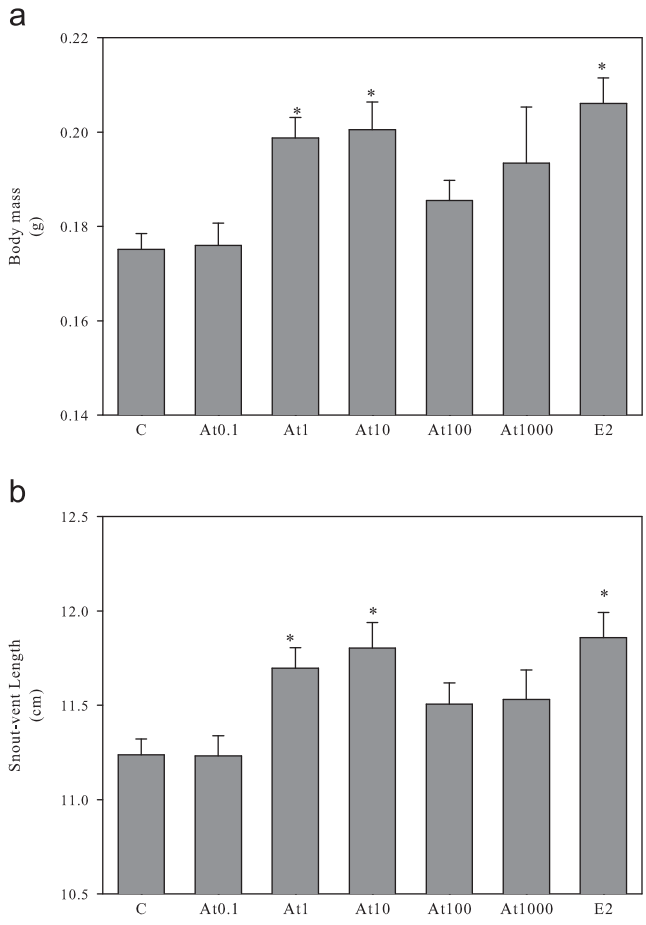

Fig. 5. (a) Body mass at completion of metamorphosis (mean ± S.E.) for control larvae of *Rhinella arenarum* and larvae exposed to atrazine and 17ß-estradiol. (b) Snout-vent length at completion of metamorphosis (mean ± S.E.) for control larvae of *Rhinella arenarumand* larvae exposed to atrazine and 17ß-estradiol. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2 = tadpoles exposed to 100 μg/L of 17ß-estradiol. * = significantly different from controls ($P < 0.05$).

indicate that the total amount of time needed to complete metamorphosis is in fact constituted of distinct "steps" or "stages"; that can individually be accelerated or decelerated by atrazine. Consequently, the global effect of atrazine on total time to metamorphosis is the result of the amplitude and direction of the change in rate observed at every stage. This means that there may be situations, as occurred in the present study with 1000 μg/L of atrazine, were the sum of the alterations in metamorphic rate cancel themselves and where the total time to metamorphosis is unaffected even though the timings of metamorphosis are altered. In view of the above, care should therefore be taken when considering studies reporting the absence of effect if total time to metamorphosis is the only parameter informed. The collection of such studies may serve to enhance the weight-of-evidence towards an absence of effect even though the results presented in these studies are not as decisive as they may seem. Knowing that atrazine is affecting development is important in cases where total time to metamorphosis is not altered as other unstudied physiological alterations may also be occurring.

The plasticity of the response to atrazine described above is an illustration of the complexity and flexibility of the metamorphic process and of the inherent variability that is generated when this process is altered by an exposure to chemicals or other environmental factors. This natural variability implies that a considerable sample size should be employed in order to allow a sensitive detection of variations in metamorphic rate (OECD, 2004; Rohr and McCoy, 2010). For instance, although the current study provides the first report of an alteration of developmental rate by a concentration of atrazine as low as 1 μg/L, seven studies have previously examined atrazine concentrations of this range; all of them failing to detect an effect. If sample size is considered, however, it emerges that only two of these seven studies were well-replicated studies (Carr et al., 2003; Kloas et al., 2009), the five other studies being based on much smaller sample sizes (Storrs and Semlitsch, 2008; Oka et al., 2008; Williams and Semlitsch, 2010; Spolyarich et al., 2010; Langlois et al., 2010). If these five studies are disregarded because the absence of effect may be due to the low power of the analysis, the weight-of-evidence towards an absence of effect shifts considerably from 7:1 to 2:1. Under this new perspective, the fact that both well-replicated studies that failed to detect an effect were conducted on *Xenopus laevis* is an important element. Indeed, as adults of *X. laevis* are aquatic and the completion of metamorphosis is not associated to an emergence of the water, it is likely that the metamorphic program of this species is much different from the one of *R. arenarum*, which need to emerge of the water before the temporary pond they inhabit dry out.

In conjunction with the important sample size used, the detection of an effect at low concentrations of atrazine may have been facilitated in the current study by the use of values corresponding to the time required for 50 percent of the

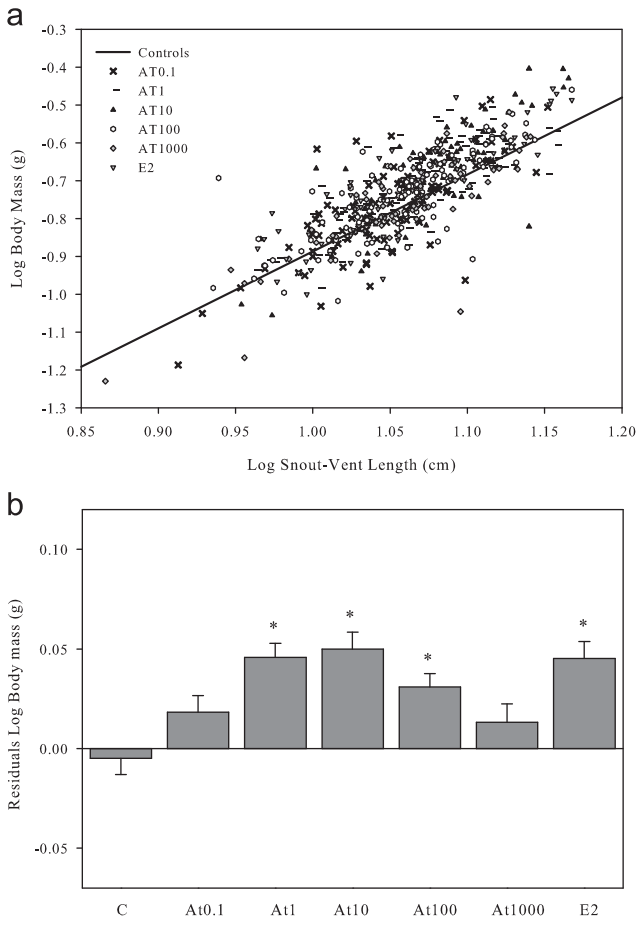

Fig. 6. (a) Distribution of body mass in function of snout-vent length for recently metamorphosed control larvae of *Rhinella arenarum* and larvae exposed to atrazine and 17b-estradiol. The regression line describes the relationship observed between body mass and snout-vent length in control animals. (b) Residual (mean ± S.E.) between measured body mass and the theoretical body mass of a control animal calculated by applying the equation of the regression line presented above to snout-vent length data of animals exposed to atrazine and 17b-estradiol. Controls are denoted by C. The number indicated at the side of the abbreviation "At" represents the concentration of atrazine in μg/L. E2 = tadpoles exposed to 100 μg/L of 17ß-estradiol. * = significantly different from controls ($P < 0.05$).

individuals to reach the stages examined instead of values representing 100 percent of the individuals. The rationale for employing such an approach is the same that is used to justify the use of parameters such as the lethal dose 50 (LD50) or the lethal concentration 50 (LC50) in toxicology: the least variability in the sigmoid curve illustrating the data is obtained at the 50 percent level of response (Rand et al., 1995). Although metamorphosis data of invertebrates or amphibians are commonly expressed in terms of 50 percent metamorphosis (Wendt, 1996; Forward et al., 2001; Balch et al., 2006), this approach has been little employed in the literature reporting on the effects of atrazine on amphibians.

Together with reducing age at metamorphosis, atrazine concentrations between 1 and 100 μg/L also caused tadpoles to transform into significantly longer and heavier metamorphs of superior body condition. As was the case for developmental rate, the concentration–response curve representing the effects of atrazine on body weight, length and condition was also non-monotonic, although in this case, the curve took the form of an inverted U. The current study is the first to report an increase of size at metamorphosis in larvae exposed to atrazine, previous studies generally reporting an absence of effect at low atrazine concentrations (Carr et al., 2003; Coady et al., 2004; Kloas et al., 2009; Langlois et al., 2010; Williams and Semlitsch, 2010) or a reduction of body size at higher concentrations of atrazine (Diana et al., 2000; Brown Sullivan and Spence, 2003; Orton et al., 2006; Choung et al., 2011; Spolyarich et al., 2010; Zaya et al., 2011). An exception to this general rule is the publication of Hayes et al. (2006), which reported a significant reduction of body weight and length at metamorphosis in tadpoles exposed to 0.1 μg/L.

Interestingly, tadpoles exposed to 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. Similar accelerations of metamorphosis in response to estradiol have previously been demonstrated by Coady et al. (2004) and Bauer-Dantoin and Meinhardt (2010), but inhibitions of developmental rate were also observed in other studies (Gray and Janssens, 1990; Hogan et al., 2008; Kloas et al., 2009; Sharma and Patiño, 2010). Although the current study was not designed to examine potential mechanisms by which atrazine might influence metamorphosis, general hypotheses can be proposed regarding potential modes of action. First, the apparent coherence between the responses to atrazine and estradiol observed in the present study would appear to suggest an estrogenic mode of action. Such a mechanism is plausible given that atrazine has been shown to exert estrogenic effects in a number of biological systems, including in amphibians (Sanderson et al., 2001; Holloway et al., 2008; Hayes et al., 2011).

A mode of action involving a steroid hormone receptor would be coherent with the non-monotonic concentration–response curves observed in the current study as non-monotonic curves are characteristic of physiological responses to estradiol and other hormones acting through steroid hormone receptors (Calabrese et al., 2007; Bauer-Dantoin and Meinhardt, 2010; Peterson Myers et al., 2009). Although the mechanisms underlying these non-monotonic concentration–response behaviors are not yet fully understood, they are possibly due to the inherently nonlinear process of receptor homodimerization implicated in steroid hormone receptor signaling (Li et al., 2007).

Aside from an interaction with the estrogen-receptor, the non-monotonic effects of atrazine on metamorphosis could also be the result of an interaction with the glucocorticoid receptor, another category of steroid hormone receptor. Indeed, in contrast with mammals and adult amphibians, amphibian larvae present the distinctive feature that hypothalamic control of TSH release, the causative factor governing anuran metamorphosis through the induction of thyroid hormones release, is mediated through the corticotropin-releasing factor (CRF) instead of the thyrotropin-releasing factor (TRF) (Denver and Licht, 1989; Shi, 2000; Okada et al., 2004). Given that the more classical function of CRF, the stimulation of the hypothalamic-pituitary-interrenal axis (HPI) responsible for the secretion of glucocorticoids and the non-specific stress response, is also operational in larval amphibians, there results an intricate relationship between the two hormonal axes (Hayes, 2000).

On the whole, the current study demonstrated that *R. arenarum* tadpoles exposed to environmentally-relevant concentrations of atrazine metamorphose earlier and at a greater size than control animals; possibly due to an estrogenic action of atrazine. As short larval period and large size at metamorphosis are generally assumed to confer greater fitness (Wilbur and Collins, 1973; Werner, 1986), the observed impacts of atrazine on larval development may offer the impression to entail limited ecological consequences. However, further investigation should be conducted before reaching such a conclusion, as the implications of age and size at metamorphosis for the fitness of individuals are complex and have been, overall, studied in a limited number of species and/or circumstances. Moreover, there remains the

possibility that other unstudied parameters are also at play to interfere any positive effects that short larval period and large size at metamorphosis may have on fitness. For example, Beck and Congdon (2000) demonstrated that, although decreases in larval duration and increases in mass at metamorphosis were advantageous in terms of locomotor performance in *Bufo terrestris*, this positive influence was weakened by the fact that the exact same characteristics were associated to increased energy expenditures for maintenance. Similarly, Gervasi and Foufopoulos (2008) found that shorter developmental times induced by desiccation were associated to lower total leukocyte number and weaker cellular immune system responses in *Rana sylvatica*. Considering the low concentrations at which atrazine was found to alter metamorphosis in *R. arenarum*, the needs to further study the ecological implications of the findings described in the present study would appear to be pressing.

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