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Atrazine exposure affects growth, body condition and liver health in *Xenopus laevis* tadpoles

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ABSTRACT

Six studies were performed regarding the effects of atrazine, the most frequently detected pesticide in fresh water in the US, on developing *Xenopus laevis* tadpoles exposed 5 days post-hatch through Nieuwkoop Faber Stage 62. The levels of atrazine tested included those potentially found in puddles, vernal ponds and runoff soon after application (200 and 400 μg/L) and a low level studied by a number of other investigators (25 μg/L). One study tested 0, 25 and 200 μg/L, another tested 0, 200 and 400 μg/L, while the remaining four studies tested 0 and 400 μg/L. During all exposures, mortality, growth, metamorphosis, sex ratio, fat body (a lipid storage organ) size and liver weights, both relative to body weight, were evaluated. In selected studies, feeding behavior was recorded, livers and fat bodies were histologically evaluated, liver glycogen and lipid content were determined by image analysis, and immunohistochemical detection of activated caspase-3 in hepatocytes was performed. The NOEC was 25 μg/L. None of these exposure levels changed sex ratios nor were intersex gonads noted, however, no definitive histological evaluation of the gonads was performed. Although a marginal increase in mortality at the 200 μg/L level was noted, this was not statistically significant. Nor was there an increase in mortality at 400 μg/L versus controls. At the 400 μg/L level, tadpoles were smaller than controls by 72 h of exposure and remained smaller throughout the entire exposure. Appetite was not decreased at any exposure level. Slowed metamorphosis was noted only at 400 μg/L in two of five studies. Livers were significantly smaller in the study that tested both 200 and 400 μg/L, yet no pathological changes or differences in glycogen or lipid stores were noted. However, livers from 400 μg/L exposed tadpoles had higher numbers of activated caspase-3 immunopositive cells suggesting increased rates of apoptosis. Fat body size decreased significantly after exposure to 200 and 400 μg/L although these organs still contained some lipid and lacked any pathology. Since this was noted across all studies, it was considered the most sensitive indicator of atrazine exposure measured. The changes noted in body and organ size at 200 and 400 μg/L atrazine indicated exposure throughout development compromised the tadpoles. Significant reductions in fat body size could potentially decrease their ability to survive the stresses of metamorphosis or reduce reproductive fitness as frogs rely on stored lipids for these processes.

1. Introduction

The presence of agricultural, pharmaceutical and industrial chemicals in our environment is ubiquitous. As these chemicals become more intensively and widely used, they are being detected in more places and at higher concentrations than before. These chemicals have been implicated in decreases in population of both endangered and sentinel frog species (Stuart et al., 2004). Exposures result in adverse affects including immunosuppression, loss of fertility and fecundity, developmental abnormalities, behavioral changes, and mortality that, in conjunction with habitat loss, climate change, and emerging infectious disease, have resulted in the amphibian population losses noted by scientists worldwide (Stuart et al., 2004).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most widely used and commonly detected herbicides in North America and receives a great deal of scientific scrutiny by both governmental and academic investigators. It is a known endocrine disruptor in mammals, birds, reptiles, fish and amphibians (Bisson and Hontela, 2002; Fan et al., 2008; Hayes et al., 2002, 2003; Holloway et al., 2008; McMullin et al., 2004; Stoker et al.,

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2002) affecting normal reproductive function and development in these organisms. In studies with *Xenopus laevis* tadpoles, changes in gonadal development were noted after exposures to low, environmentally relevant concentrations (Hayes et al., 2002, 2003; Tavera-Mendoza et al., 2001, 2002). Consequently, much research effort has been focused on studying atrazine's reproductive effects on amphibian species at these low exposure levels (Carr et al., 2003; Hayes et al., 2002, 2003; Hecker et al., 2004; Jooste et al., 2005; Kloas et al., 2009; Langlois et al., 2010; Murphy et al., 2006; Oka et al., 2008; Rohr et al., 2008). However, these studies have generated equivocal results.

In addition to the effects on gonadal development, previous studies have shown that atrazine exposure causes a variety of additional adverse effects in frogs. Using exposure levels starting as low as 0.1 μg/L, levels frequently found in ground and surface water (EPA, 2009), several investigators have reported delays in metamorphosis in tadpoles from several frog species (Coady et al., 2004; Freeman and Rayburn, 2005; LaFiandra et al., 2008; Langlois et al., 2010; Rohr et al., 2008). In contrast, many others have found that metamorphic rate was not affected after exposure to similar nominal concentrations (Carr et al., 2003; Coady et al., 2005; Hayes et al., 2002, 2003; Kloas et al., 2009; Oka et al., 2008). These differences could, in part, be explained by differences in experimental design and which species were used. Atrazine was also shown to be immunotoxic for both adult and developing northern leopard frogs after short exposures to concentrations of less than 3 μg/L, levels of atrazine most frequently found in ground water (Brodkin et al., 2007; Houck and Sessions, 2006; Rohr et al., 2008).

At less frequently reported environmental levels of atrazine, concentrations of 3–150 μg/L (Huber, 1993), investigators observed increased mortality in a number of frog species (Solomon et al., 2008; Storrs and Kiesecker, 2004). Interestingly, tadpoles exposed to 3 μg/L exhibited greater mortality rates that those exposed to 30 and 100 μg/L (Storrs and Kiesecker, 2004). Additionally, tadpoles exposed to 200 μg/L of atrazine were lighter and shorter than their unexposed counterparts (Diana et al., 2000). However, when concentrations of less than 200 μg/L were used, other investigators did not note these changes (Coady et al., 2005; Hayes et al., 2003; Kloas et al., 2009).

When exposures were to higher concentrations, atrazine was found to cause a variety of adverse effects. Allran and Karasov (2001) showed that of 2×10^4 μg/L atrazine increased respiration rates and induced anorexia in adults of Northern Leopard frogs (*Rana pipiens*). At similar concentrations, atrazine interfered with normal development (Allran and Karasov, 2001). Increased numbers of *X. laevis* tadpoles with malformations in multiple tissues and an increased presence of apoptotic cells in the kidney and midbrain were noted after exposure to these levels in early development (Lenkowski et al., 2008). Still, Tavera-Mendoza et al. (2001, 2002) reported that development of gonadal tissue in *X. laevis* tadpoles was altered when tadpoles were exposed to only 21 μg/L atrazine.

An Affymetrix microarray analysis designed to determine the differential gene expression profile of tadpoles exposed to 400 μg/L atrazine throughout development as compared to unexposed controls was conducted by our laboratory (Langerveld et al., 2009). There were changes in the expression of single genes involved with growth and metabolism, as well as immune function, dietary protein digestion and new protein synthesis. These changes in gene expression occurred in conjunction with an increased mortality rate, a reduction in metamorphic rate, and a reduction in body weight and length at the end of the exposure (Celestine, 2006; Langerveld et al., 2009). There was also a reduction in fat body size, the frog's lipid storage organ, in these animals. All of these results lead to the hypothesis that exposure to 400 μg/L atrazine throughout development resulted in an energy deficit in these tadpoles. Since this article was the first to report these types of changes due to atrazine exposure in frogs, there was an interest in determining what the mechanisms were behind this change in physiology.

Therefore, our first objective was to establish a standardized protocol that would result in the effects noted in the initial study and would augment these data with additional assays designed to provide further insight into what was happening at the organ level. This would provide a system that could be used to address our second objective, which was to begin to investigate the cellular mechanisms behind these phenotypic effects. This article deals with the first objective and describes the findings of a series of *in vivo* studies investigating atrazine's phenotypic effects in exposed *X. laevis* tadpoles. To accomplish the second objective, animals in these studies were also used in biochemical and molecular assays designed to investigate possible mechanisms responsible for the atrazine-related changes that were noted. The results from these assays are presented and discussed in a companion article (Zaya et al., 2011).

2. Methods and materials

Six separate experiments were performed and the following section describes the general protocol for all of them. The exposure levels for the studies were as follows: four studies tested 0 and 400 μg/L (Studies A, B, C, and D), one study tested 0, 25 and 200 μg/L (Study E), while the remaining study tested 0, 200 and 400 μg/L (Study F). Details regarding the specific experimental design of each study can be found in Table 1. Tabulated and graphed data from each study are available in the Supplemental Data. Western Michigan University's Institutional Animal Care and Use Committee approved all procedures (Protocol #: 06-07-01).

2.1. Exposures

A stock solution of 25 mg/L atrazine (Sigma–Aldrich, St. Louis, MO) in water was prepared. Atrazine was added to filter-sterilized ultrapure water; the mixture was sonicated and warmed to 50 °C for no more than 15 min to dissolve the atrazine. The stock solution was diluted in exposure water to make final exposure solutions with nominal concentrations of 0 (Control), 25, 200, or 400 μg/L of atrazine. To validate the preparation protocol for these exposure solutions, samples of the stock solution and a dilution of the stock, with a final concentration of 1 μg/L, were assayed by an EPA certified laboratory (KAR Laboratories, Inc., Kalamazoo, MI). Both the stock and 1 μg/L solution had concentrations of atrazine that were within ±4% (25.3 mg/L and 0.96 μg/L, respectively) of target concentrations. The concentration of 1 μg/L was chosen because it fell within the detectable range of the validated assay specifically for atrazine, assay #EPA 525.2. The stock solution was assayed by gas chromatography using a nitrogen–phosphorus detector, assay EPA 8000. Two lots of atrazine were used for all described studies.

All concentration levels were chosen because they are within ranges reported to occur during peak application seasons (Gaynor et al., 1995; Storrs and Kiesecker, 2004). The 400 μg/L level was chosen because it was the exposure concentration used in Langerveld et al. (2009) which produced changes in fat body size. The 200 μg/L level was chosen since it was a concentration approximately mid way between the high and lowest exposure concentrations. The level of 25 μg/L was chosen since it was used by a number of other investigators.

2.2. Animals

Eggs were obtained after inducing adult *X. laevis* to breed using a series of injections of human chorionic gonadotropin (Sigma–Aldrich, St. Louis, MO) according to the recommended