Research Article

Differential acute sensitivity of wild *Rana sylvatica* and laboratory *Xenopus laevis* tadpoles to the herbicide atrazine

Robert H. Floyd[1], Jenna Wade[2], D. Andrew Crain

Department of Biology, Maryville College, Maryville, Tennessee 37803
[1]Current Address: Biology Department, Jacksonville State University, AL
[2]Current Address: 840 H Wyckford Drive, Indianapolis, IN 46214

Abstract. Atrazine is one of the most used herbicides in the United States and has previously been implicated in local amphibian declines. The purpose of this study was to examine the influence of atrazine on 2 frog species—the amphibian model species *Xenopus laevis* and the wood frog *Rana sylvatica*. Tadpoles just before hindlimb emergence were exposed to either 1 part per billion (ppb) or 25 ppb atrazine in their water. Behavior was observed daily, and at the end of 15 days exposure the tadpoles were weighed, developmentally staged, and measured for head width and tail width. Differences were found among all of these for the *X. laevis* ($p < 0.002$), with the difference occurring only for the low dose animals (and not the high dose animals) for weight, stage, and tail width. Head width was significantly different for the *X. laevis* exposed to both the high and low concentrations of atrazine. No morphological differences were seen in *R. sylvatica*, but increased mortality and behavioral differences were seen in the *R. sylvatica* high dose group. These results suggest that: (1) *Xenopus laevis* may not be an adequate model for all anurans (2) low doses of atrazine have a greater influence than high doses in *X. laevis*, and (3) *Rana sylvatica* is more sensitive in lethal but not sublethal endpoints.

Introduction

The scientific community has recognized global declines in amphibian populations since the early 1990s (Blaustein and Wake, 1990). These amphibian declines are due to both natural and anthropogenic causes (Corn, 2000), and much of the anthropogenic etiology is attributed to human release and redistribution of environmental toxicants. Amphibians are particularly sensitive to aquatic-borne toxicants due to their anatomy, physiology, and ecology (Henry, 2000). The recognition that amphibians may be at elevated risk to environmental toxicants has led to the use of amphibians as sentinels of aquatic toxicity and to the standardization of experimental protocols using amphibians. Due to the ease of animal husbandry and the ability to induce breeding year-round, the African clawed frog *Xenopus laevis* has become the model amphibian used in both acute (e.g., Mitch-

Correspondence to: Robert H. Floyd, Biology Department, Martin Hall, Jacksonville State University, 700 Pelham Rd., Jacksonville, AL 36265; phone: (256) 689–0039; e-mail: jsu0286n@jsu.edu

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ell et al., 2005; Gauthier et al., 2006) and chronic (e.g., Huang et al., 2005) toxicology studies.

Xenopus laevis tadpoles have been used extensively to examine the potential detrimental effects of the herbicide atrazine. Atrazine is one of the most heavily used pesticides in the world, with over 70 million pounds being applied in the United States each year (Withgott, 2002). As a result of its ability to inhibit Photosystem II in dicots but not alter monocots, atrazine is used extensively on agricultural crops such as corn, sorghum, and sugar cane as well as on residential lawns and golf courses. Numerous studies have raised concern that chronic exposure of developing tadpoles to environmentally relevant concentrations of atrazine can have detrimental sublethal effects such as demasculinization of male *X. laevis* frogs, with males exhibiting decreased testosterone concentrations, smaller laryngeal openings, and hermaphroditism (Hayes, 2004). In addition, acute exposure of developing *X. laevis* tadpoles to environmentally relevant concentrations of atrazine causes developmental delays (Freeman and Rayburn, 2005).

One question deserving attention is how good *X. laevis* is as a model for other anurans. The purpose of this study is to compare the effect of environmentally relevant concentrations of atrazine on laboratory reared *X. laevis* tadpoles and wild wood frog (*Rana sylvatica*) tadpoles. It is hypothesized that exposure to atrazine will affect the development of both frog species in a dose-dependent manner, with higher concentrations of atrazine inhibiting growth (as indicated by body weight) and development (as indicated by developmental stage).

Methods and Materials

Animals and treatment groups

Twenty-four *Xenopus laevis* tadpoles between developmental stages 52–55 (Nieuwkoop and Faber, 1994) were obtained from Nasco (Fort Atkinson, WI) and divided equally between 3 identical containers, and an equal number of wild-collected *Rana sylvatica* tadpoles at developmental stages 33–35 (Gosner, 1960) were divided into an additional 3 containers. These stages were chosen because they have corresponding morphology and are the stages just before hind limb buds begin to form. The *R. sylvatica* used for this experiment had been taken from the wild as part of a large egg mass that was hatched in the laboratory. Once separated, all animals were housed in an incubator at 20 °C with a photoperiod of 12 hours light: 12 hours dark. Food was provided in excess daily throughout the experiment.

Using the experimental procedures of Coady et al. (2005) as a guideline for determining atrazine concentration, groups of 8 *X. laevis* and 8 *R. sylvatica* were exposed to 1 μg/L (1 part per billion) of atrazine (98% pure, Chem Service, West Chester, PA; atrazine was introduced to the water in 40 μL aliquots), 25 μg/L of atrazine, or 40 μL of 95 % ethanol. Atrazine was diluted in 95 % ethanol, and thus the control tadpoles of each species were also exposed to an equal quantity of ethanol. New dechlorinated water and new atrazine or ethanol were given to the tadpoles every 5 days for 15 days.

Endpoints measured

Observations were made daily; deceased tadpoles were removed from their containers as soon as being found lifeless. After 15 days the tadpoles were anesthetized using MS222 (0.05%, Sigma, St. Louis, MO), staged using Nieuwkoop and Faber staging for *Xenopus* and Gosner staging for *R. sylvatica*, and weighed. Head width just posterior to the eyeballs and tail width at the base of the tail were measured using calipers (see Figure 1).

Statistical analysis

One-way ANOVA followed by Fisher's post-hoc analysis (Statview Version 5.0.1, SAS Institute Inc, Cary, NC) was used to statistically ana-

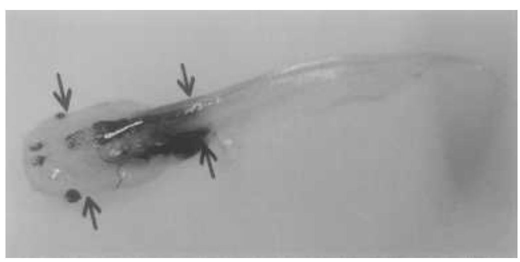

Figure 1. *Xenopus laevis* tadpole with arrows indicating where head width and tail width were measured.

Table 1. Mean and standard deviation for the stage, body weight, and tail width of *Xenopus laevis* and *Rana sylvatica* tadpoles exposed to 0, 1, or 25 µg/L of atrazine. For each species, superscripts "b" denotes statistical significance at P < 0.05 for the measured endpoint; similarly, superscript "a" indicates no significant difference from the control group.

Species	Concentration of atrazine	Stage mean/sd	Body weight (g) mean/sd	Head width (mm) mean/sd	Tail width (mm) mean/sd
Xenopus laevis	0µg/L	56.71 [a]/0.49	0.74 [a] /0.14	10.37 [a] /0.38	4.00 [a] /0.27
	1µg/L	55.38 [b]/1.06	0.44 [b] /0.19	8.40 [b] /0.986	3.09 [b] /0.62
	25µg/L	56.63 [a] /0.52	0.61 [a] /0.12	8.64 [b] /1.29	3.71 [a] /0.53
Rana sylvatica	0µg/L	38.50/0.76	0.88/0.19	9.75/0.825	4.11/0.24
	1µg/L	38.62/0.74	0.87/0.21	9.14/1.32	4.34/0.39
	25µg/L	37.67/2.31	0.91/0.55	9.20/2.86	4.27/0.59

lyze all qualitative data. Due to obvious differences in body type that naturally occur between these species these statistics were not compared between the *X. laevis* and *R. sylvatica*.

Results

After exposure to atrazine or ethanol for 15 days, one-way ANOVA and Fisher's post-hoc analysis revealed significant differences among the *Xenopus laevis* tadpoles. Differences were found among treatment groups for stage ($p = 0.003$), body weight ($p = 0.003$), head width ($p = 0.002$), and tail width ($p = 0.007$). No significant differences were found among the *Rana sylvatica* tadpoles. Means and standard deviations as well as Fisher's post-hoc results are presented in Table 1.

The most surprising results uncovered were that the smaller concentration of atrazine correlated to a significantly smaller head size and tail with whereas the larger concentration did not. In addition, the *X. laevis* exposed to smaller concentrations were also significantly earlier in developmental stage following Nieuwkoop and Faber (1994). *Xenopus laevis* average head with was significantly smaller for both concentrations of atrazine.

Whereas the *Xenopus* tadpoles showed significant morphological differences, the *R. sylvatica* tadpoles displayed noticeable behavioral differences. The *R. sylvatica* tadpoles exposed to 25 µg/L atrazine showed very little swimming activity and apparent disrupted equilibrium compared to the controls and the *X. laevis* in all concentrations. The *R. sylvatica* often had to be physically touched to induce swimming activity and frequently fell on their side when they stopped swimming. In contrast, the *X. laevis* were very active and showed no obvious evidence of disrupted equilibrium.

In addition, there was a high mortality rate in the *R. sylvatica* exposed to 25 µg/L of atrazine. Five of the 8 tadpoles in this group died by the end of the experiment, whereas there was no mortality in any other *X. laevis* or *R. sylvatica* group.

There was also one abnormally small tadpole among the *R. sylvatica* exposed to the high concentration. Despite having been of similar size prior to atrazine exposure, this particular tadpole experienced very little growth and development compared to the others in the same concentration, and, due to its especially low level of activity, it developed a wound caused by attempted predation from the other tadpoles. Figure 2 shows the smaller tadpole compared to others in the same concentration.

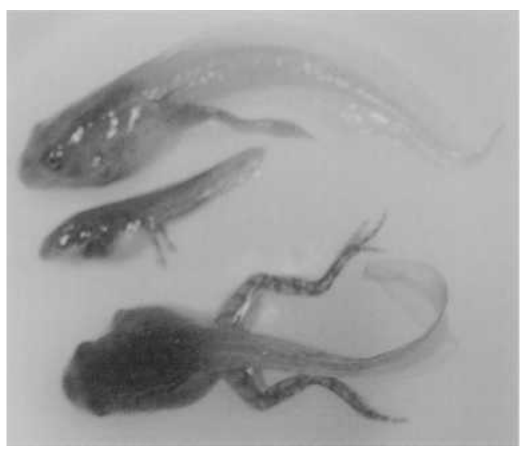

Figure 2. Digital image of 3 surviving *Rana sylvatica* tadpoles exposed to 25 µg/L of atrazine after 15 days of exposure. Despite their size differences, all three individuals were originally exposed between developmental stages 33–35 (Nieuwkoop and Faber, 1994).

Discussion

The results indicate that *Xenopus laevis* and *Rana sylvatica* respond very differently to environmental concentrations of atrazine, with *X. laevis* exhibiting morphological abnormalities and *R. sylvatica* showing behavioral changes. One part per billion (ppb) of atrazine caused morphological abnormalities in *X. laevis* stage, body weight, head width, and tail width, but only one morphological endpoint (head width) was altered by 25 ppb atrazine. No morphological effects were noted in *R. sylvatica*, but 25 ppb induced behavioral abnormalities and increased mortality. These different responses between *X. laevis* and *R. sylvatica* show that *X. laevis* is not an adequate model for determining the effect of atrazine on all anuran species.

While no morphological alterations were evident among treatment groups of *R. sylvatica*, the wood frogs appeared more sensitive to high concentrations of atrazine as 62.5 % of them died in the 25 μg/L treatment group whereas there were no mortalities in any of the other groups. The wood frog tadpoles surviving the 25 μg/L treatment showed altered swimming and sluggish behavior, which in the wild would have severe consequences for these individuals due to an inability to escape predation.

For stage, body weight, and tail width, the low dose of atrazine (1 μg/L) induced greater response than the high dose; for all of these endpoints there was no difference between the control and 25 μg/L groups, but the differences between the control and 1 μg/L animals were highly significant. While these results were unexpected and initially surprising, they are consistent with numerous other studies of the effect of atrazine on *X. laevis* in particular and toxicant effects on organisms in general. Recently, the field of toxicology has acknowledged that low doses often have greater effects than high doses, resulting in an inverted-U shaped dose-response curve (Calabrese and Baldwin, 1998). Indeed, this low-dose stimulation and high-dose inhibition may be more common than traditional dose-response models where higher dosages induce greater effects (Calabrese, 2005). While there is no single explanation for the greater effect of lower dosages, it is thought that animals are able to physiologically respond to and counteract higher doses but not lower ones, resulting in deleterious effects of only the lower doses. This "homeostatic hypothesis" is gaining support in the literature, but requires much future examination in a variety of disciplines such as physiology, developmental biology, and toxicology (Stebbing, 2003).

The present study is not the first to find that lower doses of atrazine have a greater effect than higher doses. Lower concentrations of atrazine caused greater mortality than higher doses in 3 species of frogs (including *R. sylvatica*; Storrs and Kiesecker, 2004). While we did not see such an effect on mortality, our study was only 15 days whereas Storrs and Kiesecker (2004) began to see significant mortality at approximately that time. In addition to mortality, some reproductive endpoints are affected more by lower doses of atrazine than higher doses. When American leopard frogs (*Rana pipiens*) are exposed to environmentally relevant doses of atrazine, male frogs develop poorly organized testes or testicular oocytes (becoming hermaphroditic; Hayes et al., 2003). Surprisingly, such hermaphroditism and gonadal dysgenesis is more pronounced at 0.1 ppb than at 1 ppb (Hayes et al., 2003). In the present study, 1 ppb induced morphological changes in *X. laevis* (but not *R. sylvatica*) whereas 25 ppb did not.

The results from both *X. laevis* and *R. sylvatica* warrant much future research. First, the low-dose effects on morphological endpoints in *X. laevis* should be examined further by use of more and lower dosages of atrazine. Second, studies should examine the influence of even lower dosages on *R. sylvatica* to determine if a morphological inverted-U effect would occur in this species. Third, and perhaps most interesting, the chronic behavioral effects of atrazine on *R. sylvatica* should be examined. Specifically, the question of what happens to those tadpoles that survive a high-dose atrazine exposure must be addressed. It is hoped that such future studies will elucidate the complex effects of atrazine on anurans.

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